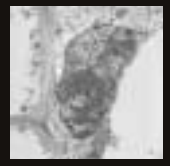
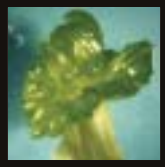
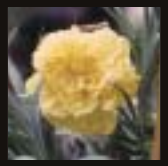
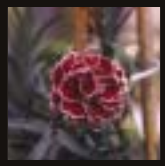
Eva Casanova






Organogènesi adventícia i nivells d'àcid indol-3-acètic i citocinines endògens en explants de pètals i fulles de clavell (*Dianthus* L.) cultivats *in vitro*

Departament de Biologia Vegetal
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Tesi Doctoral

UNIVERSITAT DE BARCELONA

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ORGANOGÈNESI ADVENTÍCIA I NIVELLS D'ÀCID INDOL-3-ACÈTIC I CITOCININES ENDÒGENS EN EXPLANTS DE PÈTALS I FULLES DE CLAVELL (*Dianthus* L.) CULTIVATS *IN VITRO*

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Memòria de la tesi presentada per

Eva Casanova i Fernández

per optar al grau de Doctora en Biologia

Directores: Ma Isabel Trillas i Gay i Ma Luisa Moysset i Agustí

Programa de doctorat del Departament de Biologia Vegetal de la Universitat de Barcelona:

"La fisiologia de les plantes i l'ambient"

Bienni 1996-1998

Vist i plau de les directores

Dra. Ma Isabel Trillas i Gay

Dra. Ma Luisa Moysset i Agustí

Barcelona, novembre del 2004

A la meva família

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Índex

Abreviatures

Les abreviatures escollides han estat les que s'utilitzen en anglès, ja que són les que es troben més habitualment en la literatura científica.

Abreviatura / Abbreviation	Català	English
ANOVA	Anàlisi de la variància	Analysis of variance
BA	N^6-benziladenina	N^6-benzyladenine
BHT	*t*-butilhidroxitoluè	*t*-butylated hydroxytoluene
bp	Parells de bases	Base pairs
BSA	Albúmina de sèrum boví	Bovine serum albumin
CaMV	Virus del mosaic de la coliflor	Cauliflower mosaic virus
CK	Citocinina	Cytokinin
CTAB	Bromur de cetiltrimetilamoni	Cetyltrimethylammonium bromide
DNA	Àcid desoxiribonucleic	Desoxyribonucleic acid
dNTP	Desoxiribonucleòtids	Desoxyribonucleotides
DHZ *	Dihidrozeatina	Dihydrozeatin
DHZR *	Ribòsid de la dihidrozeatina	Dihydrozeatin riboside
DHZRMP *	Ribòsid 5'-monofosfat de la dihidrozeatina	Dihydrozeatin riboside 5'-monophosphate
dpm	Desintegracions per minut	Disintegrations per minute
DW	Pes sec	Dry weight
EDTA	Àcid etilendiaminotetraacètic	Ethylenediaminetetraacetic acid
EGTA	Àcid etilen glicol-bis-(ß-aminoetil eter)-N, N, N', N'-tetraacètic	Ethylene glycol-bis-(ß-aminoethyl ether)-N, N, N', N'-tetraacetic acid
ELISA	Assaig de l'enzim immunoadsorbit	Enzyme-linked immunosorbent assays
FW	Pes fresc	Fresh weight
h	Hores	Hours
HPLC	Cromatografia líquida d'alta resolució	High performance liquid chromatography
IAA	Àcid indol-3-acètic	Indole-3-acetic acid
iP *	N^6-(÷^{2}-isopentenil)adenina o N^6-isopenteniladenina	N^6-(÷^{2}-isopentenyl)adenine or N^6-isopentenyladenine

iPR *	N^6-(÷^{2}-isopentenil)adenosina o N^6-isopenteniladenosina	N^6-(÷^{2}-isopentenyl)adenosine or N^6-isopentenyladenosine
iPRMP *	N^6-(÷^{2}-isopentenil)adenosina 5'-monofosfat o N^6-isopenteniladenosina 5'-monofosfat	N^6-(÷^{2}-isopentenyl)adenosine 5'-monophosphate or N^6-isopentenyladenosine 5'-monophosphate
kDa	Kilodàltons	Kilodaltons
kV	Kilovolts	Kilovolts
min	Minuts	Minutes
mRNA	RNA missatger	Messenger RNA
MS	Murashige i Skoog	Murashige and Skoog
NAA	Àcid 1-naftalenacètic	1-naphthaleneacetic acid
PCR	Reacció en cadena de la polimerasa	Polymerase chain reaction
PBS	Tampó fosfat salí	Phosphate buffer saline
RAPD	DNA polimòrfic amplificat aleatòriament	Random amplified polymorphic DNA
RH	Humitat relativa	Relative humidity
RNA	Àcid ribonucleic	Ribonucleic acid
rpm	Revolucions per minut	Revolutions per minute
SDS-PAGE	Electroforesi amb gel de poliacrilamida, amb dodecil sulfat sòdic	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tampó tris(hidroximetil)aminometà salí	Tris(hydroxymethyl)aminomethane buffer saline
TEAA	Acetat de trietilamoni	Triethylammonium acetate
Tris	Tris(hidroximetil)aminometà	Tris(hydroxymethyl)aminomethane
TDZ o Thidiazuron	N-fenil-N'-1,2,3-tiadiazol-5-ilurea	N-phenyl-N'-1,2,3-thiadiazol-5-ylurea
V	Volts	Volts
Z *	Zeatina	Zeatin
ZR *	Ribòsid de la zeatina	Zeatin riboside
ZRMP *	Ribòsid 5'-monofosfat de la zeatina	Zeatin riboside 5'-monophosphate

* Les abreviatures de les citocinines han estat les proposades per Kamínek et al. (2000).

Kamínek M, Brezinová A, Gaudinová A, Motyka V, Vanková R, Zazímalová E (2000) Purine cytokinins: a proposal of abbreviations. Plant Growth Regul. 32:253-256

INTRODUCCIÓ GENERAL

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1. El clavell

Els clavells són plantes del gènere *Dianthus* L., el qual pertany a la família de les cariofil·làcies i consta de més de 300 espècies. El nom del gènere prové dels escrits de Teofrast sobre Dios Anthos, la flor dels Déus. Els clavells silvestres es caracteritzen per les inflorescències molt simples, sovint reduïdes a una sola flor situada a l'extrem de la tija. Les flors són actinomorfes, amb cinc pètals lliures i en general més o menys profundament dentats al marge, i cinc sèpals soldats tot formant un calze llarg i estret, amb la base recoberta de dos o tres parells de bràctees imbricades. Tenen cinc estams i els dos carpels formen un únic pistil súper. Solen ser herbes vivaces, cespitoses, de tiges erectes poc o gens ramificades que duen a cada nus un parell de fulles oposades, llargues i estretes. Moltes espècies de clavells són pròpies de costers rocosos, de terraprims o fins i tot de penyes o cingles, però d'altres són plantes de prat, de marges de bosc o d'herbassars (Masalles et al. 1988, Mii et al. 1990).

1.1. Història i desenvolupament dels clavells comercials actuals

Els clavells comercials actuals provenen de l'espècie *D. caryophyllus* L., que és una herba relativament robusta nativa de l'àrea mediterrània i que s'ha cultivat al llarg de més de 2000 anys. Alguns dels cultivars de clavell moderns, però, són híbrids interespecífics que s'han obtingut per incorporar característiques d'altres espècies de clavell (Mii et al. 1990, Whealy 1992, Vainstein et al. 1991, van Altvorst 1994). Els clavells comercials solen florir al llarg de tot l'any, les flors tenen un ventall més ampli de colors, i tenen la tija més robusta que els clavells silvestres, els quals floreixen a la primavera i a l'estiu i les seves flors desprenen fortes olors, però tenen una senescència ràpida. Les flors dels clavells cultivats són més grans que les de les espècies silvestres i comprenen, no cinc pètals, sinó molts més. Són propagats vegetativament i es cultiven majoritàriament per ser venuts com a flor tallada, però també s'utilitzen com a planta de jardí o, més recentment, com a planta de test. Actualment es continuen fent grans esforços en el camp de la hibridació del clavell per tal d'aconseguir una millor qualitat i productivitat.

Els cultivars comercials de *D. caryophyllus* tenen inflorescències cimoses i per això poden ser cultivats com a clavells estàndard o com a clavells *spray* (també anomenats miniatura). Els clavells estàndard es produeixen tot eliminant les poncelles laterals i deixant la flor terminal, mentre que els *spray* es cultiven tot treient la flor terminal, amb el consegüent desenvolupament de les poncelles laterals. A principis del segle XIX va començar a França i Itàlia el desenvolupament de cultivars de clavell, cultivats com a estàndard. Posteriorment es van exportar als Estats Units, on paral·lelament també es va desenvolupar la seva hibridació. Els cultivars obtinguts per William Sim, anomenats cultivars Sim i procedents de mutacions d'un primer cultivar batejat amb el seu nom i obtingut el 1938, van tenir molt d'èxit i s'expandiren entre els hibridadors. Se n'obtingueren de tots colors i dominaren el mercat durant molts anys, en abscència d'alternatives (Holley i Baker 1963). Actualment, molts dels clavells estàndard són

creuaments entre els Sim i els Mediterranis. Els clavells *spray* els desenvolupà W. W. Thompson al 1956 als Estats Units, i seguidament s'expandiren a Europa (van Altvorst 1994). Aquesta classificació dels clavells en estàndard o *spray*, però, està basada en característiques fenotípiques (Vainstein et al. 1991, van Altvorst 1994). Els nombrosos creuaments interespecífics i intraespecífics realitzats com a mínim des del segle XVI, la hibridació per mutacions, el secretisme dels hibridadors pel que fa a l'origen dels cultivars amb èxit, els moltíssims hibridadors que hi treballen arreu del món i la poca documentació de la història de la hibridació d'aquesta espècie, compliquen l'estudi de les relacions genètiques entre els cultivars de clavells actuals.

El clavell és actualment un dels cultius de flor més importants del món, junt amb el d'altres flors tallades, com ara les roses, els crisantems i els gladiols (Horn 2002). És relativament fàcil de fer créixer i produeix un gran nombre de flors per metre quadrat. A més, té moltes de les qualitats essencials per una bona flor tallada: és una flor robusta que s'empaqueta bé, es transporta sense problemes i té una durada d'unes dues o tres setmanes després de ser collida. Durant molts anys els clavells foren la flor més exportada a nivell mundial, i actualment encara romanen en la segona posició de les taules d'exportació i importació mundials. La producció inicial de clavell provenia majoritàriament dels Estats Units i dels països mediterranis, però en l'actualitat la producció principal s'ha traslladat a països equatorials, com Colòmbia i Kènia, tot i que encara resta una bona part de la producció al voltant de la Mediterrània: Espanya, Turquia, Itàlia, Marroc i Israel. El major consumidor n'és els Estats Units, que al 2003 va importar 678 milions de tiges de clavell (60 milions de dòlars), sent la segona flor després de les roses (http://www.pathfastpublishing.com). A Espanya, la producció de clavell incrementa des del 1993, tot passant de 1.825 milions de tiges als 2.210 de l'any 2000. Paral·lelament, les exportacions de clavell també augmenten des del 1993, fins a quadruplicar-se (48.000 tones) al 1999. El principal país receptor n'és Holanda, seguida pel Regne Unit, França i Alemanya (http://www.mapya.es, Ministerio de Agricultura, Pesca y Alimentación).

1.2. La hibridació en el clavell

Un dels objectius principals dels hibridadors de qualsevol espècie cultivada és millorar-la tant quantitativament com qualitativa. A part de la millora de les característiques agronòmiques, com ara un increment de la producció, de l'arrelament o de la resistència a patògens i herbicides, en el camp de les plantes ornamentals s'han d'obtenir continuament noves varietats per respondre a la demanda del consumidor. Pel clavell, tant com a flor tallada com ornamental en test, serien desitjables canvis en els colors i les formes de la flor, un increment de la seva fragància, l'allargament de la vida de la flor tallada i modificacions en la morfologia de la planta (Mii et al. 1990, Whealy 1992, van Altvorst 1994). La morfologia de la planta pot ser modificada tant per raons agronòmiques com per raons estètiques.

1.2.1. Hibridació tradicional i hibridació molecular

La hibridació clàssica és la que s'ha usat tradicionalment per la introducció de nous trets i per crear noves varietats en moltes espècies, incloent les ornamentals, però és un procés lent i tediós. La descendència seleccionada

fenotípicament cal mantenir-la tot propagant-la vegetativament, mitjançant esqueix o per micropropagació *in vitro*, per tal d'assegurar la producció de plantes genèticament idèntiques. En aquest tipus d'hibridació, però, els gens disponibles per l'obtenció de nous trets queden limitats al genoma dels parentals, que en general han de pertànyer a la mateixa espècie per tal que el creuament sigui compatible. A més, el fet que moltes varietats siguin estèrils també impedeix la seva millora mitjançant la hibridació tradicional (Vainstein 2002).

Usant tècniques d'hibridació molecular, és a dir d'obtenció de plantes transgèniques, la planta pot mantenir tota la seva informació genètica i guanyar els trets afegits. A més, l'enginyeria genètica permet introduir tot tipus de gens, podent-nos saltar les barreres d'incompatibilitat pels creuaments. De tota manera, per enginyeria genètica només és possible introduir en les plantes característiques que siguin monogèniques o oligogèniques. La hibridació molecular no substituirà la hibridació tradicional, però sí que pot complementar-la per tal d'aconseguir característiques específiques (Vainstein 2002).

El cultiu de cèl·lules, teixits o òrgans vegetals i l'optimització de la tècnica de regeneració de plàntules són essencials per subministrar el sistema i el material per la manipulació genètica i, per tant, és el primer requeriment de la transformació d'espècies vegetals. Aquests sistemes estan força ben establerts en moltes espècies, incloent ornamentals (Zuker et al. 1998), tot i que encara hi ha espècies recalcitrants al cultiu *in vitro* (Cassells 2002). Així, l'increment en l'eficiència dels procediments de regeneració *in vitro* de plantes i també dels de transformació ha permès, en els últims anys, millorar varietats ornamentals per hibridació molecular (Zuker et al. 1998). Fins l'actualitat s'han transformat una trentena d'espècies ornamentals, entre les quals s'hi troba el clavell (Deroles 2002).

1.3. Clavells transgènics

S'han obtingut clavells amb nous colors de flor tot alterant la ruta metabòlica dels flavonoids, els pigments majoritaris en el clavell. La introducció de l'antisentit del gen de la flavanona 3-hidroxilasa n'ha atenuat o eliminat el color carbassa-vermellós del cultivar Eilat (Zuker et al. 2002). La companyia Florigene Ltd. (Austràlia) ha produït clavells amb diverses tonalitats violetes amb la introducció del gen de la flavonoid 3',5'-hidroxilasa en clavells blancs (Fukui et al. 2003). Aquests cultivars, anomenats Moondust i Moonshadow, són actualment les úniques varietats transgèniques de flor ornamental que es comercialitzen.

També s'han obtingut clavells del cultivar Eilat que expressen el gen de la linalol sintasa, absent en clavell, amb l'objectiu d'augmentar-ne la fragància. Tot i l'increment en la síntesi de linalol no s'han detectat diferències en la fragància (Lavy et al. 2002).

La prolongació de la longevitat de la flor tallada s'ha aconseguit suprimint l'expressió del gen que codifica l'ACC oxidasa, que catalitza la biosíntesi de l'etilè a partir de l'àcid 1-aminociclopropà-1-carboxílic (ACC), en els cultivars Scania, White Sim i Nora (Savin et al. 1995, Kosugi et al. 2002).

La introducció del gen *rol*C d'*Agrobacterium rhizogenes* en el cultivar White Sim ha produït canvis molt significatius en l'arquitectura de la planta, tant en la part aèria com en la subterrània (Zuker et al. 2001). El fenotip obtingut està descrit en l'apartat 4.4.

2. El cultiu *in vitro* del clavell

A finals del segle XIX es realitzaren els primers esforços per tal d'investigar la possibilitat que les cèl·lules es poguessin desenvolupar isolades de les plantes, tot donant lloc a les tècniques de cultiu de cèl·lules i teixits actuals (White 1943). El primer cultiu de teixits vegetals de creixement il·limitat s'obtingué en *Daucus carota* (pastanaga) (Gautheret 1939). A partir de petits fragments, anomenats explants, amb teixit vascular i incloent una font d'auxines en el medi s'estimulà el creixement de teixit indiferenciat. Aquest teixit, anomenat cal·lus, podia ser subcultivat indefinidament en condicions estèrils. La producció de cal·lus es considera que és un procés de desdiferenciació d'un teixit organitzat, tal i com indiquen els canvis morfològics i de l'activitat metabòlica. En un experiment clàssic, Skoog i Miller (1957) alterant la relació citocinina (CK)/auxina del medi aconseguiren la formació de rels i tiges adventícies a partir de cal·lus de *Nicotiana tabacum* (tabac) i, poc després, es trobà que cal·lus de *D. carota* regeneraren embrions somàtics que es pogueren desenvolupar fins a formar plantes senceres (Steward et al. 1958). Actualment, aquests processos morfogènics s'han pogut obtenir en moltes espècies (George 1993, Thorpe 1994, Collin i Edwards 1998), a partir de diversos explants, com ara meristemes, fragments de tija, pètal, fulla o rel, o a partir de cèl·lules o protoplasts, cultivats en un medi nutritiu amb una composició química determinada, en un ambient físic controlat i en condicions estèrils.

El cultiu *in vitro* de cèl·lules, teixits i òrgans vegetals pot tenir tant una vessant bàsica com una vessant aplicada. D'una banda, permet realitzar estudis a nivell genètic, bioquímic i fisiològic i aprofundir en el coneixement del metabolisme i del creixement i desenvolupament vegetal. D'altra banda, el cultiu *in vitro* té nombroses aplicacions, com la micropropagació, l'obtenció de plantes lliures de virus i de patògens o la conservació de material vegetal d'interès genètic, de gran interès en plantes ornamentals (Collin i Edwards 1998).

En el cas del clavell, els primers cultius *in vitro* es realitzaren ja a principis dels anys 50 per l'obtenció de plantes lliures de virus i de patògens, donada la importància econòmica d'aquest cultiu. Posteriorment, el cultiu *in vitro* també s'utilitzà per la micropropagació d'aquestes plàntules lliures de virus i patògens, per poder obtenir-ne grans quantitats de manera ràpida (Holley i Baker 1963). Amb aquesta finalitat, s'utilitzà el cultiu de meristemes, d'àpexs de tiges o de nusos (Hacket i Anderson 1967, Petru i Landa 1974, Earle i Langhans 1975, Davis et al. 1977, Jelaska i Sutina 1977, Shabde i Murashige 1977). Encara, actualment, s'utilitzen aquestes tècniques per l'obtenció de plantes mare de clavell, les quals s'utilitzen per treure'n esqueixos per la seva multiplicació vegetativa amb la certesa que siguin sans, i també per la producció d'un gran nombre d'individus uniformes a partir de material vegetal inicial limitat (Kallak et al. 1996). A més, el cultiu *in vitro* també s'utilitza per la conservació de material vegetal amb interès genètic, per l'obtenció d'híbrids somàtics amb l'isolament de protoplasts i la seva fusió (Nakano i Mii 1993b, Nakano et al. 1996), i per la regeneració de plantes a partir de cèl·lules o teixits en els que s'han donat variacions somaclonals o en els que s'han induït mutacions per mètodes físics o químics (Simard et al. 1992). El cultiu *in vitro* i l'aplicació de tècniques d'enginyeria genètica han possibilitat la regeneració de plantes de clavell transgèniques a partir de cèl·lules transformades (Zuker et al. 1999).

Les plàntules de clavell cultivades *in vitro*, però, tenen tendència a esdevenir hiperhídriques (Ziv 1991). El fenomen de la hiperhidricitat, inicialment anomenat vitrificació, consisteix en anomalies morfològiques i fisiològiques desenvolupades per les plàntules en determinades condicions de cultiu, que fan que adquireixin un aspecte vitri, translúcid i suculent, i que no permeten la seva aclimatació *ex vitro* (Ziv 1991, Debergh et al. 1992, Kevers et al. 2004).

2.1. La morfogènesi

El cultiu *in vitro* es basa en la totipotència de les cèl·lules vegetals, és a dir, en la capacitat d'aquestes cèl·lules de donar lloc a una planta sencera. Algunes tècniques de cultiu *in vitro* permeten la regeneració de plantes *de novo*, tot controlant la composició hormonal del medi de cultiu, a través de dues vies de desenvolupament, l'organogènesi i l'embriogènesi somàtica. L'organogènesi porta a la diferenciació d'estructures unipolars: primordis de tiges i de rels que originen tiges adventícies (caulogènesi) i rels adventícies (rizogènesi). L'embriogènesi somàtica porta a la formació d'embrions somàtics, tot seguint els estadis de desenvolupament dels embrions zigòtics (globular, cor, torpede i cotiledonar), però sense fecundació. A més, ambdós tipus de processos morfogènics es poden classificar com a directes, si els centres d'activitat meristemàtica es formen directament en el l'explant cultivat sense la fase de cal·lus, o com a indirectes, quan s'originen a partir d'un cal·lus (George 1993, Thorpe 1994).

En el clavell la via morfogènica més habitual és l'organogènesi, en forma de rizogènesi o caulogènesi depenent del balanç de reguladors del creixement, auxines i CKs, del medi de cultiu (Zuker et al. 1998). En general l'organogènesi sol ser directa, encara que alguns estudis han generat la fase de cal·lus per tal d'aconseguir regenerar plantes a partir de cèl·lules o protoplasts (Nakano i Mii 1992, Nakano i Mii 1993a, Nakano i Mii 1995). En condicions de cultiu molt específiques, com ara amb la presència de l'àcid 2,4-diclorofenoxiacètic, també se'n pot arribar a promoure l'embriogènesi somàtica (Frey et al. 1992, Yantcheva et al. 1998).

La formació de tiges adventícies ha estat descrita histològicament (Thorpe 1994). Els primers canvis es detecten amb l'inici de la divisió cel·lular en determinades zones de l'explant, posteriorment es formen meristemoids i aquests, seguidament, esdevenen primordis de tija, els quals ja es troben ubicats a la superfície de l'explant. Els primordis esdevenen gemmes apicals i al cap de pocs dies ja s'observen tiges vegetatives amb els corresponents primordis foliars (Thorpe 1994). En clavell es troben descripcions histològiques de l'organogènesi *in vitro*, encara que bàsicament són per esbrinar quins teixits són els que originen les tiges adventícies (Frey i Janick 1991, Simard et al. 1992).

Pel que fa a la zona de regeneració d'òrgans, la formació de tiges adventícies tant en explants de fulles com de pètals de clavell es dóna a la seva base, en nombrosos cultivars (van Altvorst et al. 1992b, Zuker et al. 1998), tal com també hem observat en pètals d'Early Sam (Fig. 1) i de White Sim (Fig. 2).

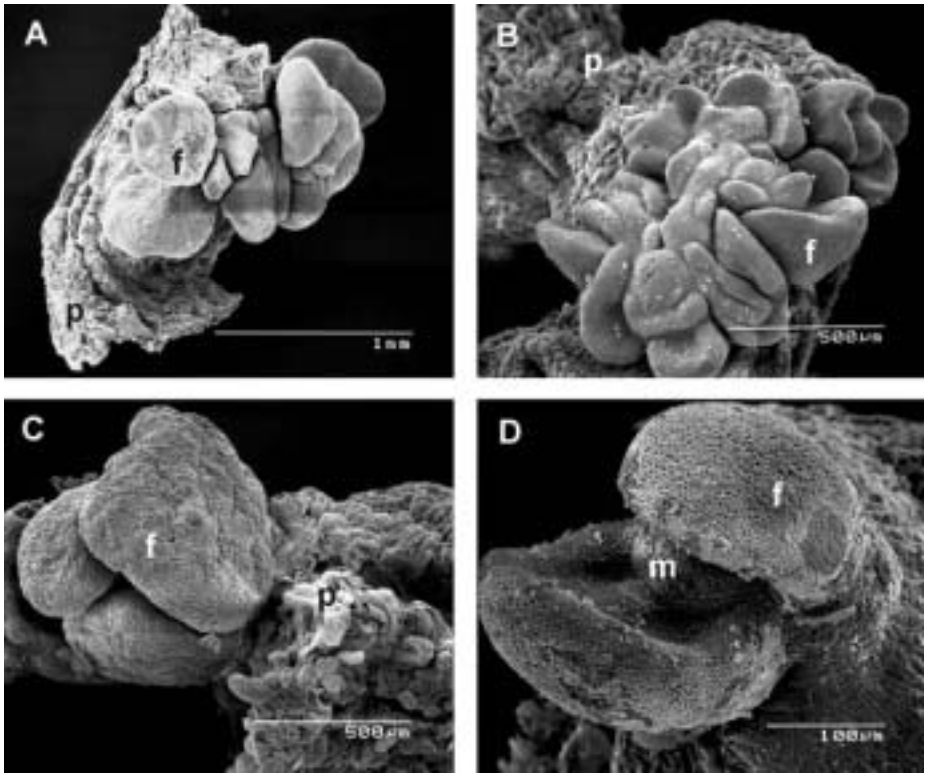

Fig. 1. Microfotografies, al microscopi electrònic de rastreig, de l'organogènesi adventícia a la base d'explants de pètals de clavell (*Dianthus caryophyllus* cv. Early Sam) al 21è dia de cultiu en medi MS amb 0.5 µM NAA i 0.5 µM TDZ (A i B) i amb 0.5 µM NAA i 5.0 µM TDZ (C i D), en les que s'observa que els primordis foliars estan perfectament formats. (p), pètal; (f) primordis foliars; (m) meristema apical.

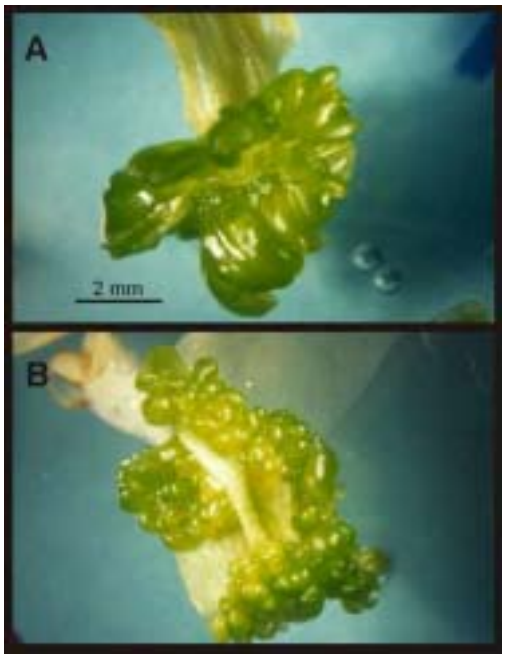

Fig. 2. Fotografies de l'observació en lupa binocular de l'organogènesi adventícia a la base d'explants de pètals de clavell (*Dianthus caryophyllus* cv. White Sim) al 22è dia de cultiu en medi MS amb 0.5 µM NAA i 0.5 µM TDZ. (A) Formació de tiges a l'extrem de la base del pètal, la part habitualment regenerativa; (B) i formació de tiges en altres parts de la base del pètal menys habituals a regenerar.

2.1.1. Factors de control de la morfogènesi adventícia

En general, el desenvolupament organitzat que porta a la regeneració de plàntules pot aconseguir-se mitjançant la selecció adequada d'un explant, una bona elecció del medi de cultiu, incloent les substàncies reguladores del creixement, i controlant-ne l'ambient físic (Thorpe 1994). Així, doncs, la inducció de la morfogènesi *in vitro* està controlada per nombrosos factors que podem agrupar en intrínsecs i extrínsecs. Els factors intrínsecs són aquells que depenen de la planta mare i de l'explant i, per tant, són els més difícils de controlar i els que solen fer que una espècie sigui recalcitrant a ser cultivada. Els factors extrínsecs es refereixen principalment a la composició del medi i a l'ambient físic del cultiu.

2.2. Efecte del genotip

Teòricament, tant en explants com en cal·lus es pot induir la formació d'òrgans i embrions tot controlant els medis de cultiu. Però l'estratègia regenerativa d'un cultiu depèn en gran part de l'espècie (Thorpe 1994), encara que també podem trobar variacions entre varietats i cultivars de la mateixa espècie. És dificil explicar perquè es troben diferències tan marcades en la capacitat regenerativa d'un explant quan els genotips de les varietats o cultivars són tan similars (Collin i Edward 1998). La presència de gens foranis (transgens), com per exemple el gen *rolC* d'*Agrobacterium rhizogenes*, que en expressar-se en la planta transgènica fossin capaços de modificar directament o indirecta la concentració hormonal endògena o bé la sensibilitat dels teixits a les hormones, podrien afectar la capacitat morfogènica dels explants o cal·lus.

2.2.1. Cultivars estudiats en el cultiu *in vitro* del clavell

Pel que fa al genotip s'han descrit diferències en l'organogènesi, usant explants de pètals, en diversos cultivars de clavell, com el cultivars Scania, Sandra, Ceris Royalette o Lisa (Frey i Janick 1991, Fisher et al. 1993), amb un únic estudi exhaustiu utilitzant fragments de fulles i pètals com a explants (van Altvorst et al. 1992b). Els cultivars dels que disposem, White Sim (estàndard), Early Sam (*spray*) i Pulcino, Inorsa R-24 i Mei Ling (híbrids interespecífics), no han estat estudiats *in vitro*, excepte el White Sim, del que se n'ha estudiat la regeneració en pètals, receptacles i tiges (Nugent et al. 1991, Fisher et al. 1993, Watad et al. 1996), però sense un estudi exhaustiu pel que fa als reguladors del creixement i la seva concentració.

2.3. Efecte de l'explant

La regeneració de plàntules *in vitro* pot obtenir-se d'explants de quasi qualsevol part de la planta mare, tant a partir d'òrgans (rels, fulles, tiges, cotilèdons, hipocòtils), com de teixits o cèl·lules específics (pol·len, teixit ovular, endosperm, mesòfil). A la pràctica, però, per cada espècie o varietat hi ha determinats explants que permeten una més bona regeneració adventícia. Els explants que contenen una proporció més elevada de cèl·lules amb capacitat

mitòtica, com ara teixits juvenils, meristemes o àpexs, poden regenerar millor (Thorpe 1994, Collin i Edward 1998). D'altra banda, la capacitat morfogènica també depèn de l'estat fisiològic de l'explant. Aquest pot dependre de l'edat de la planta mare, del seu estat ontogenètic i fins i tot de l'estació de l'any en que obtenim els explants (George 1993, Thorpe 1994, Collin i Edward 1998). És per això que és important que la planta mare, a part d'estar sana i d'haver rebut els requeriments nutricionals adequats, estigui creixent activament, i no estigui en un període de dormició (Collin i Edward 1998). Finalment, la posició de l'explant en el medi tambe és un factor important que influeix en la regeneració obtinguda (Thorpe 1994).

2.3.1. Explants utilitzats en el cultiu *in vitro* del clavell

En els primers cultius de clavell s'utilitzaven meristemes o àpexs de tiges per la seva propagació *in vitro* o per l'eliminació de virus (Petru i Landa 1974, Earle i Langhans 1975, Davis et al. 1977). Posteriorment, s'obtingueren tiges adventícies a partir del cultiu de diversos explants: fragments de tija (Roest i Bokelmann 1981, Frey i Janick 1991, Nugent et al. 1991, Nakano et al. 1994, van Altvorst et al. 1995, Watad et al. 1996), segments de fulla (Frey i Janick 1991, van Altvorst et al. 1992b, 1994, 1995, Messeguer et al. 1993, Nakano et al. 1994), gemmes axil·lars (Miller et al. 1991a, van Altvorst et al. 1995), pètals (Kakehi 1979, Gimelli et al. 1984, Leshem 1986, Frey i Janick 1991, Miller et al. 1991b, Nugent et al. 1991, van Altvorst et al. 1992b, Fisher et al. 1993, Nakano et al. 1994), anteres (Villalobos 1981), òvuls (Demmink et al. 1987) i receptacles (Miller et al. 1991b, Nugent et al. 1991, Frey i Janick 1991).

2.4. Efecte de la composició del medi de cultiu

Els constituents del medi de cultiu es poden dividir en dues classes: sals inorgàniques (macronutrients i micronutrients) i substàncies orgàniques (font de carboni i d'energia, vitamines, aminoàcids i reguladors del creixement) (Thorpe 1994, Collin i Edwards 1998). Pel que fa a les substàncies inorgàniques, existeixen diverses combinacions optimitzades, però la formulació MS (Murashige i Skoog 1962) amb alta concentració de sals, o bé formulacions derivades d'aquesta, són les més usades (Thorpe 1994). En referència a les substàncies orgàniques, l'efecte dels reguladors del creixement és el més complex. El control de l'organogènesi adventícia depèn bàsicament de la presència d'auxines i CKs (Krikorian 1995, Coenen i Lomax 1997). Aquests dos reguladors del creixement estimulen la divisió cel·lular i controlen la diferenciació i la morfogènesi (Coenen i Lomax 1997, Collin i Edwards 1998). Al 1957, ja es va demostrar que l'organogènesi adventícia de tiges i rels a partir de cal·lus de *N. tabacum* era dependent del balanç d'auxina i CK en el medi de cultiu. Mentre que una relació CK/auxina elevada promovia la producció de tiges, una relació CK/auxina baixa produïa la formació de rels, i relacions intermèdies promovien la proliferació de cal·lus (Skoog i Miller 1957) (Fig. 3). Posteriorment, un efecte similar dels diferents balanços s'anà trobant en altres espècies. Així, el sistema d'ajust de la relació CK/auxina per controlar l'organogènesi *in vitro* és, ara per ara, un procediment àmpliament establert que constitueix la base de gran part dels protocols de micropropagació actuals (Krikorian 1995, Collin i Edwards 1998).

Altres substàncies reguladores del creixement, com ara les gibberel·lines, quasi no s'utilitzen en els medis de cultiu, ja que no són essencials per la inducció d'òrgans, però actuen com a moduladors de la resposta morfogènica. L'àcid abscísic només s'utilitza en casos específics, com ara per la prevenció de la germinació precoç en embrions somàtics. I l'etilè es considera una substància nociva que s'hauria d'evitar en l'ambient del cultiu *in vitro* (Krikorian 1995).

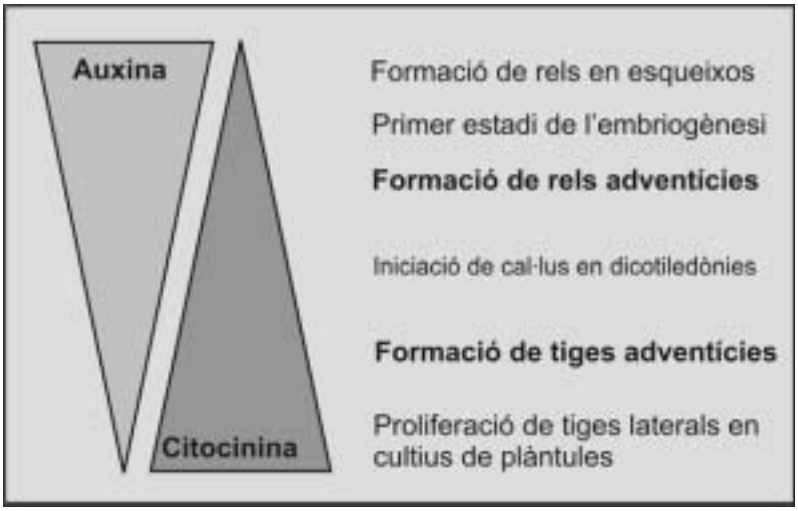

Fig. 3. Concentracions relatives d'auxina i citocinina típicament requerides pel creixement i la morfogènesi *in vitro* (adaptat de George 1993).

2.4.1. Medis de cultiu i reguladors del creixement utilitzats en el cultiu *in vitro* del clavell

Des dels primers cultius de clavell s'ha utilitzat el medi MS (Murashige i Skoog 1962), amb algunes modificacions en les substàncies orgàniques, concretament en les vitamines, pel cultiu d'explants de pètals (Gimelli et al. 1984).

Pel que fa les auxines, tot i que en estudis inicials s'havia utilitzat l'àcid indol-3-acètic (IAA) (Petru i Landa 1974, Roest i Bokelmann 1981), s'usa majoritàriament l'auxina sintètica àcid 1-naftalenacètic (NAA) (Fig. 4), ja que és l'única auxina que penetra fàcilment la membrana plasmàtica sense necessitat d'un transport actiu (Nordström et al. 2004) i no es metabolitza tan ràpidament com una auxina natural.

La N^6-benziladenina (BA) (Fig. 4), una CK aromàtica natural derivada de l'adenina (Strnad 1997), és una de les més usades com a font exògena de CK en els medis de cultiu. Encara que en els primers cultius *in vitro* d'àpexs de tija de clavell s'utilitzava la kinetina (CK sintètica també del grup de les aromàtiques) (Petru i Landa 1974, Earle i Langhans 1975, Davis et al. 1977), la BA és la més utilitzada tant en explants de fulla com de pètal (Kakehi 1979, Gimelli et al. 1984, Nugent et al. 1991, van Altvorst et al. 1992b, 1994, 1995, Fisher et al. 1993, Messeguer et al. 1993, Nakano et al. 1994).

Alguns derivats de les fenilurees, substàncies sintètiques com ara l'N-fenil-N'-1,2,3-tiadiazol-5-ilurea (TDZ o thidiazuron) (Fig. 4), són altament actius com a CKs (Mok i Mok 2001). S'ha trobat que el TDZ pot provocar una resposta més gran que la BA en l'estimulació de la formació de tiges en moltes espècies, com ara *Phaseolus vulgaris*, *Paspalum scrobiculatum* o *Hordeum vulgare* (Malik i Saxena 1992, Vikrant i Rashid 2002, Sharma et al.

2004). A més, algunes espècies llenyoses recalcitrants a ser cultivades *in vitro* s'han pogut cultivar gràcies a la presència de TDZ en el medi (revisat per Huetteman i Preece 1993, Murthy et al. 1998). En cultius de clavell *in vitro* el TDZ ha estat molt poc utilitzat (Frey i Janick 1991), tot i que estudis comparatius entre diverses CKs han mostrat que el TDZ és la CK més efectiva per la regeneració de tiges adventícies en explants de tiges i pètals de clavell dels cultivars Scania i White Sim (Nugent et al. 1991, Nakano et al. 1994, Watad et al. 1996). En explants de fulles, el TDZ no ha estat efectiu en la regeneració de tiges (Frey i Janick 1991, Nakano et al. 1994), probablement per problemes en la separació de les fulles de la planta, procés dificultós que ha estat optimitzat posteriorment per van Altvorst et al. (1994). Es pot considerar, doncs, que l'efecte del TDZ en fulles de clavell no s'ha estudiat. Es desconeix si els derivats de les fenilurees actuen de manera directa o bé indirecta tot modificant la biosíntesi i el metabolisme de les CKs endògenes (Mok i Mok 2001).

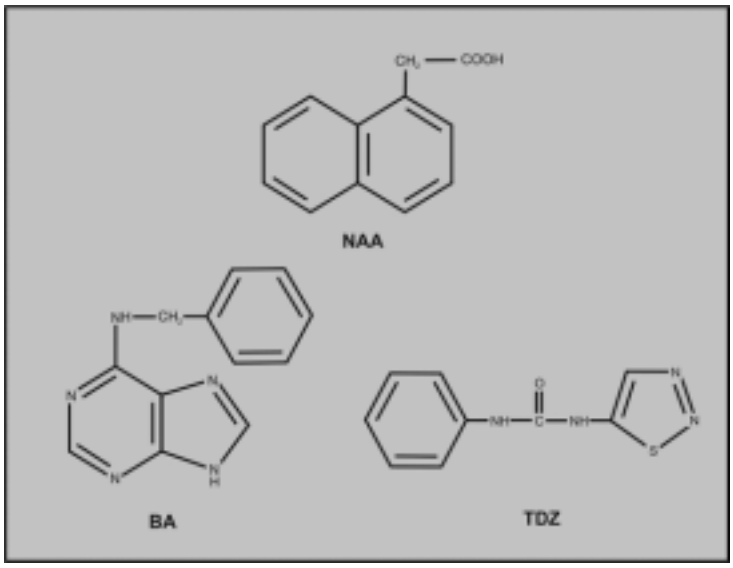

Fig. 4. Estructura química de l'auxina sintètica àcid 1-naftalenacètic (NAA) i de les CKs N^6-benziladenina (BA), natural, i N-fenil-N'-1,2,3-tiadiazol-5-ilurea (TDZ), sintètica.

2.5. Efecte de l'ambient físic del cultiu

Els factors que efecten l'ambient físic del cultiu són, d'una banda, els que incideixen en l'estat hídric del cultiu, com el grau de solidificació del medi i la humitat relativa (RH) del pot de cultiu (Ziv 1991). D'altra banda tindríem la composició de l'atmosfera del cultiu, la temperatura i la llum (George 1993, Thorpe 1994).

2.5.1. Efecte de la concentració d'agent gelificant

Els medis de cultiu poden ser líquids o es poden solidificar. Per solidificar o, més ben dit, gelificar el medi se sol usar agar o gelrite, que són polisacàrids complexos (George 1993, Thorpe 1994). La concentració d'agent gelificant, tot i formar part de la composició del medi, l'afecta de manera física tot solidificant-lo en major o menor grau. Això afecta

la disponibilitat d'aigua (mesurada a través del potencial hídric del medi) i, com a conseqüència, dels components del medi (Ziv 1991, Fujiwara i Kozai 1995, Smith i Spomer 1995). Alguns cultius que creixen en medi líquid esdevenen hiperhídrics, i l'addició d'agents gelificants pot ajudar a millorar la seva qualitat (Ziv 1991, Debergh et al. 1992). En l'actualitat la hiperhidricitat en el cultiu de teixits continua sent un problema encara no resolt (Kevers et al. 2004). En l'intent de reduir la hiperhidricitat amb l'addició d'agents gelificants, també es redueix el creixement i la taxa de propagació de moltes espècies cultivades *in vitro* (Debergh et al. 1981, Pasqualetto et al. 1988, Turner i Singha 1990).

2.5.1.1. Concentracions d'agar utilitzades en el cultiu *in vitro* del clavell

El clavell és un cultiu amb tendència a la hiperhidricitat (Mii et al. 1990, Ziv 1991). L'agar és, pràcticament, l'únic agent gelificant usat en el cultiu de clavell. El gelrite només s'ha usat en comptades ocasions (Nakano et al. 1994, Miller et al. 1991a, b), probablement perquè tendeix a generar un nombre més elevat de tiges hiperhídriques que l'agar (Turner i Singha 1990, Franck et al. 2004). Les concentracions d'agar més utilitzades en clavell oscil·len entre els 6 i els 8 g l^{-1} (Petru i Landa 1974, Roest i Bokelmann 1981, Gimelli et al. 1984, Frey i Janick 1991, Nugent et al. 1991, van Altvorst et al. 1992b, 1994, 1995). L'augment de la concentració d'agar en cultius de plàntules de clavell disminueix el nombre de tiges hiperhídriques, però també redueix el creixement i la taxa de propagació de les plàntules (Ziv et al. 1983, Hakkaart i Versluijs 1983, Yadav et al. 2003). L'efecte dels agent gelificants en la morfogènesi d'òrgans adventicis ha estat molt poc estudiat (Brown et al. 1979, Bornman i Vogelman 1984, Castro-Concha et al. 1990, Owens i Wozniak 1991) i, fins al moment, no s'ha fet cap estudi del seu efecte en l'organogènesi adventícia en el clavell.

2.5.2. Efecte de la humitat relativa

La RH dels pots de cultiu tancats hermèticament sol ser propera al 100% (George 1993). Aquesta podria ser, també, una de les causes de la hiperhidricitat obtinguda en el cultiu d'algunes espècies (Ziv 1991, Fujiwara i Kozai 1995). És per això que alguns autors han estudiat l'efecte de la disminució de la RH en el cultiu, la qual redueix la hiperhidricitat i, paral·lelament, també redueix el creixement de les plàntules *in vitro*. Aquesta disminució de la RH s'ha aconseguit usant dessecants (Ziv et al. 1983) o ventilant els pots de cultiu tot usant taps no hermètics (Debergh et al. 1992, Sallanon i Maziere 1992, Majada et al. 1997).

2.5.2.1. Control de la humitat relativa en el cultiu *in vitro* del clavell

La disminució de la RH en cultius de plàntules senceres de clavell en medis sòlids ha aconseguit disminuir-ne la hiperhidricitat, però a la vegada se n'ha reduït el seu creixement i la taxa de propagació (Ziv et al. 1983, Majada et al. 1997). En canvi, els medis líquids amb una RH per sota del 100% han permès una disminució de la

hiperhidricitat, però no del creixement i de la taxa de propagació (Ziv et al. 1983, Majada et la. 1997). L'efecte de la RH en la morfogènesi adventícia, però, no ha estat estudiada ni en el clavell ni en cap altra espècie.

2.5.3. Efecte d'altres factors físics

L'atmosfera del cultiu afecta el desenvolupament dels cultius, principalment la concentració dels gasos etilè, O_2 i CO_2 (George 1993, Thorpe 1994). En plàntules de clavell i d'*Actinidia deliciosa* cultivades *in vitro* l'etilè efecta negativament el seu creixement i taxa de propagació (Fal et al. 1999, Arigita et al. 2003), i en clavell també incrementa la seva hiperhidricitat (Fal et al. 1999). En canvi, en *Gardenia jasminoides*, l'increment de CO_2 en l'atmosfera del cultiu, junt amb la disminució de sacarosa en el medi, incrementa els nivells d'autotrofia de les plàntules i millora la seva posterior aclimatació *ex vitro* (Serret et al. 1997).

La temperatura controla el creixement i el desenvolupament *in vivo* i *in vitro* de les plantes, però no sol ser un factor que s'examini. En general, els cultius es mantenen a una temperatura constant, entre 20 i 30ºC, però s'hauria de determinar una temperatura òptima per cada espècie cultivada i cada procés estudiat (George 1993, Thorpe 1994).

Tot i que la majoria d'investigadors tampoc no avaluen el seu efecte, la llum influeix en la morfogènesi *in vitro* (George 1993, Thorpe 1994). D'una banda, els requeriments d'energia lumínica influeixen, combinats amb altres factors, en els nivells d'autotrofia de plàntules de *G. jasminoides* (Serret et al. 1997, Serret i Trillas 2000). D'altra banda, s'ha demostrat l'efecte de la qualitat de la llum i del fotoperíode en l'embriogènesi somàtica d'*Araujia sericifera* (Torné et al. 2001) i en l'organogènesi del clavell (dades no publicades).

3. Auxines i citocinines en l'organogènesi

El procés de l'organogènesi *in vitro* ha estat estudiat en poques espècies, com *N. tabacum* (Attfield i Evans 1991a, b, Dhaliwal et al. 2003), *Begonia* x *erythrophylla* (Burritt i Leung 1996) o *Saintpaulia ionantha* x *confusa* (Lo et al. 1997a, b). Aquests estudis confirmen els resultats de Christianson i Warnick (1983) en *Convolvulus arvensis*, en els que es van proposar tres fases en aquest procés. En la primera, fase d'adquisició de competència, l'explant adquireix l'habilitat (no la capacitat) de respondre a una inducció organogènica a través del procés de desdiferenciació, durant el qual les cèl·lules activen l'expressió de gens que codifiquen cinases dependents de ciclines i ciclines, que regulen la progressió del cicle cel·lular, per tal de poder reentrar en la mitosi (Sugiyama 1999). En la segona fase, la d'inducció organogènica, les cèl·lules competents adquireixen la determinació de seguir una via de desenvolupament determinada i, com a resultat de la divisió d'aquestes cèl·lules, es formen meristemoids. Ambdues fases requereixen la presència d'auxines i CKs en el medi. En la tercera fase, la de determinació o diferenciació morfològica, els teixits poden continuar el seu desenvolupament independentment de reguladors del creixement exògens (Thorpe 1994, Sugiyama 1999). Aquestes tres fases són el resultat de canvis en l'expressió gènica que, alhora, estan regulats per senyals endògens i ambientals (Sugiyama 1999). En *Arabidopsis thaliana*,

planta model per estudis genètics, s'estan estudiant quins gens estan implicats en cadascuna de les fases de l'organogènesi (Ozawa et al. 1998, Zhao et al. 2002).

L'organogènesi adventícia *in vitro* és una eina valuosa per l'estudi de la regulació hormonal d'aquest procés morfogènic, i la seva interacció amb altres senyals. No obstant, està en discussió si el desenvolupament de les tiges en cultiu *in vitro* és totalment comparable al de la planta sencera, ja que en els cultius els nivells hormonals s'han optimitzat per obtenir els efectes desitjats i no necessàriament per mimetitzar situacions fisiològiques (Howell et al. 2003). En el cultiu de teixits, els reguladors del creixement, auxines i CKs, del medi tenen un papar bàsic en la manipulació de l'organogènesi adventícia (Skoog i Miller 1957) i ho fan en concert amb altres factors (Thorpe 1994), entre els quals s'inclouen les pròpies hormones endògenes de l'explant. De fet, l'estímul dels reguladors del creixement exògens pot provocar, en alguns casos, canvis en la concentració d'hormones endògenes, per tant és important conèixer si els reguladors del creixement actuen per si mateixos o bé alterant la biosíntesi o el catabolisme de les hormones endògenes. Es troben diversos articles on es quantifica la concentració d'auxines i CKs endògenes durant l'organogènesi en explants d'*Actinidia deliciosa* i *Pinus pinea* i l'embriogènesi en *Corylus avellana*, induïdes amb la presència de la CK BA (Feito et al. 1995, Centeno et al. 1997, Valdés et al. 2001, Centeno et al. 2003). En canvi, hi ha molt pocs estudis de com estan afectades les hormones endògenes en casos d'inducció de morfogènesi adventícia amb TDZ, substància amb efecte de CK (Mok et al. 1982, Ricci et al. 2001). Durant el procés de formació de cal·lus (Capelle et al. 1983) i en processos embriogènics, s'ha trobat que el TDZ actua incrementant els nivells d'algunes CKs endògenes (Murthy et al. 1995, Hutchinson et al. 1996, Hutchinson i Saxena 1996, Victor et al. 1999). L'efecte de CK del TDZ podria ser a través de la inhibició de l'activitat CK oxidasa, tal com s'ha descrit en cal·lus de *Glycine max* (Hare i van Staden 1994). El TDZ també pot mimetitzar l'efecte de les auxines en la inducció de l'embriogènesi i, en alguns casos, pot incrementar-ne la seva concentració (Murthy et al. 1998). Fins el moment, no s'ha avaluat l'efecte del TDZ en el contingut d'auxines i CKs endògenes en un procés d'organogènesi adventícia.

La implicació de les auxines i CKs endògenes en l'organogènesi es tractarà a l'apartat 3.4. Abans, però, exposem breument les característiques generals de cadascun d'aquests grups d'hormones i com interaccionen entre si.

3.1. Les auxines

Les auxines són compostos hormonals amb diferent estructura química que promouen l'allargament cel·lular. A més, també estimulen altres processos com l'activitat mitòtica del càmbium, la biosíntesi d'etilè, la diferenciació del xilema, la formació de rels tant en cultiu de teixits com *in vivo*, el desenvolupament de fruits i també promouen la dominància apical (Cleland 1999, Crozier et al. 2000, Leyser 2003).

La principal auxina en la majoria de plantes és l'àcid indol-3-acètic (IAA). Se sintetitza principalment en zones on hi ha divisió cel·lular, com en meristemes apicals, primordis foliars, fulles joves, llavors en desenvolupament i fruits en creixement, on s'hi troba en quantitats elevades (Cleland 1999). Observacions recents indiquen que també se sintetitzen a les arrels (Ljung et al. 2001).

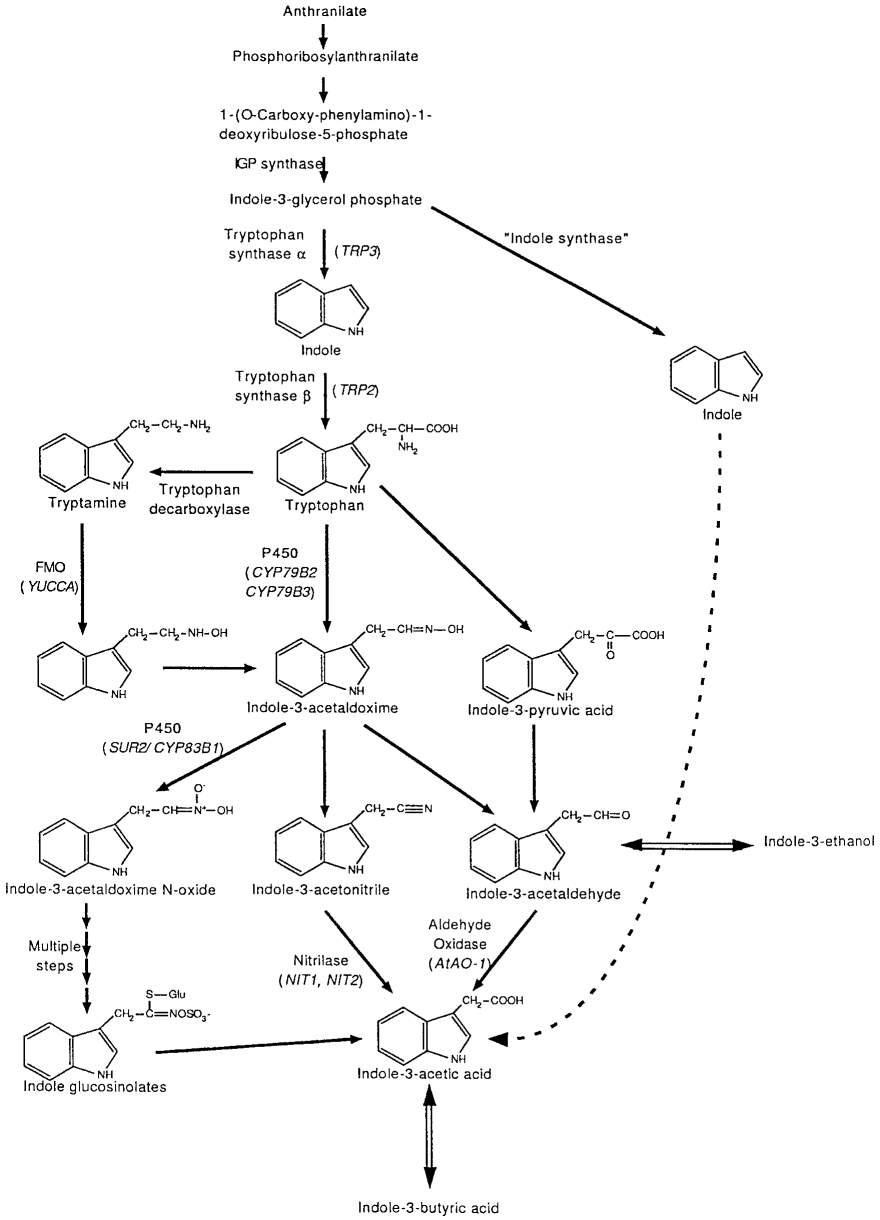

Fig. 5. Rutes biosintètiques de l'àcid indol-3-acètic (IAA). L'IAA se sintetitza a partir del triptòfan a través de tres vies, la de l'àcid indol-3-pirúvic, la de l'indol-3-acetaldoxima i la de la triptamina, i també a partir de l'indol (línia discontínua). Els noms dels gens d'*Arabidopsis* implicats estan indicats en cursiva. La línia discontínua indica la via independent del triptòfan, a partir de l'indol. Les dobles fletxes representen vies que també poden contribuir a la síntesi d'IAA. IGP, indol-3-glicerol; FMO, flavin monooxigenasa; P450, citocrom P450 (Ljung et al. 2002).

La biosíntesi de l'IAA (Fig. 5) té lloc a partir de diferents precursors, el triptòfan i probablement l'indol. La biosíntesi dependent del triptòfan es dóna a través de diverses vies: la de l'àcid indol-3-pirúvic, la de l'indol-3-acetaldoxima i la de la triptamina, tot sent la primera la més comú. La via de l'indol-3-acetaldoxima és important en algunes famílies com les brassicàcies, les gramínies i les musàcies, i ha estat caracteritzada en *Arabidopsis* (Bandurski et al. 1995, Crozier et al. 2000, Bartel et al. 2001, Ljung et al. 2002, Zazímalová i Napier 2003). La importància de la ruta dependent del triptòfan davant de la independent varia al llarg del cicle vital i també en resposta a senyals ambientals. S'ha suggerit que les plantes utilitzen la ruta independent del triptòfan per mantenir

nivells basals d'IAA i estimulen les vies dependents del triptòfan en situacions d'estrés i durant l'embiogènesi (Ljung et al. 2002).

L'IAA s'inactiva de manera irreversible per descarboxilació de la cadena lateral o bé per oxidació de l'anell indòlic. Un altre tipus d'inactivació és la conjugació de la cadena lateral amb aminoàcids com l'aspàrtic, a la qual en algunes espècies com *Vicia faba*, *Lycopersicon esculentum* i *Daucus carota* s'hi afegeix l'oxidació de l'indol. La conjugació de l'anell indòlic amb glucoses també desactiva l'IAA de manera irreversible. En canvi, la conjugació de la cadena lateral amb glucosa tot formant O-glucòsids d'IAA és un procés reversible. Per tant, la hidròlisi d'aquests conjugats pot donar lloc a l'alliberament d'IAA actiu (Bandurski et al. 1995, Crozier et al. 2000, Bartel et al. 2001, Ljung et al. 2002, Zazímalová i Napier 2003).

L'IAA es transporta polarment i també de manera no polar a través del floema (Zazímalová i Napier 2003). El transport polar, que és actiu, direccional i extravascular, genera gradients apical-basals i radials d'IAA, amb nombroses implicacions en el creixement i desenvolupament de les plantes i en les respostes a estímuls gravitròpics i fototròpics. Així, l'IAA actua com a senyal posicional que regula la inciació i posició dels primordis foliars (fil·lotaxi) en el meristema apical, la diferenciació dels teixits vasculars i l'establiment de la polaritat apical-basal i de la simetria bilateral de l'embrió (Vogler i Kuhlemeier 2003).

3.2. Les citocinines

Les CKs també regulen molts processos al llarg del cicle vital de la planta. Estimulen la divisió cel·lular, provoquen expansió foliar, promouen el desenvolupament dels cloroplasts a partir d'etioplasts, endarrereixen la senescència de les fulles i inhibeixen la formació de rels i la formació del xilema, entre d'altres efectes (Cleland 1999, Crozier et al. 2000).

Les CKs naturals són derivats de l'adenina amb una cadena lateral en la posició N^6, de tipus isoprenoide, encara que també pot ser un compost aromàtic (Mok i Mok 2001). De CKs de tipus isoprenoide trobem la N^6-(÷^{2}-isopentenil)adenina o N^6-isopenteniladenina (iP), la zeatina (Z) i la dihidrozeatina (DHZ), mentre que la BA és una CK aromàtica. La Z sembla que és la CK més comú en les plantes (Davies 1995), encara que en algunes lleguminoses predominen les CKs tipus DHZ (Crozier et al. 2000). Les CKs actives es considera que són les bases lliures, iP, Z i DHZ, i probablement els ribòsids respectius, iPR, ZR i DHZR, amb una ribosa en la posició N^9 (McGaw i Burch 1995, Crozier et al. 2000). Es discuteix si els ribòsids 5'-monofosfat (o ribòtids) tenen activitat de CKs per si mateixos o després de la seva ràpida interconversió en ribòsids i bases lliures de CK (Crozier et al. 2000).

Les CKs es troben en les plantes en forma de molècules lliures o com a components de tRNAs. Són abundants en els àpexs de les rels i de les tiges i en embrions en desenvolupament. Se sintetitzen principalment als àpex de les rels, i el seu transport és bàsicament xilemàtic de les rels cap a la tija. Però també són sintetitzades per les tiges, com ara en el meristema apical, en el càmbium i en llavors en desenvolupament, i poden ser transportades pel floema (Davies 1995, Cleland 1999, Nordström et al. 2004).

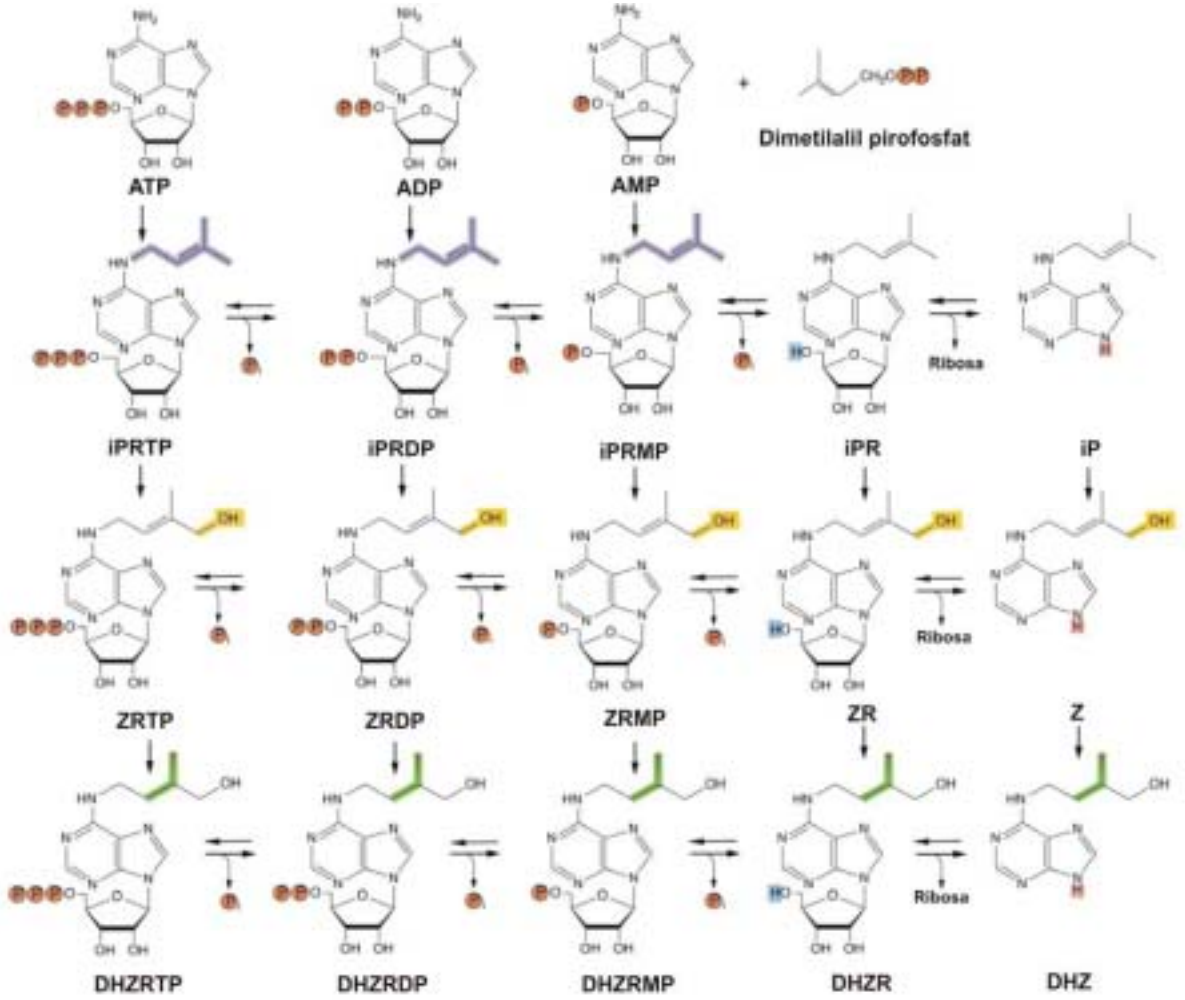

Fig. 6. Ruta biosintètica de les principals CK naturals. iPRTP, N^6-isopenteniladenosina 5'-trifosfat; iPRDP, N^6-isopenteniladenosina 5'-difosfat; iPRMP, N^6-isopenteniladenosina 5'-monofosfat; iPR, N^6-isopenteniladenosina; iP, N^6-isopenteniladenina; ZRTP, ribòsid 5'-trifosfat de la zeatina; ZRDP, ribòsid 5'-difosfat de la zeatina; ZRMP, ribòsid 5'-monofosfat de la zeatina; ZR, ribòsid de la zeatina; Z, zeatina; DHZRTP, ribòsid 5'-trifosfat de la dihidrozeatina; DHZRDP, ribòsid 5'-difosfat de la dihidrozeatina; DHZRMP, ribòsid 5'-monofosfat de la dihidrozeatina; DHZR, ribòsid de la dihidrozeatina; DHZ, dihidrozeatina.

La biosíntesi de CKs tipus isoprenoide es produeix a partir de modificacions bioquímiques de l'adenina. En *Agrobacterium tumefaciens*, s'inicia amb la síntesi del ribòsid 5'-monofosfat de la iP (iPRMP), mitjançant la unió d'adenosina 5'-monofosfat (AMP) amb el dimetilalil pirofosfat (o isopentenil pirofosfat). Aquesta reacció està catalitzada per l'enzim IPT (dimetilalil pirofosfat : 5'-AMP-isopentenil transferasa o, més abreujadament, isopentenil transferasa) codificat pel gen *ipt* (Zazímalová et al. 1999, Crozier et al. 2000). Recentment, en el genoma d'*Arabidopsis* s'han descobert nou gens *IPT* (Kakimoto 2001, Takei et al. 2001), cadascun dels quals presenta una expressió específica en determinats teixits o parts d'òrgans (Miyawaki et al. 2004). Els IPT d'*Arabidopsis* tenen més afinitat per l'ADP o l'ATP que per l'AMP. Així en les plantes, la primera reacció de la síntesi de CKs formada en plantes és la formació dels ribòsids 5'-difosfat o 5'-trifosfat de la iP (iPRDP o iPRTP), a partir dels quals s'originen les CKs de tipus iP, Z i DHZ (Fig. 6). En la ruta de biosíntesi de les CKs la iP dóna lloc a la Z amb la hidroxilació de la

cadena lateral, catalitzada per una *trans*-hidroxilasa. La Z es transforma en DHZ mitjançant la reducció d'aquesta cadena, amb l'acció de la zeatina reductasa. De la mateixa manera, l'iPR i els seus ribòsids 5'-fosfat poden convertir-se en ZR i els seus ribòsids 5'-fosfat, i aquests, tot i no ser substrats de la zeatina reductasa, possiblement puguin convertir-se en DHZR i els seus ribòsids 5'-fosfat. A la vegada, els tres tipus de CKs en forma de bases lliures, els ribòsids i els ribòsids 5'-fosfat s'interconverteixen entre si fàcilment, mitjançant reaccions catalitzades pels mateixos enzims del metabolisme de l'adenina (McGaw i Burch 1995, Chen 1997, Zazímalová et al. 1999, Crozier et al. 2000, Mok i Mok 2001). A més d'aquesta via dependent de l'iPRMP, hi ha estudis en *Arabidopsis* que demostren que hi ha una ruta biosintètica alternativa, independent de l'iPRMP, en la que es formaria directament el ribòsid 5'-monofosfat de la Z (ZRMP) a partir d'AMP i un compost encara no identificat (Astot et al. 2000).

Hi ha dues vies d'inactivació de les CKs tipus isoprenoide. Una seria la seva oxidació catalitzada per CK oxidases, que hidrolitzen la molècula en l'adenina i la cadena lateral, i que tenen com a substrat CKs amb la cadena lateral isoprenoide insaturada (iP, iPR, Z i ZR). Les CKs tipus DHZ, doncs, no són substrat de la CK oxidasa. L'altra via d'inactivació seria la seva conjugació amb alanina en la posició N^9 de l'adenina de la CK, o amb glucosa en les posicions N^3, N^7 i N^9 tot formant N-glucòsids. Si la unió de la glucosa és en el grup hidroxil de la cadena lateral de les CKs Z, ZR, DHZ i DHZR es formen els O-glucòsids, que es consideren biològicament actius, ja sigui per si mateixos o perquè són substrat de η-glucosidades, les quals poden catalitzar la hidròlisi d'aquest tipus de glucòsid, tot alliberant la CK (McGaw i Burch 1995, Zazímalová et al. 1999, Crozier et al. 2000, Mok i Mok 2001).

3.3. Interacció entre auxines i citocinines

Les auxines i les CKs actuen per si mateixes i interaccionant entre si regulant el creixement i el desenvolupament vegetal. Les interaccions poden ser sinèrgiques, antagòniques o additives, mostrant la complexitat d'acció d'aquestes hormones (Coenen i Lomax 1997). En molts casos, els seus efectes són el resultat d'una modificació del balanç entre ambdues hormones. En la planta sencera, el balanç entre CKs i auxines controla la diferenciació del xilema (Aloni 1995) i la dominància apical (Cleland 1999). De la mateixa manera la relació entre CKs i auxines del medi regula l'organogènesi *in vitro* (Skoog i Miller 1957, Cleland 1999).

Les auxines i les CKs també poden controlar el desenvolupament de la planta tot regulant-se les unes a les altres (Swarup et al. 2002). Un increment d'auxines, ja sigui per aplicació exògena (Zhang et al. 1995) o per sobreproducció degut a la transformació amb els gens *iaaM* i *iaaH* (biosíntesi d'auxines) d'*A. tumefaciens* (Eklöf et al. 2000), produeix una disminució de les CKs actives en *N. tabacum*. S'ha suggerit que les auxines podrien actuar estimulant l'activitat CK oxidasa, induint la conjugació de les CKs (Coenen i Lomax 1997, Swarup et al. 2002), o bé inhibint la síntesi de CKs per la via independent a l'iPRMP (Nordström et al. 2004). En canvi, un increment de CKs pot produir tant un augment com una disminució en els nivells d'IAA. D'una banda, l'aplicació exògena de CKs en *Zea mays* i *P. vulgaris* i la transformació amb l'*ipt* (biosíntesi de CKs) d'*A. tumefaciens* en *Nicotiana glutinosa* ha provocat un increment d'IAA lliure, i s'ha proposat que les CKs inhibien els enzims que conjuguen l'IAA amb l'aspartat (Coenen i Lomax 1997). D'altra banda, estudis recents amb plantes de *N. tabacum* i *Arabidopsis*

transformades amb l'*ipt* mostren una disminució en els nivells d'IAA, respecte les plantes no transformades (Eklöf et al. 2000, Nordström et al. 2004).

3.4. Implicació de les auxines i les citocinines en el cicle cel·lular i l'organogènesi

L'organogènesi adventícia en cultiu *in vitro* és el resultat de l'adquisició de competència de les cèl·lules per poder proliferar, la reactivació del cicle cel·lular i la posterior diferenciació de teixits i formació d'òrgans. És per això que, del gran ventall de processos controlats per auxines i CKs, ens centrarem en la regulació del cicle cel·lular i l'organogènesi de tiges i rels.

3.3.1. Regulació del cicle cel·lular

La recerca de factors que estimulessin les cèl·lules a dividir-se portà al descobriment de les CKs (Miller et al. 1955). Tot i que són les CKs les que típicament s'han considerat com a inductores i necessàries per la divisió cel·lular (Schmülling 2001), ja que les cèl·lules d'un cultiu de teixits en general no es divideixen sense l'aplicació de CKs exògenes, les auxines també són necessàries per la divisió cel·lular, però moltes vegades en els cultius les concentracions endògenes són suficients. Així, en cultius *in vitro*, la divisió cel·lular és estimulada per les CKs en combinació amb les auxines (Davies 1995, Cleland 1999).

Estudis d'immunolocalització de CKs a nivell tissular en planta sencera han mostrat que es troben en zones amb activitat mitòtica. Alguns d'aquests estudis, demostren un acúmul de CKs en el meristema apical de les tiges en diverses espècies (Sossountzov et al. 1988, Dewitte et al. 1999, Jacqmard et al. 2002). En cultiu *in vitro*, s'ha publicat la localització de CKs en zones de divisió cel·lular durant el procés d'embriogènesi somàtica (Ivanova et al. 1994, Kärkönen i Simola 1999). Fins el moment, però, no hi ha estudis de localització de CKs en un procés d'organogènesi adventícia.

El cicle cel·lular de les plantes, com en altres eucariotes, consta de quatre fases i la seva progressió està controlada en les transicions G_1-S i G_2-M (Francis i Sorrell 2001, Kakimoto 2003, Dewitte i Murray 2003). Anàlisis quantitatives i aplicacions exògenes han demostrat que les CKs i les auxines són necessàries pel reinici (desdiferenciació) i la regulació del cicle cel·lular. D'una banda, els protoplasts de *Petunia hybrida* requereixen l'auxina àcid 2,4-diclorofenoxiacètic i la CK BA per reiniciar el cicle cel·lular (Tréhin et al. 1998). I els protoplasts de *N. tabacum* requereixen CKs per l'inici de la fase S (Jacqmard et al. 1994). D'altra banda, en explants d'aquesta espècie en procés de desdiferenciació, l'IAA hi ha incrementat de manera transitòria coincidint amb l'inici de la mitosi (Boucheron et al. 2002). I en cultius de cèl·lules BY-2 de *N. tabacum* amb divisió sincronitzada, les CKs tipus Z han incrementat a la fase S i a la fase M (Redig et al. 1996, Laureys et al. 1998, Laureys et al. 1999, Dobrev et al. 2002), però s'ha trobat que els nivells endògens d'IAA no han variat al llarg del cicle cel·lular (Redig et al. 1996).

La regulació del cicle cel·lular és molt complexa i s'ha conservat al llarg de l'evolució. Els principals conductors del cicle cel·lular són les cinases dependents de ciclina (CDKs), les quals conjuntament amb les seves subunitats reguladores, les ciclines (CYC), i sent sotmeses a processos de fosforilació-desfosforilació regulen el

cicle cel·lular eucariòtic. A la figura 7 hi trobem la representació d'un model força acceptat de la regulació hormonal de les transicions G_1-S i G_2-M en cèl·lules vegetals (Stals i Inzé 2001). Les CKs i les auxines regulen les transicions G_1-S i G_2-M tot controlant l'activitat CDK. Durant la fase G_1 aquestes hormones promouen l'expressió de les ciclines tipus D (CYCD) i d'una CDKA, complex que activa el pas G_1-S (Stals i Inzé 2001, Boniotti i Griffith 2002). En concret, les CKs incrementen l'expressió de la CYCD3, una ciclina tipus D localitzada en zones de divisió cel·lular com ara el meristema apical o primordis foliars (Frank i Schmülling 1999, Kakimoto 2003, Dewitte i Murray 2003). Pel pas G_2-M, les auxines i les CKs activen la transcripció de CDKs i de ciclines tipus A i B (Stals i Inzé 2001, Boniotti i Griffith 2002). Les auxines regulen positivament l'expressió de la CDK més abundant que controla pas de la fase G_2 a la M, la CDC2 (cicle de divisió cel·lular 2), i les CKs activen la CDC25, la qual activa la cinasa CDC2 tot desfosforilant-la (D'Agostino i Kieber 1999, Frank i Schmülling 1999, Landrieu et al. 2004).

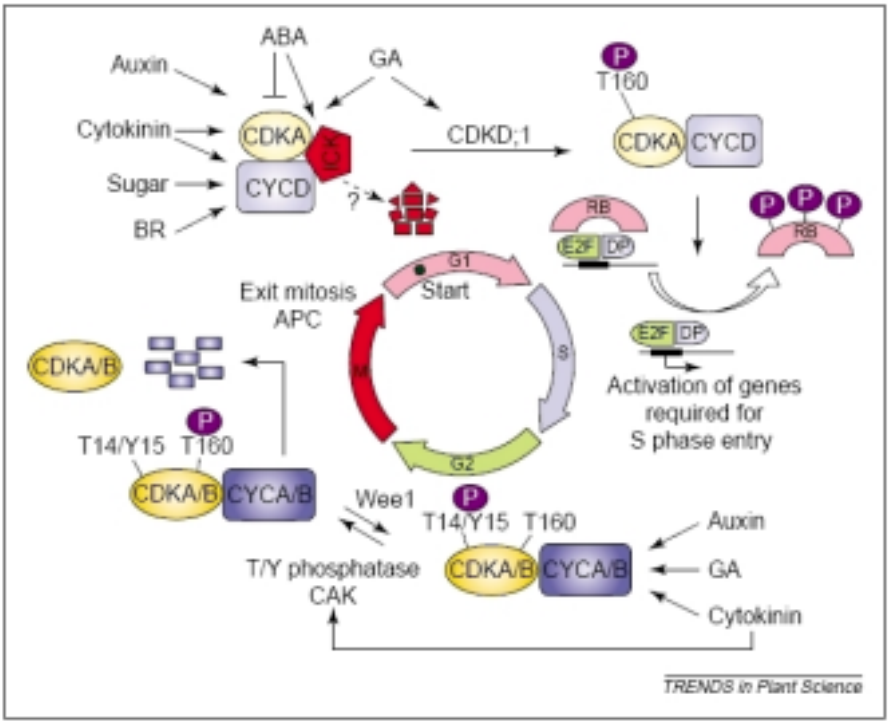

Fig. 7. Model per les transicions G_1-S i G_2-M en cèl·lules vegetals (Stals i Inzé 2001). Per la transició G_1-S es promou l'expressió de ciclines tipus D (CYCD) i la seva subunitat catalítica, una cinasa A dependent de ciclina (CDKA), per part d'auxines, CKs, brassionosteroids (BR) i sacarosa. Aquest complex s'activa amb la dissociació del factor inhibidor ICK, induït per l'àcid abscísic (ABA), i la fosforilació de la CDKA mitjançant una altra cinasa activada per les gibberel·lines (GA). Al final de la fase G_1 aquest complex CDKA-CYCD hiperfosforila la proteïna retinoblastoma (RB), la qual allibera el complex E2F-DP. Els factors E2F-DP lliures promouen la transcripció de gens necessària per l'entrada a la fase S. Pel pas de la fase G_2 a la M, les auxines i les CKs, juntament amb les gibberel·lines, activen la transcripció de CDKs (CDKA i CDKB) i de ciclines (CYCA i CYCB) tipus A i tipus B. Els complexes CDK-CYC s'activen amb una desfosforilació i l'acció d'una cinasa activadora de CDKs (CAK). Finalment un complex promotor de l'anafase (APC) promou la proteolisi de les ciclines tipus B i s'acaba el procés de la mitosi. Estudis recents indiquen que l'àcid jasmònic pot aturar el cicle cel·lular en les fases G_1 i G_2 (Swiatek et al. 2002).

Estudis en suspensions cel·lulars, protoplasts, explants i durant el desenvolupament vegetal han indicat que la competència de les cèl·lules a desdiferenciar-se i reiniciar la mitosi està associada a la capacitat d'expressar la

CDC2 i ciclines tipus B, juntament amb la participació d'altres factors (Hemerly et al. 1993, Tréhin et al. 1998, Jelenska et al. 2000, Boucheron et al. 2002). Un cop les cèl·lules han esdevingut competents amb la transcripció del *CDC2*, l'activació de la CDC25 induïda per les CKs permet l'inici de la mitosi.

3.3.2. Regulació de la formació de tiges i rels

Els experiments en cultiu de teixits de Skoog i Miller (1957) ja demostraren que el balanç entre CKs i auxines exògenes regulava la formació d'òrgans en el cultiu *in vitro* de cal·lus de *N. tabacum*. Des de llavors, en cultius *in vitro*, s'han induït tiges i rels adventícies de manera habitual amb l'addició de CKs i auxines.

La major part d'estudis d'organogènesi s'han fet amb aplicacions de reguladors del creixement i amb l'obtenció plantes mutants o transgèniques amb gens com ara l'*ipt* o els *iaa* d'*A. tumefaciens*. Per estudiar la relació de les CKs amb el desenvolupament de tiges *in vitro*, no només s'ha estudiat amb transgènics amb l'*ipt* d'*A. tumefaciens* (Zhang et al. 1995), sinó també amb sobreexpressors d'alguns dels propis gens *IPT* de les plantes, en *Arabidopsis* (Sun et al. 2003), o amb mutants com ara l'*hoc* (*high shoot-organogenic capacity*) de la mateixa espècie (Catterou et al. 2002). Explants d'aquestes plantes transgèniques i mutants mostren una capacitat de generar tiges adventícies en abscència de CKs en el medi de cultiu i mostren un fenotip típic de sobreexpressió de CKs. L'estudi de mutants del receptor de CKs CRE1, en canvi, mostra que els manca la capacitat de formació de tiges en cultiu (Inoue et al. 2001). D'altra banda, l'estudi dels diversos mutants *arf* (*aberrant lateral root formation*) d'*Arabidopsis* ha permès conèixer la implicació de les auxines i alguns gens en la formació de rels adventícies i de rels laterals (Celenza et al. 1995). Es té poc coneixement de la manera com les CKs i les auxines promouen la formació de tiges i rels, tot i que un dels mecanismes de les CKs per activar el procés de formació de tiges en cultiu *in vitro* seria mitjançant l'activació de determinats gens (Howell et al. 2003).

De manera anàloga al cultiu *in vitro*, la regulació de l'organogènesi en la planta sencera també s'ha estudiat amb aplicacions exògenes i amb l'obtenció plantes mutants o transgèniques amb nivells alts o baixos d'hormones. En plantes de diverses espècies transformades amb l'*ipt* s'hi ha trobat un acúmul de CKs, principalment del tipus Z (Zazímalová et al. 1999). Plantes de *N. tabacum* amb aquest gen tenen fulles menudes amb la senescència endarrerida, molta ramificació lateral i perden la dominància apical (Schmülling et al. 1989). La sobreexpressió d'un dels gens *IPT* (*AtIPT8*) d'*Arabidopsis*, que contràriament a l'*ipt* d'*A. tumefaciens* produeix alts nivells de CKs tipus iP, genera plàntules amb arrels curtes, hipocòtils allargats i cotilèdons verd foscos (Sun et al. 2003). En canvi, plantes de *N. tabacum* i *Arabidopsis* transgèniques amb la CK oxidasa, amb baixos nivells de CKs, tenen el meristema apical reduït i la part aèria amb el creixement endarrerit, mentre que el sistema radicular està molt desenvolupat (Werner et al. 2001, 2003). D'altra banda, en relació a les auxines, la transformació de plantes de *N. ta*bacum amb els gens *iaa*, amb la consegüent sobreproducció d'auxines endògenes, provoca enanisme, fulles petites, la formació de rels adventícies i l'increment de la dominància apical (Sitbon et al. 1992).

Així, la regulació hormonal de l'organogènesi en la planta sencera confirma la hipòtesi de Skoog i Miller (1957) pels cultius *in vitro*. Un increment de CKs, i per tant un increment en el balanç CKs/auxines, promou el creixement de les gemmes laterals tot reduint la dominància apical, mentre que un increment en les auxines promou

la dominància apical i la formació i desenvolupament de les rels (Cleland 1999). Tanmateix, estudis recents en plantes de *N. tabacum* expressores dels gens *ipt* i *iaaM* i *iaaH* mostren que per algunes respostes, a part de la relació CKs/auxines, també són importants els seus nivells absoluts (Eklöf et al. 2000).

4. Clavells transformats amb el gen *rolC* d'*Agrobacterium rhizogenes*

Els oncogens d'*A. tumefaciens* o d'*A. rhizogenes*, introduïts de manera natural en les plantes que infecten, són capaços d'intervenir en el metabolisme de les hormones endògenes de la planta o bé afectar la sensibilitat del teixits a les hormones, per això poden ser molt útils pel millorament de certs trets agronòmics o fins i tot per produir noves morfologies en la planta.

4.1. Els oncogens d'*A. tumefaciens* i *A. rhizogenes*

Tant l'*A. tumefaciens* com l'*A. rhizogenes* (família de les rizobiàcies) són bacteris edàfics patògens que produeixen efectes morfogènics en plantes dicotiledònies. L'*A. tumefaciens* indueix tumors teratogènics o indiferenciats al coll de la tija (corona) de les plantes produint la malaltia del tumor o de l'agalla de corona (*crown gall*). L'*A. rhizogenes* indueix la proliferació de rels en la zona d'infecció produint la malaltia de les rels velloses (*hairy root*), tot i que en alguns casos pot induir la formació de tumors sense el desenvolupament de rels (Gaudin et al. 1994, Binns i Costantino 1998, Meyer et al. 2000).

Els gens d'*Agrobacterium* implicats en la tumorogènesi i en la rizogènesi de les plantes infectades s'anomenen oncogens (Binns i Costantino 1998). Estan ubicats en el plasmidi Ti (inductor de tumors) en l'*A. tumefaciens* i en el plasmidi Ri (inductor de rels) en l'*A. rhizogenes* (Fig. 8A), concretament en el T-DNA (DNA transferit a les cèl·lules hostes de la planta). Els oncogens d'*A. tumefaciens* estan implicats en la biosíntesi d'auxines (*iaaM* i *iaaH*) o de CKs (*ipt*), tot incrementant els seus nivells en les plantes infectades, mentre que els d'*A. rhizogenes* (*aux1*, *aux2* i gens *rol*), molt menys estudiats, poden estar implicats bé en el metabolisme d'auxines, CKs o d'altres hormones com les gibberel·lines, o bé en la resposta de les cèl·lules de la planta a aquestes hormones (Binns i Costantino 1998, Meyer et al. 2000). En el T-DNA també hi ha els gens relacionats amb la síntesi d'opines (Fig. 8A), derivats d'aminoàcids conjugats amb sucres fosfodièsters, les quals són usades per les soques patògenes com a substàncies específiques pel seu creixement. Les opines s'utilitzen per classificar les soques d'*A. rhizogenes*: agropina, manopina, cucumopina i mikimopina (Trovato i Linhares 1999, Meyer et al. 2000).

4.2. El fenotip rels velloses (*hairy root*) causat per *A. rhizogenes*

En moltes espècies es poden regenerar plantes de les rels velloses produïdes per la infecció d'*A. rhizogenes*, cultivades *in vitro*. Aquestes plantes regenerades tenen un fenotip característic: la dominància apical es redueix, tant en la tija com en les arrels, els entrenusos són més curts, les fulles són més petites i arrugades, es produeixen rels

adventícies i tenen floració tardana i fertilitat reduïda (Tepfer 1984). Per analogia al nom de la malaltia aquests símptomes se'ls anomena fenotip de les rels velloses. Els principals responsables d'aquest fenotip són els gens *rol* (*root loci* o locus de les rels), anomenats *rolA*, *rolB*, *rolC* i *rolD*, que es troben ubicats en el T_L-DNA (regió esquerra del T-DNA) del plasmidi Ri d'*A. rhizogenes* (White et al. 1985, Slightom et al. 1986) i que també són essencials per l'establiment de la malaltia de les rels velloses. Els gens *aux*, ubicats en el T_R-DNA (regió dreta del T-DNA) del plasmidi Ri, no tenen efecte en la inducció de rels velloses (Trovato i Linhares 1999, Meyer et al. 2000) (Fig. 8B).

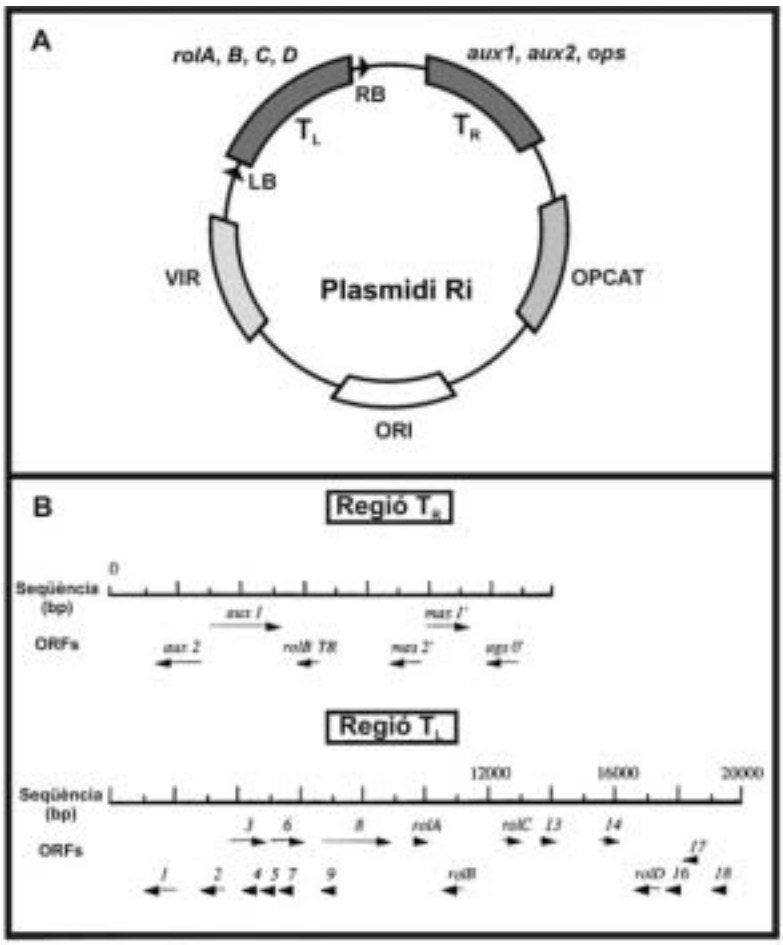

Fig. 8. (A) Plasmidi Ri de les soques tipus agropina d'*Agrobacterium rhizogenes* (adaptat de van der Salm et al. 1996) i (B) organització funcional del seu T-DNA (adaptat de Chriqui et al. 1996). T_L, regió esquerra del T-DNA, que conté els gens *rolA*, *B*, *C* i *D*; T_R, regió dreta del T-DNA, amb els gens de la síntesi d'auxines (*aux1* and *aux2*) i de la síntesi d'opines (*ops*); RB, extrem dret del T_L-DNA; LB, extrem esquerre del T_L-DNA; VIR, regió amb els gens de virulència; ORI, origen de replicació; OPCAT, gens del catabolisme de les opines.

4.3. Plantes ornamentals amb flor transgèniques amb els gens *rol*

L'increment de la importància econòmica de les plantes ornamentals amb flor arreu de món mostra un bon futur per la hibridació d'aquest tipus de plantes (Horn 2002). La transformació d'ornamentals amb l'*A. rhizogenes* o amb els seus gens *rol* tindria, en principi, una vessant aplicada, ja que aquests gens han resultat ser útils per millorar certes característiques agronòmiques i també per produir noves formes en les plantes transformades, tant en les parts

aèries com subterrànies, i fins i tot n'han alterat el procés de floració (Christey 2001). L'estudi d'aquestes plantes, però, també pot ajudar a conèixer millor la capacitat dels oncogens d'*A. rhizogenes* d'afectar processos de diferenciació.

Fins al moment s'han transformat algunes espècies vegetals amb els gens *rolA*, *rolB*, *rolC* i *rolD*, sols o combinats, i amb *A. rhizogenes*, però la informació està força dispersa. Christey (1997) feu un recull de totes les espècies transformades amb *A. rhizogenes* fins aquell moment, que n'eren 53. En el recull del 2001 el nombre d'espècies s'amplià fins a 79, i també hi incorporà algunes de les espècies transformades amb els gens *rol* (Christey 2001). Cal dir que entre aquestes plantes transformades, les ornamentals n'eren una minoria. En canvi, les transformacions més recents, majoritàriament amb *A. rhizogenes*, amb la combinació *rolABC* o amb el gen *rolC* sol, moltes són en plantes ornamentals, ja que el fenotip obtingut pot ser interessant tant agronòmicament com comercial. Així, manca un recull de totes les plantes ornamentals tranformades tant amb *A. rhizogenes* com amb els gens *rol*, per tal de posar de manifest canvis fenotípic comuns entre línies transgèniques de diferents espècies. Això podrà ser útil per trobar quins gens o quina combinació gènica són adequats per obtenir trets determinats interessants per altres espècies que es vulguin manipular genèticament.

4.3.1. Fenotips de plantes transgèniques amb el gen *rolC*

Els gens *rol*, sols o combinats entre si, s'han usat per transformar plantes de diferents espècies vegetals, i de les quals en modifiquen el creixement i el desenvolupament. D'entre els gens *rol*, el gen *rolC* és el més usat pels investigadors, possiblement perquè és el que menys malformacions provoca en la planta transgènica.

Els primers estudis de les alteracions fenotípiques causades pel *rolC* foren en *N. tabacum* (Schmülling et al. 1988, Nilsson et al. 1993, Scorza et al. 1994). Aquestes plantes transgèniques tenen la dominància apical reduïda, són plantes nanitzades, amb la morfologia foliar alterada, la floració avançada i la viabilitat del pol·len reduïda o, a vegades, presenten esterilitat masculina. Alteracions semblants també han estat descrites en plantes d'altres espècies transformades amb *rolC*, com *Solanum tuberosum* (Fladung 1990, Schmülling et al. 1993), *Atropa belladonna* (Kurioka et al. 1992), *Populus tremula* x *Populus tremuloides* (Nilsson et al. 1996, Fladung et al. 1997) i *Pyrus communis* (Bell et al. 1999). El clavell ha estat una de les primeres plantes ornamentals transformades amb el gen *rolC* (Ovadis et al. 1999, Zuker et al. 2001), però també s'han transformat plantes d'altres espècies ornamentals com *Osteospermum ecklonis* (Giovannini et al. 1999), *Petunia* (Winefield et al. 1999), *Chrysanthemum morifolium* (Mitiouchkina i Dolgov 2000) i *Pelargonium* x *domesticum* (Boase et al. 2004).

4.4. Plantes de clavells transgèniques amb el gen *rolC*

Els clavells del cultivar White Sim amb el gen *rolC* utilitzats en els nostres estudis foren obtinguts al laboratori del Dr. Vainstein (Departament d'Horticultura, Facultat d'Agricultura, Universitat Hebrea de Jerusalem, Israel) l'any 1997 i, posterioment, foren caracteritzats fenotípicament (Zuker et al. 2001). Tant la construcció del plasmidi binari pAM*rolC*

(Fig. 9) per transformar aquests clavells com el mètode de transformació estan descrits per Zuker et al. (1999, 2001).

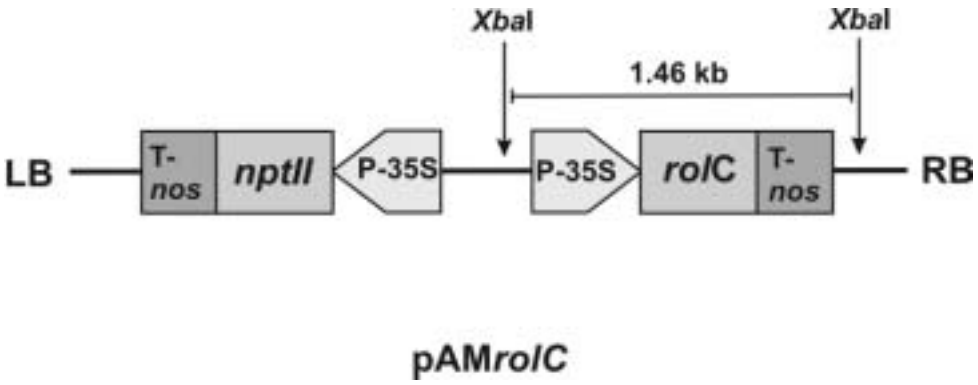

Fig. 9. El T-DNA del plasmidi binari pAM*rolC* amb el gen *rolC* d'*Agrobacterium rhizogenes*. RB, extrem dret del T-DNA; LB, extrem esquerre del T-DNA; *nptII*, gen de la neomicina fosfotransferasa II; P-35S, promotor de l'RNA 35S del virus del mosaic de la coliflor; T-*nos*, finalitzador del gen de la nopalina sintasa.

4.4.1. Fenotip dels clavells transgènics amb *rolC*

En clavell, l'expressió del *rolC* amb el promotor constitutiu 35S provoca una reducció en la dominància apical, amb el conseqüent augment de la ramificació i, per tant, del nombre potencial d'esqueixos. A diferència de plantes d'altres espècies transformades amb aquest gen, les plantes de clavell només tenen un lleuger enanisme que és pràcticament imperceptible, la morfologia foliar no està alterada i el nombre de tiges florals incrementa fins a tres vegades. A més, el *rolC* provoca un increment en l'arrelament dels esqueixos (Ovadis et al. 1999, Zuker et al. 2001). Pel que fa a la reducció de la fertilitat que sol provocar el *rolC*, com que el cultivar transformat, el White Sim, ja té esterilitat masculina, totes les alteracions morfològiques obtingudes són avantatjoses.

4.5. Nivells hormonals en plantes transgèniques amb els gens *rol*

Contràriament als oncogens del T-DNA del plasmidi Ti, els efectes dels quals en la diferenciació poden ser explicats per un desequilibri hormonal causat per la síntesi d'auxines i CKs, els oncogens del T-DNA del plasmidi Ri exerceixen el seu efecte a través d'un mecanisme molt més complex (Binns i Costantino 1998, Trovato i Linhares 1999, Meyer et al. 2000). Els gens *rol* indueixen, per si mateixos, un patró complex de modificacions morfogèniques i de desenvolupament (Trovato i Linhares 1999). Aquestes modificacions, algunes de les quals s'han descrit a l'apartat 4.2, són semblants als efectes biològics provocats per algunes hormones, com les auxines, CKs o gibberel·lines, però es desconeix si els gens *rol* efecten el seu metabolisme, la sensibilitat dels teixits a aquestes hormones (Binns i Costantino 1998) o el seu mecanisme d'actuació és diferent. Tot i que es disposa d'estudis per intentar explicar els efectes bioquímics de les proteïnes Rol d'*A. rhizogenes*, els resultats encara són preliminars per

poder elaborar un model que permeti entendre la causa dels fenotips de les plantes transformades amb aquests gens (Trovato i Linhares 1999, Meyer et al. 2000) .

En el cas concret del gen *rolC*, alguns estudis indiquen que pot tenir un efecte en les hormones endògenes de la planta transformada, ja que la seva presència afavoreix la ramificació d'algunes espècies (Schmülling et al. 1988), indicant un efecte de CK reconegut àmpliament, i fins i tot pot incrementar la seva capacitat d'arrelament (Ovadis et al. 1999, Zuker et al. 2001), indicant un nou efecte del gen *rolC*, un efecte d'auxina. Es desconeix si aquest efecte dual del *rolC* en plantes de clavell es manifesta en l'organogènesi de tiges i rels adventícies en cultiu *in vitro*.

Estruch et al. (1991a) va demostrar que la proteïna RolC presentava una activitat η-glucosidasa amb la capacitat d'hidrolitzar *in vitro* els glucòsids de les CKs i d'alliberar-ne les CKs actives. Però fins el moment, les dades dels nivells de CKs en plantes *rolC* són contradictòries, ja que incrementen, disminueixen o es mantenen estables depenent de l'espècie i del teixit analitzat (Nilsson i Olsson 1997, Meyer et al. 2000). Els nivells de l'auxina IAA, en canvi, no varien en plantes de *N. tabacum* o *S. tuberosum* transformades amb aquest gen (Nilsson et al. 1993, Schmülling et al. 1993). Pel que fa a les plantes de clavell transformades amb el gen *rolC* no es disposa de cap estudi que n'analitzi les hormones endògenes.

OBJECTIUS

OBJECTIUS

Objectiu general

L'objectiu general d'aquesta tesi és estudiar l'efecte de factors intrínsecs (genotip -diversos cultivars i línies transgèniques amb el *rolC*- i explant) i de factors extrínsecs (reguladors del creixement i agent gelificant del medi, i humitat relativa del cultiu) en l'organogènesi adventícia en explants de clavell (*Dianthus* L.). L'organogènesi s'avaluarà tant macroscòpicament, comptant les rels i les tiges adventícies, com microscòpicament, amb estudis histològics i ultrastructurals. S'estudiarà la implicació de les auxines i les citocinines endògenes en aquest procés organogènic tot analitzant canvis tant en la seva concentració com en la seva localització. Aquest objectiu global es desglossa en objectius específics.

Objectius específics

1. Estudiar la relació genètica entre vuit cultivars de clavell, *Dianthus caryophyllus* L. (White Sim, Red Sim, Pallas, Bianca Neve i Early Sam) i híbrids interespecífics (Pulcino, Inorsa R-24 i Mei Ling).

2. Avaluar l'efecte del genotip (cultivars White Sim, Early Sam, Pulcino, Inorsa R-24 i Mei Ling), l'explant (pètals i fulles) i la composició de reguladors de creixement dels medis de cultiu (l'auxina àcid 1-naftalenacètic i les citocinines N^6-benziladenina i thidiazuron (TDZ)), en l'organogènesi adventícia de tiges i rels.

3. Analitzar la implicació de l'auxina endògena àcid indol-3-acètic (IAA) i les citocinines endògenes del tipus isoprenoide N^6-isopenteniladenina (iP), zeatina (Z), dihidrozeatina (DHZ) i els seus ribòsids (iPR, ZR i DHZR) en la regeneració de tiges induïda pel TDZ en explants de pètals del cultivar White Sim.

4. Estudiar l'efecte de la humitat relativa en l'atmosfera de cultiu i la concentració d'agar del medi en l'organogènesi adventícia i el grau d'hiperhidricitat de tiges regenerades en explants de pètals dels cultivars White Sim i Early Sam.

5. Valorar l'efecte del gen *rolC*, d'*Agrobacterium rhizogenes*, en l'organogènesi adventícia de tiges i rels en explants de pètals i fulles de quatre línies de clavell del cultivar White Sim transgèniques amb aquest gen.

6. Determinar l'efecte del gen *rolC* en el contingut d'IAA i de citocinines endògenes del tipus isoprenoide (iP, iPR, Z, ZR, DHZ, DHZR) en bases de pètals i fulles de tres línies transgèniques del cultivar White Sim, i estudiar la implicació d'aquestes hormones en la capacitat organogènica d'aquests explants.

7. Realitzar una revisió bibliogràfica de la influència dels gens *rolA*, *B*, *C* i *D*, sols o combinats, en els fenotips obtinguts en plantes ornamentals amb flor transformades amb aquests gens.

RESULTATS

CAPÍTOL 1

Relació genètica entre cultivars de clavell amb l'ús de RAPDs

Relació genètica entre cultivars de clavell amb l'ús de RAPDs

Resum

La relació genètica de diversos cultivars de clavell (*Dianthus* L.) ha estat examinada utilitzant l'anàlisi del DNA polimòrfic amplificat aleatòriament (RAPD). Les bandes polimòrfiques obtingudes ens han permès obtenir un dendograma basat en les relacions genètiques entre els cultivars. Els cultivars pertanyents a l'espècie *D. caryophyllus* han estat agrupats tots junts, sense separació entre els cultivars estàndard i *spray*, classificats fenotípicament. La major part dels híbrids interespecífics han estat separats del grup *D. caryophyllus* en el dendograma, excepte el cultivar Pulcino.

Genetic relatedness in carnation cultivars using RAPDs

Abstract

The genetic relatedness of several carnation cultivars (*Dianthus* L.) was surveyed using random amplified polymorphic DNA (RAPD) analysis. The polymorphic bands obtained allowed us to construct a dendogram based on the genetic relationship between the cultivars. All the *D. caryophyllus* cultivars were grouped together, with no separation between standard and spray phenotypically classified cultivars. Most of the interspecific hybrids were separated from the *D. caryophyllus* group in the dendogram, except the cultivar Pulcino.

Abbreviations: RAPD, random amplified polymorphic DNA; PCR, polymerase chain reaction.

Introduction

Carnation types

The carnation (*Dianthus caryophyllus* L.) originated in the Mediterranean coastal regions and has been cultivated for 2000 years. The wild carnation plants, with 15 pairs of chromosomes, flowers in spring and winter, and their flowers have a strong scent and a fast senescence. After many years of crosses and selection of individuals with desirable characteristics, hundreds of cultivars have been developed for flower commercialization, few of which being tetraploid (Holley and Baker 1963). These numerous inter and intraspecific crosses, mutation breeding, the secrecy of the breeders regarding the origin of successful cultivars and the scarcity of documentation on the breeding history of this species hinder the evaluation of the genetic relationships between modern carnations.

At present, the commercial *D. caryophyllus* cultivars can be divided into two main groups: standard carnations and spray carnations. However, this grouping is based on phenotypic features. There are also interspecific hybrids, maily with a dwarf phenotype, thus called pot carnations, that have been obtained to incorporate characteristics from other carnation species (Vainstein et al. 1991, van Altvorst 1994). All the commercial cultivars are propagated vegetatively.

Standard carnations

Standard carnations are produced by disbudding all lateral flower buds and leaving the terminal flower. This type of carnation has to be disbudded continuously in order to maintain the size of the flower and its quality. The oldest carnation type is the standard carnation with a long, heavy stem. Around 1800, the development of carnation

cultivars started in the south of France and the north of Italy. In 1852, the first carnation seedlings were imported into the United States and since then carnation breeding has also been carried on in that country. William Sim, in 1938, obtained a cultivar that was named after him. This cultivar and its mutants, called Sim cultivars, were very successful and brought about an increase in carnation breeders. Many colors were available and, in absence of alternatives, Sim cultivars dominated the market for many years (Holley and Baker 1963). Their tendency to split flowers and their great sensitivity to the soil fungus *Fusarium oxysporum* caused a return to the Mediterranean carnations, developed in France and Italy. Nowadays, most of the standard carnation cultivars are crosses between Sim and Mediterranean carnations (van Altvorst 1994).

Spray carnations

In contrast to standard carnations, in spray carnations the terminal flower bud is removed and lateral buds are left. Spray carnations were developed by W. W. Thompson in the United States, in 1956, and they were introduced in Europe in 1964 (van Altvorst 1994).

RAPD analysis

The classical approach to differentiating plant cultivars is based on phenotypic characters, mainly morphological and biochemical traits. Although they are extremely useful, morphological traits can be influenced by environmental conditions or determined by plant phenology (Gallego and Martínez 1996). The use of proteins as molecular markers offers many advantages, although environment can influence results. The DNA-based markers allow a direct comparison of the genetic material of individual plants avoiding the environmental influences on gene expression. There are several techniques which are widely used in plant genetic studies, such as the RFLP (restriction fragment length polymorphism), the VNTR (variable number of tandem repeats), the AFLP (amplified fragment length polymorphism) and the RAPD (random amplified polymorphic DNA) (Newbury and Ford-Lloyd 1993, Debener 2002).

The RAPD technique consists of using primers with arbitrary sequences to amplify, by PCR (polymerase chain reaction), DNA fragments, which are resolved by electrophoresis. For each genotype the amplified sequences are different, thus some of the bands of the DNA electrophoresis are polymorphic since they appear only in some of the genotypes. The RAPD methodology is reliable and compared with other techniques of DNA analysis has advantages in speed, technical simplicity, and high frequency of identification of polymorphisms. Very small amounts of DNA are required, and prior knowledge of target sequences or the use of radioactively labelled probes are not necessary (Newbury and Ford-Lloyd 1993, Debener 2002). The RAPD method presents two main applications. On one hand, DNA fragments obtained from amplification with specific primers, or a combination of primers, may be molecular markers linked to a genetic locus controlling a specific phenotypic feature (Scovel et al. 1998). On the other hand, molecular markers are also used for genetic differentiation of varieties and analyses of phylogenetic and genetic relatedness between species and varieties (Debener 2002), as is the case in our study. In fact, cultivars of

many species, such as the rose, poinsettia, *Rubus*, kiwifruit, vanilla or rice have been classified by using the RAPD technique (Gallego and Martínez 1996, Ling et al. 1997, Graham et al. 1997, Jan et al. 1999, Palombi and Damiano 2002, Besse et al. 2004, Wu et al. 2004).

Our objective was to study the genetic relatedness of eight carnation cultivars (*D. caryophyllus* L. and interspecific hybrids), by using RAPDs.

Fig. 1. Carnation cultivars studied: A, White Sim (standard carnation, Sim type) and D, Pallas (standard carnation, Mediterranean type); B, Early Sam (spray carnation); C, Pulcino, E, Mei Ling and F, Inorsa R-24 (interspecific hybrids).

Material and methods

Plant material

Carnation plants were grown under standard greenhouse conditions, over a natural photoperiod, in 4.7 L-pots (19 cm in diameter, 18 cm high) with peat:perlite (2:1, v/v), irrigated daily with Hoagland nutrient solution (Hoagland and Arnon 1938). Eight cultivars of carnation were analysed. White Sim and Red Sim (standard carnations, Sim type), Pallas and Bianca Neve (standard carnations, Mediterranean type), and Early Sam (spray carnation) are *D. caryophyllus* L. cultivars, whereas Pulcino (micro type), Inorsa R-24 (pot carnation) and Mei Ling (Chinese type) are interspecific hybrids (Fig. 1).

Plants were grown in the greenhouse of the *Servei de Camps Experimentals* of the University of Barcelona, except White Sim and Red Sim cultivars, which were grown in the greenhouse of the Faculty of Agriculture of the Hebrew University of Jerusalem (Rehovot, Israel).

DNA extraction

Young leaves were removed from the eight cultivars, and they were immediately frozen in liquid nitrogen and lyophilized. DNAs were extracted using a procedure with cetyltrimethylammonium bromide (CTAB) (Murray and Thompson 1980), with several modifications. Lyophilized tissues (40 mg per sample) were ground with liquid nitrogen using a mortar and pestle, transferred to Eppendorf tubes and incubated in 1.5 ml of the extraction buffer (2% w/v CTAB, 100 mM Tris HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 1% v/v η-mercaptoethanol) at 55ºC for 20-30 min. The tubes were swirled frequently to ensure powder dispersion into solution. After the incubation, the solutions were mixed thoroughly with 0.5 ml of chlorophorm:isoamyl alcohol (24:1) to form an emulsion and were centrifuged at 13.000 *g* for 10 min. The superior aqueous phases were carefully decanted and transferred to new tubes. The chlorophorm:isoamyl alcohol step was repeated and each superior aqueous phase was distributed into two new tubes. Then, 1.2 ml (twice the total volume) of 1% w/v CTAB, 50 mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0) and 1% v/v η-mercaptoethanol were added to each tube, and after 30 min the solutions were centrifuged at 13000 *g* for 10 min. The supernatants were decanted and discarded and the pellets were dissolved with 0.5 ml of 1 M CsCl. Then the DNAs were precipitated by adding 1 ml of 96% ethanol at –30ºC to the tubes, gently mixing until a white-colored fiber was formed. Tubes were kept at –30ºC, overnight to purify the DNAs which were recovered as pellets by centrifugation at 13000 *g* for 10 min. The DNAs were washed twice with 1ml of 80% ethanol, the solutions were gently mixed, centrifuged at 13000 *g* for 10 min and the ethanol was decanted. The tubes were transferred to a speed-vacuum (Hetovac VR-1, Denmark) for 10-20 min to dry the samples. After complete drying, the DNAs were dissolved with 30 µl of sterile distilled water (the solutions were gently mixed and then incubated at 37ºC, overnight). Genomic DNA samples were stored at 4ºC.

Table 1. Primers used in the RAPD analyses (Operon Technologies, Alameda, Calif., USA) and number of the corresponding polymorphic bands obtained from the eight carnation cultivars.

Primer	Sequence	Polymorphic fragments	Primer	Sequence	Polymorphic fragments
I11	5'-ACATGCCGTG-3'	3	K16	5'-GAGCGTCGAA-3'	6
I14	5'-TGACGGCGGT-3'	4	K20	5'-GTGTCGCGAG-3'	8
I18	5'-TGCCCAGCCT-3'	7	L3	5'-CCAGCAGCTT-3'	3
J6	5'-TCGTTCCGCA-3'	7	L6	5'-GAGGGAAGAG-3'	0
J9	5'-TGAGCCTCAC-3'	7	L9	5'-TGCGAGAGTC-3'	3
J12	5'-GTCCCGTGGT-3'	8	L12	5'-GGGCGGTACT-3'	4
J15	5'-TGTAGCAGGG-3'	0	M5	5'-GGGAACGTGT-3'	5
J18	5'-TGGTCGCAGA-3'	2	M10	5'-TCTGGCGCAC-3'	4

RAPD analyses

RAPD analyses were performed using 16 arbitrary decamer primers (Operon Technologies, Alameda, Calif., USA) (Table 1) and a programmable thermal cycler (PTC-100, M.J. Research Inc., Watertown, Mass., USA). PCR reactions were carried out in a 25-µl volume containing 25 ng of the genomic DNA, 100 µM of each dNTP, 40 µM of primer, 10 mM Tris HCl (pH 8.2), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 unit of *Taq* (*Thermus aquaticus*) DNA polymerase (Advanced Biotechnologies Ltd., UK). *Taq* DNA polymerase acts optimally at 72ºC. The reaction mixture was overlaid with 30 µl of mineral oil and subjected to PCR. The PCR conditions were those used by Scovel et al. (1998): 94ºC for 30 s followed by 40 cycles of 1 min at 94ºC, 1 min at 35ºC and 2 min at 72ºC, and finally a last step of 5 min at 72ºC. The RAPD products were resolved on a 1.5% (w/v) agarose gel in TBE buffer (0.13 M Tris base, 0.07 M boric acid and 2.45 mM EDTA, pH 8.4). The fragments of the DNA of Lambda bacteriophagus digested with *Eco*RI + *Hind*III were used as size markers. Electrophoreses were performed at 85 V. Gels were stained with ethidium bromide and were photographed under ultraviolet light. The gels obtained with the RAPD amplification of genomic DNA of carnation using the primers J12, K20, L12 and M10 are displayed in the Figure 2.

Statistical analysis

For each primer, the PCR polymorphic fragments visualized in the electrophoreses were sequentially designated as one variable (Table 1), which were scored for subsequent analysis on the basis of presence (1) or absence (0) of the amplified products for each cultivar. With these data, the squared Euclidean distance was used to calculate a dissimilarity matrix with all the eight cultivars (Table 2). The dendogram, or cluster tree, grouping the cultivars was

obtained with the hierarchical clustering procedure, and the coefficients of the cluster agglomeration were rescaled to numbers between 0 and 25. This hierarchical cluster multivariate analysis was performed with the statistical software SPSS 11.5 (SPSS Inc., Chicago, IL.).

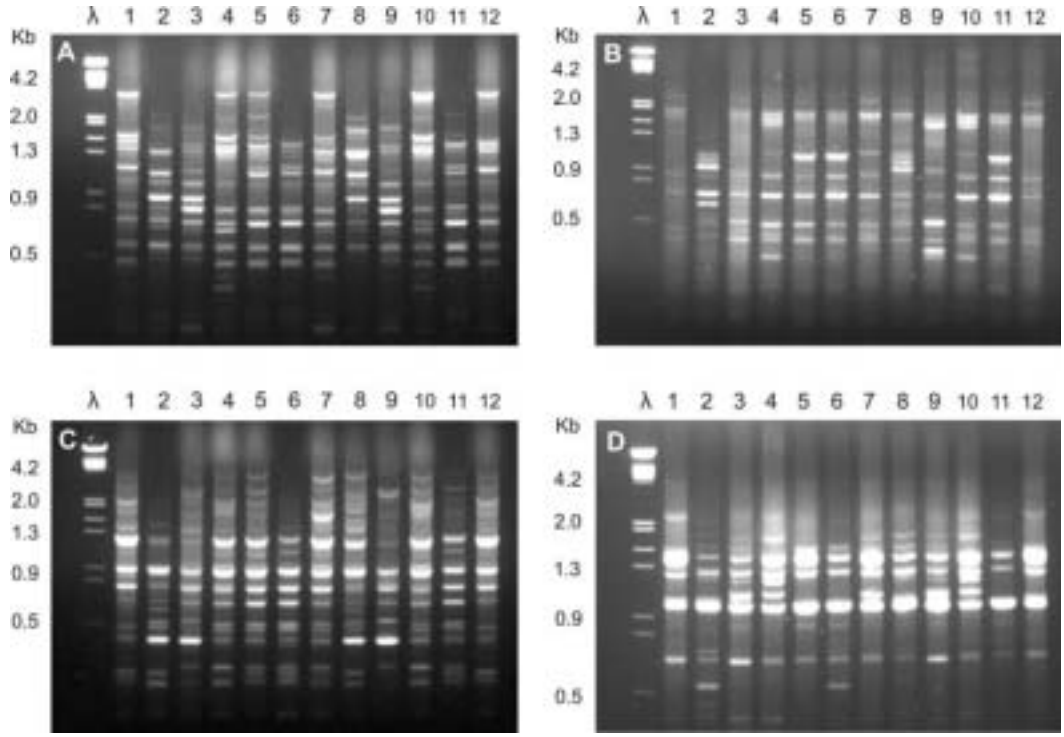

Fig. 2. RAPD amplification of genomic DNA of carnation using the primers (A) J12, (B) K20, (C) L12 and (D) M10. Molecular weight markers of the first lane, ς, correspond to the DNA of Lambda bacteriophagus digested with *Eco*RI + *Hind*III (fragments of 21.2, 5.0, 4.2, 3.5, 2.0, 1.9, 1.5, 1.3, 0.9, 0.8, 0.5 kb, from top to bottom). The following lanes correspond to the carnation cultivars: 1, White Sim; 2, Inorsa R-24; 3, Mei Ling; 4, Early Sam; 5, Pallas; 6, Pulcino; 7, Bianca Neve; 8, Inorsa R-24; 9, Mei Ling; 10, Early Sam; 11, Pallas; 12, and Red Sim.

Table 2. Dissimilarity matrix of the eight cultivars calculated with the squared Euclidean distance by using the 71 variables (polymorphic bands).

	White Sim	Inorsa R-24	Mei Ling	Early Sam	Pallas	Pulcino	Bianca Neve	Red Sim
White Sim		36	36	33	30	34	26	2
Inorsa R-24	36		34	41	42	42	32	36
Mei Ling	36	34		27	34	34	32	36
Early Sam	33	41	27		25	29	25	33
Pallas	30	42	34	25		6	24	32
Pulcino	34	42	34	29	6		26	32
Bianca Neve	26	32	32	25	24	26		26
Red Sim	2	36	36	33	32	32	26	

Results

Classification of the carnation cultivars

The 16 primers used for the DNA amplification of the eight cultivars studied produced 71 polymorphic DNA bands (Table 1), used to establish genetic relatedness between the cultivars. Their relative genetic relatedness is displayed in a dendogram that groups all of them (Fig. 3). The closest cultivars are White Sim and Red Sim. Pallas and Pulcino are also very close, whereas Bianca Neve and Early Sam have as much separation between them as between them and Pallas-Pulcino group. All four are linked to the Sim group, forming a group of six cultivars, five of which are *D. caryophyllus* L., and one, Pulcino, is an interspecific hybrid. Mei Ling and Inorsa R-24, two interspecific hybrids, are genetically the furthest separated both from one another and from the rest of the cultivars (Fig. 3). It is worth noting that, since carnation plants are vegetatively propagated, the plants of the same cultivar should have identical genotypes, unless somatic mutation has taken place.

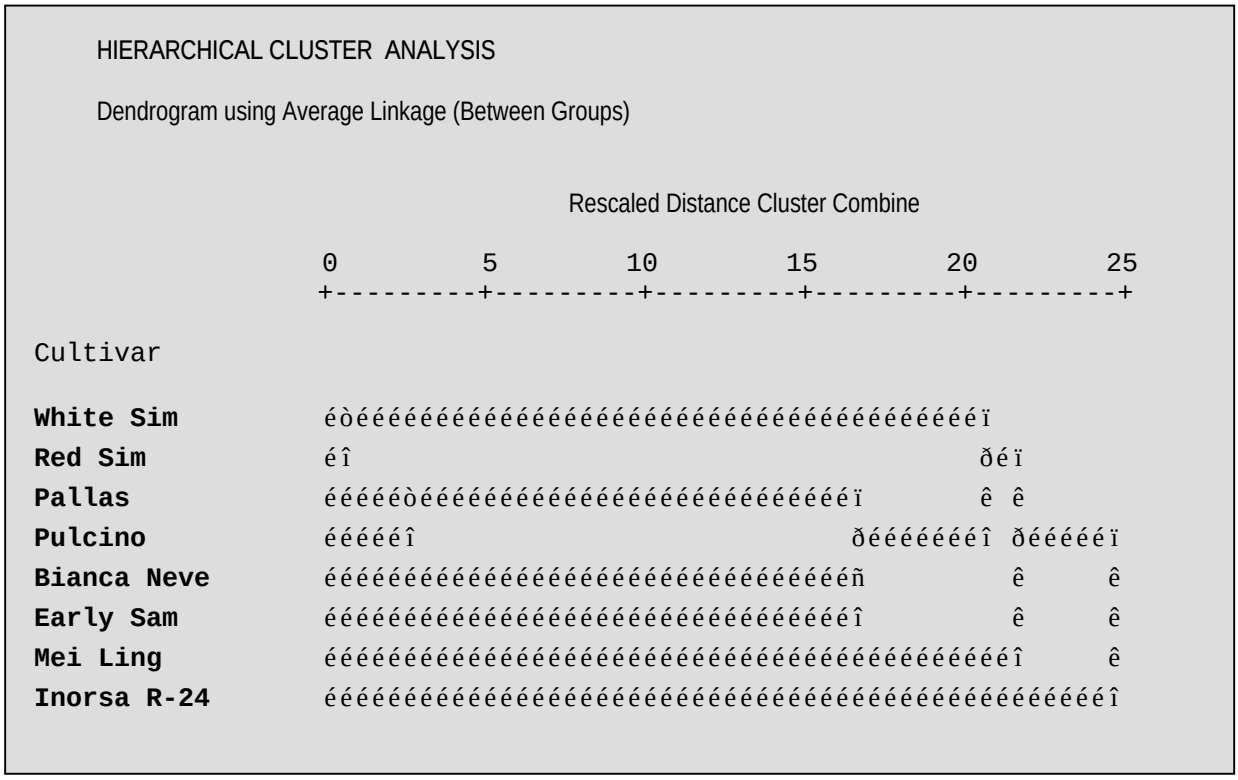

Fig. 3. Dendrogram with the relative genetic distances between the eight carnation cultivars (*Dianthus caryophyllus* L. and interspecific hybrids): White Sim and Red Sim (standard carnations, Sim type), Pallas and Bianca Neve (standard carnations, Mediterranean type), Early Sam (spray carnation), and Pulcino, Mei Ling and Inorsa R-24 (interspecific hybrids).

Discussion

The RAPD technique allowed us to find the genetic relatedness between eight carnation cultivars by using several primers to amplify their DNA. In fact, the higher the number of primers used, the closer to the real genetic relatedness the dendogram would be. However, the 71 polymorphic bands obtained seem to have reflected quite accurately what is known about these cultivars. White Sim and Red Sim cultivars, standard carnations of Sim type, differ from each other by few genes, mainly those related to their pigmentation (Holley and Baker 1963, Amir Zuker, personal communication). This is shown in the dendogram, in which they are the most closely related cultivars.

There is an intimate relationship between Pallas (standard carnation of Mediterranean type) and Pulcino (micro type carnation, interspecific hybrid), both of which have a similar phenotype and yellow flowers. This might be due to the process used to obtain Pulcino (interspecific hybrid of *D. caryophyllus* with *D. knappii*), since it is highly probable that crosses with Pallas or a relative were done in order to stabilize the yellow, a color that is very difficult to obtain in this species (Flavio Sapia, breeder of Rosenet, San Remo, Italy, personal communication). Bianca Neve, although it is a Mediterranean standard carnation like Pallas, probably comes from a more distant line, since white carnations usually come from Violet or Pesco lines (F. Sapia, personal communication). That would explain its relative distance from Pallas.

Early Sam, with flowers of a dark pink color, belongs to the spray category and comes from a mutation of Sam's Pride, obtained by W. W. Thompson in the United States (Gideon Scovel, breeder of Shemi, Israel, and Gijs van Leewven, breeder of West Select B.V., The Netherlands, personal communications). It is worth noting that this spray cultivar is closer to the Mediterranean group than are Sim cultivars, although both Mediterranean and Sim cultivars belong to standard category. Standard and spray carnations, all of them *D. caryophyllus*, would not be two genetic lines (G. Scovel, personal communication). In fact, spray carnations were originally selected from a population of standard carnations. The results would show that these traditional classifications, based on the phenotype, do not always correspond to genetic classifications. Similar to our results, other Mediterranean (Manon, Tanga, Galil and Sanderosa) and spray (Gus Royalette, White Royalette, Silvery Pink, Prestige and Lior) carnation cultivars, analysed by microsatellite RFLPs, were found to be as close genetically within each category as between the two categories (Vainstein et al. 1991).

Finally, the two interspecific hybrids, Mei Ling and Inorsa R-24, are separate from the *D. caryophyllus* group and separate from one another. Both of them are dwarves and thus phenotypically different from all the other cultivars. Mei Ling belongs to the Chinese carnation type, *D. chinensis* being the predominant species. It is a result of a breeding program begun in 1949, and Mei Ling was obtained in 1976 in San Remo, Italy (F. Sapia and Gijs van Leewven, breeder of West Select B.V., The Netherlands, personal communications). Inorsa R-24, a carnation obtained in the IRTA research center (Cabrils, Barcelona) by Ramon Messeguer, comes from an autofecundation of the tetraploid Pico variety (R. Messeguer, personal communication), which was obtained by crossing *D. allwoodii* and a Diantini carnation (tetraploid carnations, interspecific hybrids) (Messeguer 1987). In agreement with our results, other carnation hybrids (Gepo, Pinki, Cuscino de Amore and Vaso White), phenotypically classified in a 'dwarf'

category, also showed the least similarity, both within this category and with the standard and spray categories (Vainstein et al. 1991).

In general the RAPD markers allowed us to group the carnation cultivars according to their genetic characteristics. Logically, the interspecific hybrids were found genetically separated from the *D. caryophyllus* group, except the cultivar Pulcino. Within the latter group there was no genetic separation between the Mediterranean standard carnations and the spray carnations, since this is a phenotypic classification. However, the Sim standard carnations, which belong to a same genetic line, formed a clearly differentiated subgroup within the *D. caryophyllus* group.

From the eight cultivars analysed, five were chosen to study their regenerative capacity *in vitro*: one standard carnation (White Sim), one spray carnation (Early Sam) and three interspecific hybrids (Pulcino, Inorsa R-24 and Mei Ling).

References

Besse P, Da Silva D, Bory S, Grisoni M, Le Bellec F, Duval MF (2004) RAPD genetic diversity in cultivated vanilla: *Vanilla planifolia*, and relationships with *V. tahitensis* and *V. pompona*. Plant Sci. 167:379-385

Debener T (2002) Molecular markers as a tool for analyses of genetic relatedness and selection in ornamentals. In: Vainstein A (ed) Breeding For Ornamentals: Classical and Molecular Approaches (pp. 329-345). Kluwer Academic Publishers, Dordrecht, The Netherlands

Gallego FJ, Martínez I (1996) Molecular typing of rose cultivars using RAPDs. J. Hort. Sci. 71:901-908

Graham J, Iasi L, Millam S (1997) Genotype-specific regeneration from a number of *Rubus* cultivars. Plant Cell Tiss. Org. Cult. 48:167-173

Hoagland DR, Arnon DI (1938) The water-culture method for growing plants without soil. Univ. Calif. Agric. Exp. St. Circ. No 347

Holley WD, Baker R (1963) Carnation Production. Including the History, Breeding, Culture and Marketing of Carnations. M.C.Brown, Dubuque, Iowa, USA

Jan CH, Byrne DH, Manhart J, Wilson H (1999) Rose germplasm analysis with RAPD markers. HortSci. 34:341-345

Ling J-T, Sauve R, Gawel N (1997) Identification of poinsettia cultivars using RAPD markers. HortSci. 32:122-124

Messeguer R (1987) Genètica de la variació isoenzimàtica al clavell (*Dianthus caryophyllus* L.): aplicació a la identificació de varietats comercials. Doctoral Thesis. Universitat Autònoma de Barcelona, Spain

Murray MG, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucl. Acids Res. 8:4321-4325

Newbury HJ, Ford-Lloyd BV (1993) The use of RAPD for assessing variation in plants. Plant Growth Regul. 12:43-51

Palombi MA, Damiano C (2002) Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). Plant Cell Rep. 20:1061-1066

Scovel G, Ben-Meir H, Ovadis M, Itzhaki H, Vainstein A (1998) RAPD and RFLP markers tightly linked to the locus controlling carnation (*Dianthus caryophyllus* L.) flower type. Theor. Appl. Genet. 96:117-122

Vainstein A, Hillel J, Lavi U, Tzuri G (1991) Assessment of genetic relatedness in carnation by DNA fingerprints analysis. Euphytica 56:225-229

van Altvorst AC (1994) Shoot regeneration and *Agrobacterium*-mediated transformation of carnation. Doctoral Thesis. Catholic University Nijmegen, The Netherlands

Wu C-J, Cheng Z-Q, Huang X-Q, Yin S-H, Cao K-M, Sun C-R (2004) Genetic diversity among and within populations of *Oryza granulata* from Yunnan of China revealed by RAPD and ISSR markers: implications for conservation of the endangered species. Plant Sci. 167:35-42

CAPÍTOL 2

Nivells i immunolocalització de citocinines endògenes en l'organogènesi de tiges induïda pel thidiazuron en clavell

Nivells i immunolocalització de citocinines endògenes en l'organogènesi de tiges induïda pel thidiazuron en clavell

Resum

S'ha avaluat la capacitat del regulador del creixement vegetal thidiazuron (TDZ), un derivat de la fenilurea amb una alta activitat com de citocinina (CK), per promoure l'organogènesi en pètals i fulles de diversos cultivars de clavell (*Dianthus* L.), combinat amb l'àcid 1-naftalenacètic (NAA). La implicació de l'auxina endògena àcid indol-3-acètic (IAA) i les CKs del tipus isoprenoide també s'ha estudiat. La diferenciació de tiges s'ha trobat que depèn de l'explant, del cultivar i del balanç de reguladors del creixement. El TDZ sol (0.5 i 5.0 μM), així com de manera sinèrgica amb l'NAA (0.5 i 5.0 μM), promou l'organogènesi de tiges en pètals, i és més actiu que la N^6-benziladenina. En pètals del cultivar White Sim, el TDZ indueix la proliferació cel·lular de manera dependent a la concentració i, al 7è dia de cultiu, la proporció de regions meristemàtiques en aquests pètals permet la predicció de la capacitat de regenerar tiges al cap de 30 dies de cultiu. La immunolocalització dels ribòsids de les CKs, la N^6-isopenteniladenosina, el ribòsid de la zeatina (ZR) i el ribòsid de la dihidrozeatina (DHZR), en pètals organogènics mostra que es troben molt concentrats en l'extrem dels primordis de tija que s'hi han format i en les regions amb capacitat proliferativa. Tots ells deuen tenir un rol en la proliferació cel·lular, i possiblement en la diferenciació, durant el procés organogènic. Després de set dies de cultiu dels pètals White Sim, l'NAA sembla que pot ser la causa dels canvis trobats en els nivells d'IAA i DHZR, mentre que el TDZ podria ser responsable dels increments significatius en la N^6-isopenteniladenina (iP) i el ZR. El ZR és induït per baixes concentracions de TDZ (0.0-0.005 μM), mentre que la iP, que va lligada a una massiva proliferació cel·lular i a un inici en la diferenciació de tiges, està associada a nivells alts de TDZ (0.5 μM). A més dels

canvis observats en la quantificació i en la localització *in situ* de les hormones endògenes durant l'organogènesi de tiges induïda pel TDZ, proposem que el TDZ també promou el creixement de manera directa, a través de la seva pròpia activitat biològica. Pel que sabem, aquest és el primer estudi que avalua l'efecte del TDZ en les hormones endògenes en un procés organogènic.

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Levels and immunolocalization of endogenous cytokinins in thidiazuron-induced shoot organogenesis in carnation

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Summary

We evaluated the capacity of the plant growth regulator thidiazuron (TDZ), a substituted phenylurea with high cytokinin-like activity, to promote organogenesis in petals and leaves of several carnation cultivars (*Dianthus* spp.), combined with 1-naphthaleneacetic acid (NAA). The involvement of the endogenous auxin indole-3-acetic acid (IAA) and purine-type cytokinins was also studied. Shoot differentiation was found to depend on the explant, cultivar and balance of growth regulators. TDZ alone (0.5 and 5.0 μmol/L) as well as synergistically with NAA (0.5 and 5.0 μmol/L) promoted shoot organogenesis in petals, and was more active than N^6-benzyladenine. In petals of the White Sim cultivar, TDZ induced cell proliferation in a concentration-dependent manner and, on day 7 of culture, the proportion of meristematic regions in those petals allowed the prediction of shoot regeneration capacity after 30 days of culture. Immunolocalization of CK ribosides, N^6-(Δ^2-isopentenyl)adenosine, zeatin riboside (ZR) and dihydrozeatin riboside (DHZR), in organogenic petals showed them to be highly concentrated in the tips of bud primordia and in the regions with proliferation capacity. All of them may play a role in cell proliferation, and possibly in differentiation, during the organogenic process. After seven days of culture of White Sim petals, NAA may account for the changes found in the levels of IAA and DHZR, whereas TDZ may be responsible for the remarkable increases in N^6-(Δ^2-isopentenyl)adenine (iP) and ZR. ZR is induced by low TDZ concentrations (0.0–0.005 μmol/L), whereas iP, that correlates with massive cell proliferation and the onset of shoot differentiation, is associated with high TDZ levels (0.5 μmol/L). In addition to the changes observed in quantification and *in situ* localization of endogenous phytohormones during TDZ-induced shoot organogenesis, we propose that TDZ also promotes growth directly, through its own biological activity. To our knowledge, this study is the first to evaluate the effect of TDZ on endogenous phytohormones in an organogenic process.

Key words: cytokinin – *Dianthus caryophyllus* – immunocytochemistry – indole-3-acetic acid – *in vitro* culture – meristematic activity – organogenesis – thidiazuron

Abbreviations: BA = N^6-benzyladenine. – CK = cytokinin. – DHZ = dihydrozeatin. – DHZR = dihydrozeatin riboside. – IAA = indole-3-acetic acid. – iP = N^6-(Δ^2-isopentenyl)adenine. – iPR = N^6-(Δ^2-isopentenyl)adenosine. – NAA = 1-naphthaleneacetic acid. – Thidiazuron or TDZ = N-phenyl-N′-1,2,3-thiadiazol-5-ylurea. – Z = zeatin. – ZR = zeatin riboside

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Introduction

The carnation (*Dianthus caryophyllus* L.) is the leading commercial cut-flower crop worldwide in terms of the number of stems sold (Jensen and Malter 1995). Carnation breeders aim to produce new varieties, but the heterozygosity of this plant severely restricts conventional breeding procedures (Woodson 1991). Genetic engineering, combined with tissue culture techniques, can provide an alternative method for improving quality traits (Zuker et al. 1999, Jain et al. 2001, Casanova et al. 2003). Several procedures that lead to adventitious shoot regeneration from a range of carnation explants have been reported (Zuker et al. 1998). However, the organogenic response is not only affected by the explant source, but also by the genotype and the balance of plant growth regulators (Frey and Janick 1991).

The induction of organogenesis from plant tissues cultured *in vitro* generally requires cell exposure to auxins and cytokinins (CKs), which are involved in cell division and differentiation (Coenen and Lomax 1997). Thus, in tissue culture systems, active plant growth regulators within the explant are derived from endogenous biosynthesis and exogenous sources. The naturally occurring auxin is the indole-3-acetic acid (IAA), whereas the active endogenous CKs are considered to be free-base forms of adenine derivatives, N^6-(Δ^2-isopentenyl)adenine (iP), zeatin (Z) and dihydrozeatin (DHZ), and their ribosides (iPR, ZR and DHZR, respectively). The CK bases are easily interconverted with their 9-ribosides (McGaw and Burch 1995). In the accepted pathway of CK biosynthesis, iP is the precursor of Z, which, in turn, leads to DHZ through the reduction of the N^6-side chain (Chen 1997). Regarding synthetic substances, 1-naphthaleneacetic acid (NAA) is one of the most used auxins in tissue culture systems, since it is not photodegraded or oxidized in the medium before entering the tissue. In carnation tissue cultures, N^6-benzyladenine (BA) is the most used CK (van Altvorst et al. 1992, 1994, Fisher et al. 1993, Nakano et al. 1994). In contrast, thidiazuron (TDZ), a substituted phenylurea that exhibits high CK-like activity (Mok et al. 1982, Visser et al. 1992), has only been used by few authors in carnation cultures *in vitro* (Frey and Janick 1991, Casanova et al. 2003), although comparative studies have shown TDZ to be the most effective CK for adventitious shoot regeneration from carnation petals (Nakano et al. 1994). There is still a debate as to whether phenylurea derivatives exert a direct or indirect effect on the metabolism of endogenous CKs (Mok and Mok 2001). An auxin-like effect is also exhibited by TDZ, since it replaces auxins in the induction of embryogenesis (Visser et al. 1992, Murthy et al. 1998). The few available studies on the effect of TDZ on endogenous hormone levels have used several experimental systems, calli and somatic embryogenesis, and various plant species (Capelle et al. 1983, Murthy et al. 1995, Hutchinson et al. 1996, Victor et al. 1999). TDZ may promote an increase in the overall CK pool by increasing synthesis, decreasing catabolism or releasing active CKs from nonactive forms. Immunolocalization of endogenous hormones provides a complementary vision of their involvement in morphogenic processes (Sotta et al. 1992, Dewitte and van Onckelen 2001).

TDZ-induced morphogenesis in carnation explants is a suitable experimental system for studying organogenesis and the morphoregulatory role of TDZ. Here, we aim to examine the involvement of endogenous auxin and CK levels on TDZ-induced adventitious shoot regeneration in carnation explants. To our knowledge, this is the first report on the effect of TDZ on endogenous phytohormones during an organogenic process. Moreover, immunocytochemical location of endogenous CKs at early stages of the organogenic process provides additional information of the putative roles of CKs in shoot organogenesis *in vitro*.

Materials and Methods

Plant material

Five cultivars of carnation were used. White Sim (Sim category) and Early Sam (spray) are *Dianthus caryophyllus* L. cultivars, whereas Pulcino (micro type), Inorsa R-24 (pot carnation) and Mei Ling (chinese type) are interspecific hybrids. Plants were grown in standard greenhouse conditions, under a natural photoperiod. Once they reached the flowering stage, young flower buds were removed and used as the petal explant source. Cuttings from vegetative shoots were harvested and used as the leaf explant source. Both sources of explants were stored at 4 °C, for a maximum of 15 days.

Tissue culture

Flower buds were surface-sterilised for 5 s with 96 % (v/v) ethanol. Cuttings were rinsed in 70 % (v/v) ethanol, then sterilised for 8 min in 1.2 % (w/v) sodium hypochlorite and rinsed three times for 10 min in sterile water. Petals were excised and basal parts were placed in MS basal medium (Murashige and Skoog 1962) with 30 g L^{-1} sucrose, 2 mg L^{-1} glycine, 50 mg L^{-1} myo-inositol and 0.5 mg L^{-1} thiamine-HCl (Gimelli et al. 1984). Between 10–14 leaves per cutting were removed from stems, following van Altvorst et al. (1994), and basal parts were placed in MS medium with 30 g L^{-1} sucrose, 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine-HCl and 0.1 mg L^{-1} thiamine-HCl (Murashige and Skoog 1962). Before autoclaving (121 °C for 20 min), plant growth regulators were added, pH was adjusted to 5.8 and media were solidified with 8 g L^{-1} agar (High Gel Strength, Sigma). To evaluate shoot and root regeneration from petal explants, media were supplemented with NAA (0.0, 0.5 or 5.0 µmol/L) plus TDZ (0.0, 0.5 or 5.0 µmol/L) (Frey and Janick 1991) and for leaf explants, with NAA (0.5 µmol/L) plus BA (0.0, 0.5, 2.5 or 5.0 µmol/L) or TDZ (0.5 µmol/L), or with no plant growth regulators at all. Petal and leaf explants were cultured in 500-mL glass vessels containing 100 mL of solid medium (15–35 explants per vessel). Each experiment, one vessel per treatment, was performed in triplicate. To minimise the variability caused by the heterogeneous regeneration capacity intrinsic to each flower bud, petals from the same bud were randomly distributed among the media. Similarly, leaves from the same cutting were also distributed among the media. All the cultures were kept in a growth chamber at 25 ± 1 °C in a 16-h photoperiod

using cool white fluorescent tubes (TLD 58W/33, Philips, France, 80 μmol m^{-2} s^{-1}).

Shoot and root regeneration was assessed using a stereomicroscope after 30 days in culture, and it was considered to be the number of shoots or roots produced per explant.

Histological analysis

Histological studies were performed with fresh White Sim petals (day 0) and in petals cultured for 3, 5 and 7 days in media with 0.5 μmol/L NAA plus 0.0, 0.005, 0.05 or 0.5 μmol/L TDZ (Casanova et al. 2003). The bottoms of petal bases were fixed in 2 % paraformaldehyde and 2.5 % glutaraldehyde in 100 mmol/L phosphate buffer, pH 7.4, under vacuum at 4 °C for 4–6 days, postfixed in 1 % OsO_4 in the same buffer, dehydrated in an acetone series and embedded in Spurr's resin (Spurr 1969). Transversal sections (1 μm thick) were stained with 0.5 % methylene blue and observed under an Olympus BH2-UMA light microscope. Images were taken with a JVC TK1270 color video camera connected to a computer with a Matrox video card. The percentage of the area of meristematic regions was quantified using the «Imat» software, developed by the *Serveis Científico-Tècnics* of the University of Barcelona. The experiment was carried out with 50 explants per vessel (one vessel per medium). Five samples were analysed per day and medium. The remaining petals were used to assess shoot regeneration. The regeneration experiment was performed in triplicate.

Determination of phytohormone levels

Endogenous phytohormone levels of White Sim petals were analysed using 100 mg of dry weight per sample. Petals were cultured in the same media as used for histological analysis. The bottoms of the bases of fresh petals and of petals cultured for 7 days were frozen in liquid nitrogen, powdered and lyophilised, prior to the measurement of IAA and iP, Z, DHZ and their ribosides (iPR, ZR and DHZR), following Fernández et al. (1995). The phytohormones were extracted with 25 mL of 80 % (v/v) methanol containing 10 mg L^{-1} of *t*-butylated hydroxytoluene. The radiolabelled standards [8-^{14}C] BA (2.0 GBq $mmol^{-1}$) and 3-[5(n)-^{3}H] IAA (999 GBq $mmol^{-1}$) were added to determine purification losses. The extracts were evaporated under vacuum (30 °C) and the remaining aqueous solutions were cleared by centrifugation. The supernatants were adjusted to pH 3 with diluted acetic acid and passed through equilibrated Sep-Pak C_{18} cartridges (Waters Associates). The hormones retained were eluted with 10 mL of 80 % (v/v) methanol, dried under vacuum (Savant SC-200), redissolved in 2 mL of 10 mmol/L phosphate buffer saline (PBS), pH 7.4, and placed into an immunoaffinity column against iP, iPR, Z, ZR, DHZ and DHZR. IAA, which flowed through the column, was collected by adding 30 mL of PBS to the column. After the application of 30 mL of distilled water, that was discarded, the retained CKs were eluted with 30 mL of pure methanol. The PBS fraction, with the IAA, was acidified to pH 3 with HCl, re-extracted four times with diethyl ether and the samples were dried under a N_2 stream. The methanolic eluate, with the CKs, was dried under vacuum (Savant SC-200) and redissolved in the HPLC starting solvent. The six CKs were separated by reverse-phase HPLC on a Kromasil 100 C18–5 μm (150 × 4.6 mm) column (Teknokroma) using a Waters 600 liquid chromatograph connected to a Waters 996 UV detector monitored at 270 nm. The mobile phase was acetonitrile and triethylammonium acetate 40 mmol/L, at pH 7. CKs were eluted from the column by a linear gradient from 5 % to 20 % acetonitrile (v/v) over 40 min and 1.5-mL fractions were collected. Kinetin (2 μg) was added to each sample as an internal standard to verify CK retention times. The dried HPLC CK fractions were resuspended in 25 mmol/L Tris-buffer, pH 7.5, and quantified by ELISA using three polyclonal rabbit antibodies: anti-ZR to measure Z and ZR; anti-DHZR to measure DHZ and DHZR and anti-iPR to measure iP and iPR. IAA samples were methylated prior to their quantification by ELISA using anti-IAA monoclonal antibodies (Agdia®). All samples were analysed in triplicate and the measurements were also performed in triplicate.

Immunocytochemical labelling of cytokinins

Immunolocalization of CKs was performed in White Sim petals cultured for 7 days in media with 0.5 μmol/L NAA plus 0.5 μmol/L TDZ. Samples were collected at the same time than for phytohormone quantification. Petals were processed following Sossountzov et al. (1988) with modifications. Tissues were dropped in 20 mmol/L sodium metaperiodate dissolved in 50 mmol/L carbonate-bicarbonate buffer, pH 9.6, at room temperature for 2 h, and then in 2 mmol/L sodium borohydride in Tris-buffer, pH 7.6, for 1 h. Samples were fixed in 4 % paraformaldehyde and 0.1 % glutaraldehyde in 100 mmol/L phosphate buffer, pH 7.4, under vacuum at 4 °C for 4–6 days, dehydrated in an acetone series and embedded in Spurr's (1969) resin. Thin transversal sections (1 μm) were etched for 10 min in 10 % hydrogen peroxide, and rinsed in distilled water. The sections were then incubated in the block solution (0.1 mol/L phosphate buffer saline (PBS), pH 7.4, with 1 % (w/v) bovine serum albumin (BSA) and 0.05 % (v/v) Tween-20) at room temperature for 15 min. Then they were incubated in the primary antibodies diluted in the block solution in a moist chamber at 4 °C overnight. After washing the sections in the block solution, they were incubated with Protein A conjugated to colloidal gold (5 nm) diluted 1 : 85 in 0.1 mol/L PBS, pH 7.4, with 5 % BSA and 0.05 % Tween-20, at room temperature for 1 h. Sections were then washed with 0.1 mol/L PBS, pH 7.4, and rinsed with distilled water. Gold-stained sections were submitted to a silver-enhancement reaction for 14 min, using a British BioCell International kit (UK). Metallic silver precipitation around gold particles led to a shiny signal when observed at the light microscope using reflected light. Sections were finally slightly counterstained with 0.5 % methylene blue. Controls for the specificity of the immunolabelling were the replacement of the antibodies by PBS-BSA.

Polyclonal antibodies anti-iPR, anti-ZR and anti-DHZR (OlChemIm Ltd., Czech Republic), were used at concentrations of 0.996 μg/μL, 1.436 μg/μL and 0.884 μg/μL, respectively. The three antibodies recognised their respective free bases, ribosides, ribotides and N9-glucosides, with minimal cross-reaction between CK types. Since the periodate-borohydride reaction allows endogenous CK ribosides to couple to cellular proteins (Sossountzov et al. 1988, Sotta et al. 1992), our labelling indicated the riboside forms of CKs.

Statistical analyses

Differences in the regeneration capacity between media, within each cultivar and explant, and differences in hormone concentrations between media were tested using one-way ANOVA. In all cases, Duncan's multiple range test was applied when one-way ANOVA showed significant differences ($p < 0.05$). The area of meristematic regions in petal explants over time in the media was analysed using the repeated measures multivariate analysis. On day 7, differences between

media were tested using one-way ANOVA and Duncan's multiple range test. All statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, Ill.).

Results

Effect of genotype and plant growth regulators on shoot regeneration in petal explants

The main regenerative part of the carnation petals was the bottom of the basal region, but sometimes, in White Sim, shoot growth occurred on the lateral zone or on the surface of the explants. Organogenesis was direct, since no callus was observed.

The cultivars of carnation differed in their potential for organogenesis depending on the concentrations of growth regulators used. None of the five cultivars showed morphogenic responses in growth regulator-free medium. In both White Sim and Early Sam, shoot formation increased with the concentrations of NAA and TDZ, which acted synergistically (Table 1). TDZ alone regenerated shoots only in White Sim. The interspecific hybrid cultivars, Mei Ling, Inorsa R24 and Pulcino, exhibited minor shoot regeneration but showed a similar behaviour in reference to NAA and TDZ (data not shown). The highest rate of shoot regeneration was obtained in 5 μmol/L NAA plus 5 μmol/L TDZ for all the cultivars (Table 1), which was 1.20 (± 0.31), 0.33 (± 0.14) and 0.18 (± 0.08) shoots per petal, in Mei Ling, Inorsa R24 and Pulcino, respectively. However, because of the high degree of vitrification of regenerated shoots of White Sim, the most responsive cultivar, its optimum concentration of plant growth regulators was 0.5 μmol/L NAA plus 0.5 μmol/L TDZ. Thus, in subsequent experiments with White Sim petals, lower doses of TDZ (0.0–0.5 μmol/L) were tested. No roots were obtained from the petal cultures, except in a few White Sim and Early Sam petals cultured in 5 μmol/L NAA alone or combined with TDZ (data not shown).

Table 1. Adventitious shoot regeneration from petals of White Sim and Early Sam carnation cultivars (*Dianthus caryophyllus* L.) after 30 days cultured in MS solid media at a range of NAA and TDZ concentrations.

Media (μmol/L)		Shoots per petal (no.)[a] Cultivars	
NAA	TDZ	White Sim[b]	Early Sam[b]
0.0	0.0	0.00±0.00 a	0.00±0.00 a
	0.5	2.58±0.40 b	0.00±0.00 a
	5.0	4.15±0.37 c	0.12±0.05 a
0.5	0.0	0.01±0.01 a	0.00±0.00 a
	0.5	4.59±0.60 c	0.40±0.19 ab
	5.0	5.73±0.56 c	0.24±0.08 ab
5.0	0.0	0.19±0.07 a	0.06±0.03 a
	0.5	7.35±0.88 d	0.86±0.26 b
	5.0	8.81±0.96 d	2.56±0.08 c

[a] Values represent the mean ± SE. The total number of explants per treatment (medium) was 105–115 for White Sim and 90–95 for Early Sam.

[b] Within each cultivar, distinct letters denote significant differences (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test.

Effect of genotype and plant growth regulators on shoot and root regeneration in leaf explants

The morphogenic response of carnation leaves, both in shoot and root regeneration, was stronger in White Sim than in Early Sam. However, both cultivars showed a similar behaviour. The basal region was the regenerative part of leaves. Adventitious shoots and roots were obtained from leaves of both cultivars when cultured in medium without plant growth regulators (Table 2), in contrast to the results in the petal. In media with 0.5 μmol/L NAA and increasing BA concentrations, shoot regeneration increased progressively in both cultivars. Maximum root regeneration was obtained in 0.5 μmol/L NAA medium, and decreased at higher BA concentrations. Medium with TDZ gave the maximum number of shoots in both cultivars (Table 2). TDZ was more effective in inducing shoot organogenesis and inhibiting root formation in leaf explants, 9- to 10-fold in White Sim and 4- to 6-fold in Early Sam, than BA at the same concentration (0.5 μmol/L). However, leaves cultured in TDZ showed callus-forming tissue and gave highly vitrified shoots. The optimum medium to maximise unvitrified shoot regeneration from leaf explants was 0.5 μmol/L NAA plus 5.0 μmol/L BA.

Effect of TDZ concentration on the development of meristematic regions during the induction of shoot organogenesis in petal explants

Cross sections of bases of fresh White Sim carnation petals (day 0) showed no meristematic regions (Fig. 1 A), as epidermal and parenchymal cells displayed slight cytoplasmic staining, indicating a low metabolic activity. After few days of culture (3–5 days), isolated groups of cells became meristematic (Figs. 1 B, C). These proliferating cells, which are small and densely stained, arose mainly from the adaxial epidermis and subepidermis (Figs. 1 B, C) and occasionally from cells near the vascular bundles. A detail of a meristematic region, with most of the cells dividing periclinally and anticlinally, of small size, thin wall, lack of vacuole, abundant cytoplasm and prominent nucleus, can be observed in Figure 2. The size of meristematic regions increased with the number of days in culture (Fig. 1) and became significant on day 7, as shown by the repeated measures analysis. On that day, shoot primordia were observed in explants cultured in 0.5 μmol/L NAA plus 0.5 μmol/L TDZ (Fig. 1 D).

Table 2. Adventitious shoot and root regeneration from leaves of White Sim and Early Sam carnation cultivars (*Dianthus caryophyllus* L.) after 30 days cultured in MS solid media at a range of NAA, BA and TDZ concentrations.

Media (μmol/L)			Shoots per leaf (no.)[a] Cultivar		Roots per leaf (no.)[a] Cultivar	
NAA	BA	TDZ	White Sim[b]	Early Sam[b]	White Sim[b]	Early Sam[b]
0.0	0.0	0.0	0.23±0.08 a	0.05±0.03 a	0.60±0.14 b	0.25±0.09 ab
0.5	0.0	0.0	0.52±0.24 a	0.22±0.08 ab	2.75±0.34 c	2.03±0.24 c
0.5	0.5	0.0	0.47±0.15 a	0.43±0.18 ab	0.30±0.10 ab	0.50±0.16 b
0.5	2.5	0.0	2.11±0.48 b	0.54±0.18 ab	0.13±0.08 a	0.01±0.01 a
0.5	5.0	0.0	3.57±0.64 c	0.74±0.20 b	0.01±0.01 a	0.05±0.05 a
0.5	0.0	0.5	4.27±0.72 c	1.69±0.31 c	0.03±0.02 a	0.08±0.05 a

[a] Values represent the mean ± SE. The total number of explants per treatment (medium) was 75–85 for each cultivar.
[b] Within each cultivar, distinct letters denote significant differences (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test.

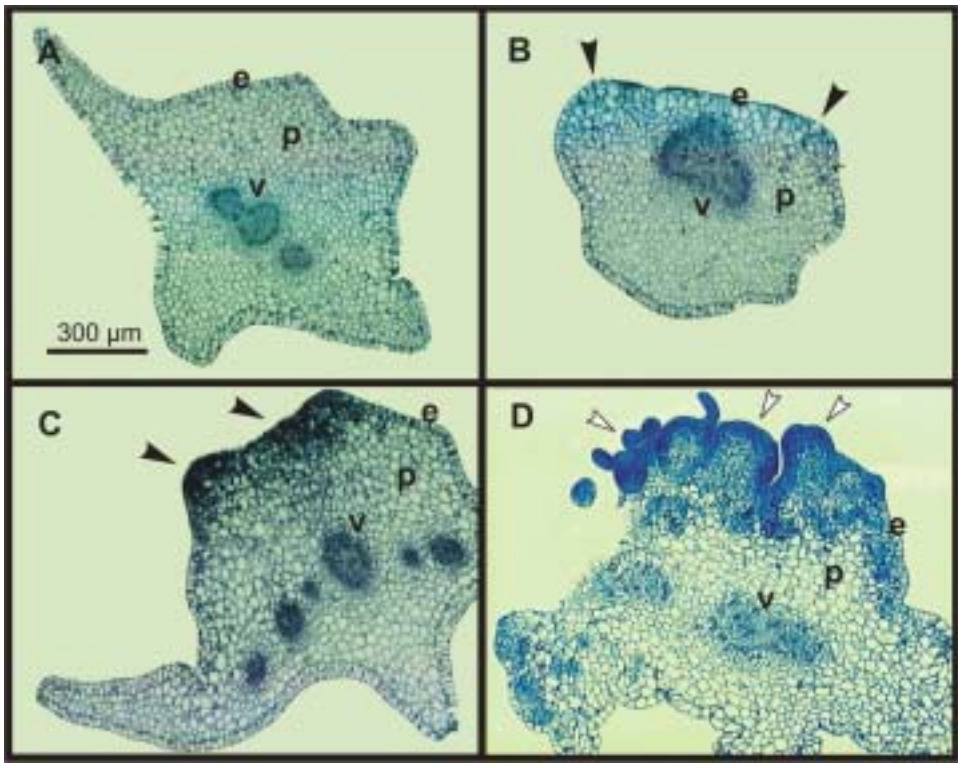

Figure 1. Transversal sections of the basal region of carnation petals (*Dianthus caryophyllus* L. cv. White Sim). (A), fresh petal; (B), (C), (D), petals after 3, 5 and 7 days of culture in MS medium supplemented with 0.5 μmol/L NAA and 0.5 μmol/L TDZ. (e), epidermis; (p), parenchyma; (v), vessels. The adaxial face of the petals is shown in the upper part of the photographs. Black arrow heads indicate meristematic areas; white arrow heads indicate shoot primordia.

On day 7, the proportion of meristematic regions depended on the TDZ concentration in the medium, increasing from 0, at which cell division was not detected, to 0.5 μmol/L of TDZ (Table 3). Moreover, on day 7 the percentage of the area of meristematic regions per petal was directly and linearly related to the number of shoots obtained per petal on day 30 ($R^2 = 0.99$, $p = 0.001$) (Table 3). Thus, we were able to estimate the shoot regeneration capacity of petal explants on day 7 of culture. Therefore, day 7 was used to analyse the levels and localisation of endogenous hormones in White Sim petal explants.

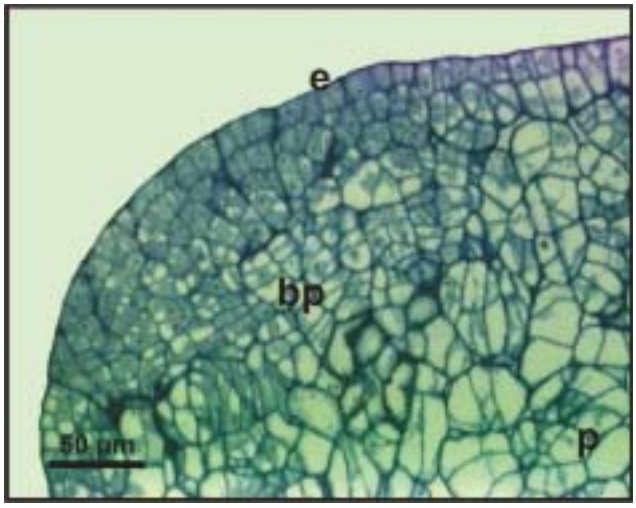

Figure 2. Meristematic region of the basal part of a carnation petal (*Dianthus caryophyllus* L. cv. White Sim) cultured for 5 days in MS medium supplemented with 0.5 µmol/L NAA and 0.5 µmol/L TDZ. (bp), bud primordium; (e), epidermis; (p), parenchyma.

Table 3. Meristematic regions (after 7 days) and adventitious shoot regeneration (after 30 days) in petal bases of carnation (*Dianthus caryophyllus* L. cv. White Sim) cultured in MS solid media at a range of NAA and TDZ concentrations.

Media (µmol/L)		Area of meristematic regions (%)[a]	Shoots per petal (no.)[b]
NAA	TDZ	Day 7	
0.5	0.0	0.00± 0.00 a	0.11±0.05 a
0.5	0.005	1.82± 0.75 a	0.19±0.07 a
0.5	0.05	16.18± 5.44 ab	1.70±0.28 b
0.5	0.5	36.74±15.26 b	4.07±0.54 c

[a] Values represent the mean ± SE of 5 histological sections. Distinct letters denote significant differences between media (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test.

[b] Values represent the mean ± SE of 130–140 explants. Distinct letters denote significant differences between media (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test.

Effect of NAA and TDZ on endogenous phytohormone levels during the induction of shoot organogenesis in petal explants

ZR (203 pmol g^{-1} dry weight) was the most abundant CK in the bases of fresh White Sim petals (day 0), followed by iP (140 pmol g^{-1}) and Z (100 pmol g^{-1}). DHZ (79 pmol g^{-1}) and its riboside (83 pmol g^{-1}) had similar levels and iPR was the lowest (26 pmol g^{-1}) (Fig. 3). On day 7 of culture, all petal explants showed a total amount of CKs, the sum of iP, Z, DHZ and their ribosides, similar to that of fresh petals. However, significant changes in the levels of specific CKs were detected. The levels of iP in petals cultured in 0.5 µmol/L NAA plus 0.5 µmol/L TDZ medium doubled those of fresh petals (Fig. 3 A). Thus, in this medium iP was more abundant than ZR. ZR increased in petals cultured in media with NAA alone (0.5 µmol/L) or with NAA plus 0.005 µmol/L TDZ (Fig. 3 B), whereas petals cultured with higher TDZ concentrations had the same levels of ZR as the fresh petals. In petals cultured in any of the media, the amount of DHZR decreased to half of

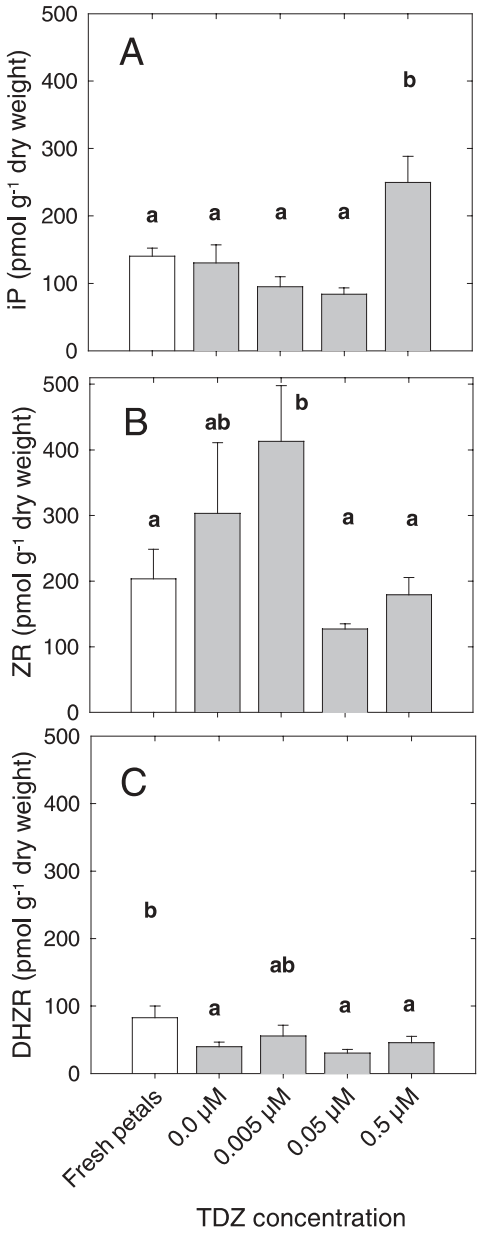

Figure 3. Content of (A) iP, (B) ZR and (C) DHZR in fresh carnation petal bases (*Dianthus caryophyllus* L. cv. White Sim), and after 7 days in MS media supplemented with 0.5 µmol/L NAA and a range of TDZ concentrations. Values represent the mean ± SE. Distinct letters denote significant differences (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test. (iP), N^6-(Δ^2-isopentenyl)adenine; (ZR), zeatin riboside; (DHZR), dihydrozeatin riboside.

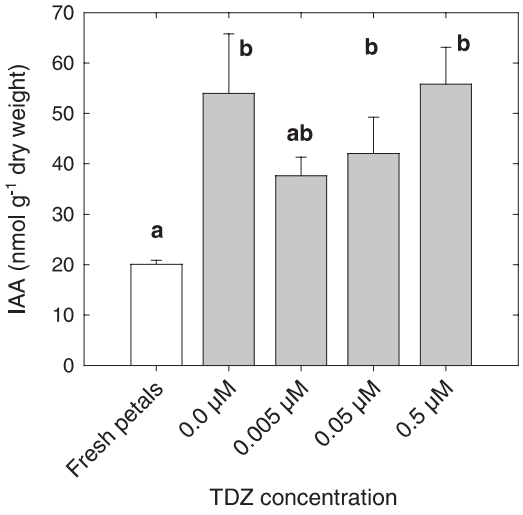

Figure 4. IAA content in fresh carnation petal bases (*Dianthus caryophyllus* L. cv. White Sim), and after 7 days in MS media supplemented with 0.5 µmol/L NAA and a range of TDZ concentrations. Values represent the mean ± SE. Distinct letters denote significant differences (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test. (IAA), indole-3-acetic acid.

that of fresh petals (Fig. 3 C). No changes were detected in the levels of iPR, Z and DHZ in any of the media.

IAA significantly accumulated (2.5-fold) in petals cultured in NAA (0.5 µmol/L) for seven days (Fig. 4). The addition of TDZ (0.005, 0.05 and 0.5 µmol/L) did not induce remarkable changes in the endogenous auxin content (Fig. 4). Owing to this IAA increase, the ratio IAA/total CKs in all cultured petals was higher than in fresh petals. In the latter, this ratio was 32, whereas after seven days of culture, it was 2–3 times higher and TDZ-independent.

Immunolocalization of endogenous cytokinins during the induction of shoot organogenesis in petal explants

Remarkable labelling of CK ribosides, using anti-iPR, anti-ZR or anti-DHZR antibodies, was observed in the tips of developing bud primordia of White Sim petals cultured for 7 days in 0.5 µmol/L NAA plus 0.5 µmol/L TDZ (Figs. 5 B, C, D). Moreover, quite strong labelling of the three CK ribosides was also observed in all of the adaxial part, from the epidermis to the vessels. Subepidermis of lateral parts, between adaxial and abaxial faces, also presented immunoreactivity, although in a lesser extent than in the adaxial face. The rest of the petal showed very few silver marks. Control sections, with the primary antibody omitted, showed no labelling (Fig. 5 A).

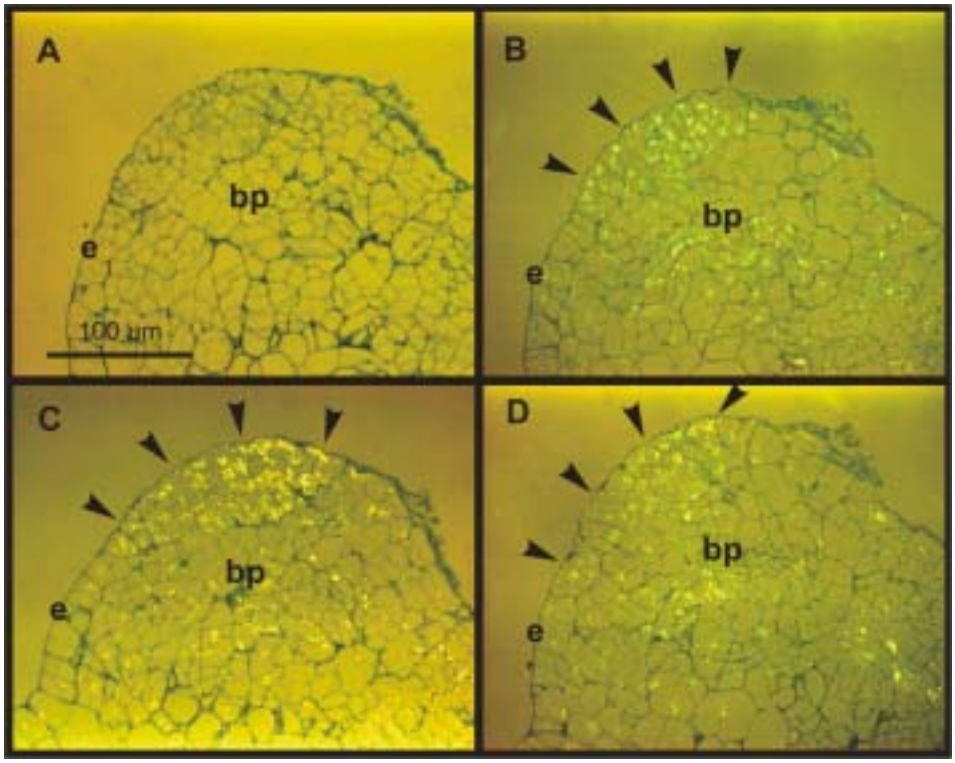

Figure 5. Immunolocalization of CK ribosides in transversal sections of the basal region of carnation petals (*Dianthus caryophyllus* L. cv. White Sim) cultured for 7 days in MS medium with 0.5 µmol/L NAA and 0.5 µmol/L TDZ, using protein A-gold and silver-enhancement method (Materials and methods). Sections were incubated with (B) anti-iPR, (C) anti-ZR and (D) anti-DHZR. Control sections (A) were incubated without the anti-CK antibodies. (bp), bud primordium; (e), epidermis. Black arrow heads indicate the CK labelling mainly in the tip of the bud primodium.

Discussion

Our results show that organogenesis in carnation depends on the type and balance of growth regulators, the explant used and the genotype (cultivar). In leaves, TDZ is more effective than BA in inducing shoot organogenesis, which is consistent with data on other carnation cultivars and other species (Nakano et al. 1994, Malik and Saxena 1992). This greater effectiveness might be due to the slow metabolism of TDZ in tissue culture systems (Mok and Mok 1985). TDZ alone is highly effective in inducing shoot organogenesis from White Sim petals, as reported for other cultivars and species (Frey and Janick 1991, Ricci et al. 2001), although auxins may be required. Moreover, TDZ acts synergistically with NAA in the shoot regeneration from petals, a response that has not been reported for other cultivars (Frey and Janick 1991). The TDZ plus NAA treatments that maximize shoot regeneration both in leaves and petals also increase shoot vitrification, an abnormality that is often associated with carnation (Genkov et al. 1997). Thus, for each cultivar and explant, the plant growth regulators should be optimised to maximize the number of unvitrified shoots. Leaf explants regenerate shoots and roots in a growth regulator-free medium, whereas petals do not. Therefore, the explant affects the shoot and root regeneration capacity, as reported elsewhere (Frey and Janick 1991, van Altvorst et al. 1992). White Sim explants, like other Sim cultivars (Frey and Janick 1991, van Altvorst et al. 1992), show the highest organogenic potential of all the cultivars tested.

Shoot formation in leaves is restricted to the basal zone, in agreement with other findings (van Altvorst et al. 1992). However, TDZ treatment increases the organogenic surface in White Sim petals, since shoots arise not only from the bottom of the bases, as reported in most studies on carnation petals (Zuker et al. 1998), but also from other parts of the petal. Similar results were obtained with BA but using liquid media (Fisher et al. 1993). In this study, shoot primordia is mainly undergone by epidermal and subepidermal cells of the adaxial surface, although other authors have found shoot primordia coming from cells near the vascular region (Frey and Janick 1991).

Our results in White Sim petal explants show that TDZ, in the presence of NAA, stimulates cell proliferation in a concentration-dependent manner, as reported for CKs (Francis and Sorrell 2001). This effect is noticeable after seven days of culture, when the meristematic activity of petals strongly correlates with the number of shoots obtained from these explants after 30 days. On day 7, immunolocalizations of iPR, ZR and DHZR in the meristematic tips of bud primordia, and also in the adaxial face of the petals, with cells that may still retain proliferative capacity, are consistent with the role played by the CKs in the control of cell division (Ivanova et al. 1994, Jacqmard et al. 1994, Francis and Sorrell 2001). Establishing an analogy between carnation adventitious bud primordia and apical bud primordia, iP- and Z-type CKs are also present in vegetative apical tissues of tomato and *Sinapis alba* (Sossountzov et al. 1988, Jacqmard et al. 2002). Similarly, CKs are detected in tobacco apical shoot meristem (Dewitte et al. 1999). Moreover, studies with transgenic tobacco plants with reduced CK levels show they are required to maintain the cell division cycle in the apical meristem, and might be involved in cell differentiation (Werner et al. 2001). Thus, we suggest that the accumulation of CKs during TDZ-induced shoot organogenesis indicates they may play a role in organogenesis of bud primordia, mainly in cell proliferation but also at the beginning of differentiation. However, this CK concentration has not been detected quantitatively, since quantification considers dividing regions and non dividing regions, and so local CK changes have been masked.

Measurement of hormone levels on day 7 shows that the increase in IAA and the decrease in DHZR compared with fresh petals may be due to exogenous NAA, since they are not dependent on the TDZ concentration in the media. Accordingly, one week of TDZ treatment did not alter IAA levels during embryogenesis in peanut (Murthy et al. 1995), but increased them in geranium (Hutchinson et al. 1996). Translocation of auxin has been shown to be essential for TDZ-induced embryogenesis (Hutchinson et al. 1996). The outstanding increase in ZR of petals induced by low doses of TDZ may result from its ribotide, since TDZ promotes the conversion of CK ribotides to ribosides in callus tissues (Capelle et al. 1983). This increase in ZR has been observed in petals without, or with few meristematic cells that might undergo proliferation. In fact, Z-type CKs have also been related to mitotic processes in several systems (Nishinari and Syono 1980, Redig et al. 1996, Laureys et al. 1998, Dobrev et al. 2002). Petals cultured with elevated concentrations of TDZ show a high degree of cell proliferation and the onset of shoot differentiation, which are related to an increase in iP levels. iP, together with Z and DHZ, also increased during TDZ-induced somatic embryogenesis in geranium and peanut hypocotyls (Hutchinson et al. 1996, Victor et al. 1999). This iP increase may be due to the TDZ-induced inhibition of CK oxidase activity, proposed as one explanation of the CK-like activity of phenylureas (Hare and van Staden 1994).

In addition to induce changes in endogenous CKs, we cannot exclude the possibility that TDZ promotes growth through its own biological activity (Mok and Mok 1985), since common CK-specific binding receptors for both adenine-type and phenylurea CKs have been discovered (Nagata et al. 1993, Yamada et al. 2001). Our results also support this hypothesis, since very few organogenic responses have been observed in the absence of TDZ. Moreover, cell proliferation and shoot regeneration, both CK-regulated processes, have increased with TDZ concentration. This report provides new insights into the effect of TDZ on endogenous CKs during organogenesis and their involvement in cell proliferation and differentiation.

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References

Capelle SC, Mok DWS, Kirchner SC, Mok MC (1983) Effects of thidiazuron on cytokinin autonomy and the metabolism of N^6-(Δ^2-isopentenyl)[8-^{14}C]adenosine in callus tissues of *Phaseolus lunatus* L. Plant Physiol 73: 796–802

Casanova E, Zuker A, Trillas MI, Moysset L, Vainstein A (2003) The *rolC* gene in carnation exhibits cytokinin- and auxin-like activities. Sci Hort 97: 321–331

Chen C-M (1997) Cytokinin biosynthesis and interconversion. Physiol Plant 101: 665–673

Coenen C, Lomax TL (1997) Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci 2: 351–356

Dewitte W, van Onckelen HA (2001) Probing the distribution of plant hormones by immunocytochemistry. Plant Growth Regul 33: 67–74

Dewitte W, Chiappetta A, Azmi A, Witters E, Strnad M, Rembur J, Noin M, Chriqui D, van Onckelen HA (1999) Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. Plant Physiol 119: 111–121

Dobrev P, Motyka V, Gaudinová A, Malbeck J, Trávnícková A, Kamínek M, Vanková R (2002) Transient accumulation of *cis*- and *trans*-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. Plant Physiol Biochem 40: 333–337

Fernández B, Centeno ML, Feito I, Sánchez-Tamés R, Rodríguez A (1995) Simultaneous analysis of cytokinins, auxins and abscisic acid by combined immunoaffinity chromatography, high performance liquid chromatography and immunoassay. Phytochem Anal 6: 49–54

Fisher M, Ziv M, Vainstein A (1993) An efficient method for adventitious shoot regeneration from cultured carnation petals. Sci Hort 53: 231–237

Francis D, Sorrell DA (2001) The interface between the cell cycle and plant growth regulators: a mini review. Plant Growth Regul 33: 1–12

Frey L, Janick J (1991) Organogenesis in carnation. J Amer Soc Hort Sci 116: 1108–1112

Genkov T, Tsoneva P, Ivanova I (1997) Effect of cytokinins on photosynthetic pigments and chlorophyllase activity in *in vitro* cultures of axillary buds of *Dianthus caryophyllus* L. J Plant Growth Regul 16: 169–172

Gimelli F, Ginatta G, Venturo R, Positano S, Buiatti M (1984) Plantlet regeneration from petals and floral induction *in vitro* in the Mediterranean carnation (*Dianthus caryophyllus* L.). Riv Ortoflorofrutt It 68: 107–121

Hare PD, Van Staden J (1994) Inhibitory effect of thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant Cell Physiol 35: 1121–1125

Hutchinson MJ, KrishnaRaj S, Saxena PK (1996) Morphological and physiological changes during thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) hypocotyl cultures. Int J Plant Sci 157: 440–446

Ivanova I, Todorov IT, Atanassova L, Dewitte W, van Onckelen HA (1994) Co-localization of cytokinins with proteins related to cell proliferation in developing somatic embryos of *Dactylis glomerata* L. J Exp Bot 45: 1009–1017

Jacqmard A, Detry N, Dewitte W, van Onckelen HA, Bernier G (2002) *In situ* localisation of cytokinins in the shoot apical meristem of *Sinapis alba* at floral transition. Planta 214: 970–973

Jacqmard A, Houssa C, Bernier G (1994) Regulation of cell cycle by cytokinins. In: Mok DWS, Mok MC (eds) Cytokinins: Chemistry, Activity, and Function. CRC Press, Boca Raton pp 197–215

Jain A, Kantia A, Kothari SL (2001) *De novo* differentiation of shoots buds from leaf-callus of *Dianthus caryophyllus* L. and control of hyperhydricity. Sci Hort 87: 319–326

Jensen MH, Malter AJ (1995) Protected agriculture, a global review. World Bank Technical Paper 253: 144–146

Laureys F, Dewitte W, Witters E, van Montagu M, Inzé D, van Onckelen HA (1998) Zeatin is indispensable for the G_2-M transition in tobacco BY-2 cells. FEBS Lett 426: 29–32

Malik KA, Saxena PK (1992) Regeneration in *Phaseolus vulgaris* L.: High-frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. Planta 186: 384–389

McGaw BA, Burch LR (1995) Cytokinin biosynthesis and metabolism. In: Davies PJ (ed) Plant Hormones: Physiology, Biochemistry and Molecular Biology. Kluwer Academic Publishers, Dordrecht pp 98–117

Mok DWS, Mok MC (2001) Cytokinin metabolism and action. Ann Rev Plant Physiol Mol Biol 52: 89–118

Mok MC, Mok DWS (1985) The metabolism of [^{14}C]-thidiazuron in callus tissues of *Phaseolus lunatus*. Physiol Plant 65: 427–432

Mok MC, Mok DWS, Armstrong DJ, Shudo K, Isogai Y, Okamoto T (1982) Cytokinin activity of *N*-phenil-*N*′-1,2,3-thidiazol-5-ylurea (thidiazuron). Phytochemistry 21: 1509–1511

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Murthy BNS, Murch SJ, Saxena PK (1995) Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): Endogenous growth regulator levels and significance of cotyledons. Physiol Plant 94: 268–276

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. In Vitro Cell Dev Biol 34: 267–275

Nagata R, Kawachi E, Hashimoto Y, Shudo K (1993) Cytokinin-specific binding protein in etiolated mung bean seedlings. Biochem Biophys Res Comm 191: 543–549

Nakano M, Hoshino Y, Mii M (1994) Adventitious shoot regeneration from cultured petal explants of carnation. Plant Cell Tiss Org Cult 36: 15–19

Nishinari N, Syono K (1980) Identification of cytokinins associated with mitosis in synchronously cultured tobacco cells. Plant Cell Physiol 21: 383–393

Redig P, Shaul O, Inzé D, van Montagu M, van Onckelen HA (1996) Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett 391: 175–180

Ricci A, Carra A, Torelli A, Maggiali CA, Vicini P, Zani F, Branca C (2001) Cytokinin-like activity of N′-substituted N-phenylureas. Plant Growth Regul 34: 167–172

Sossountzov L, Maldiney R, Sotta B, Sabbagh I, Habricot Y, Bonnet M, Miginiac E (1988) Immunocytochemical localization of cytokinins in Craigella tomato and a sideshootless mutant. Planta 175: 291–304

Sotta B, Stroobants C, Sossountzov L, Maldiney R, Miginiac E (1992) Immunochemistry applied to cytokinins: Techniques and their validation. In: Kamínek M, Mok DWC, Zazímalová E (eds) Physiology and Biochemistry of Cytokinins in Plants. SPB Academic Publishing bv, The Hague, The Netherlands pp 455–460

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electronmicroscopy. J Ultrasctruct Res 26: 31–43

van Altvorst AC, Koehorst HJJ, Bruinsma T, Dons JJM (1994) Improvement of adventitious shoot formation from carnation leaf explants. Plant Cell Tiss Org Cult 37: 87–90

van Altvorst AC, Koehorst HJJ, Bruinsma T, Jansen J, Custers JBM, de Jong J, Dons JJM (1992) Adventitious shoot formation from *in vitro* leaf explants of carnation (*Dianthus caryophyllus* L.). Sci Hort 51: 223–235

Victor JMR, Murthy BNS, Murch SJ, KrishnaRaj S, Saxena PK (1999) Role of endogenous purine metabolism in thidiazuron-induced somatic embryogenesis of peanut (*Arachis hypogaea* L.). Plant Growth Regul 28: 41–47

Visser C, Qureshi JA, Gill R, Saxena PK (1992) Morphoregulatory role of thidiazuron. Plant Physiol 99: 1704–1707

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci 98: 10487–10492

Woodson WR (1991) Biotechnology of floricultural crops. HortScience 26: 1029–1033

Yamada K, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol 42: 1017–1023

Zuker A, Ahroni A, Tzfira T, Ben-Meir H, Vainstein A (1999) Wounding by bombardment yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Mol Breed 5: 367–375

Zuker A, Tzfira T, Vainstein A (1998) Genetic engineering for cut-flower improvement. Biotechnol Adv 16: 33–79

CAPÍTOL 3

Efecte de la concentració d'agar en el medi i del tancament del recipient de cultiu en l'organogènesi de tiges adventícies de clavell i la seva hiperhidricitat

Efecte de la concentració d'agar en el medi i del tancament del recipient de cultiu en l'organogènesi de tiges adventícies de clavell i la seva hiperhidricitat

Resum

Les plàntules de clavell (*Dianthus caryophyllus* L.) cultivades *in vitro* sovint desenvolupen anomalies morfològiques i fisiològiques, fenomen anomenat hiperhidricitat, que impedeix la seva supervivència *ex vitro*. Quan s'ha incrementat la concentració d'agar en el medi de creixement (de 0 a 12 g l^{-1}), així reduint la disponibilitat d'aigua, s'ha trobat una disminució en la hiperhidricitat de les tiges adventícies regenerades a partir dels pètals de clavell. Aquesta disminució ha anat acompanyada per una reducció progressiva en el contingut d'aigua de les tiges, en el seu pes i en el nombre de capes de cèl·lules de les fulles i el tamany d'aquestes cèl·lules. Tanmateix, el nombre de tiges regenerades també ha disminuït (4 vegades de 2 a 12 g l^{-1}). De manera similar, en tubs ventilats, amb una humitat relativa més baixa que en els tubs hermèticament tancats, l'organogènesi de tiges ha disminuït juntament amb el contingut d'aigua de les tiges. Per tant, la humitat relativa i la disponibilitat d'aigua en els recipients de cultiu afecta de manera important l'organogènesi de tiges adventícies en clavell i influeix en la hiperhidricitat d'aquestes tiges.

Casanova E, Moysset L, Trillas MI. Effect of agar concentration of the medium and vessel closure on the organogenesis of adventitious carnation shoots and their hyperhydricity. Plant Cell Rep. (enviat).

Effect of agar concentration of the medium and vessel closure on the organogenesis of adventitious carnation shoots and their hyperhydricity

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Carnation plantlets (*Dianthus caryophyllus* L.) cultured *in vitro* often develop morphological and physiological anomalies, a phenomenon called hyperhydricity, which impair their survival *ex vitro*. When the agar concentration of the growth medium was increased (from 0 to 12 g l^{-1}), thus reducing water availability, the hyperhydricity of adventitious shoots regenerated from carnation petals decreased. This was accompanied by a progressive fall in the water content of the shoots, their weight and the number of leaf cell layers and the size of these cells. However, the number of regenerated shoots also decreased (4-fold from 2 to 12 g l^{-1} agar). Similarly, in ventilated tubes, with lower relative humidity than tightly closed tubes, shoot organogenesis diminished with shoot water content. Therefore, relative humidity and water availability in culture vessels greatly affect adventitious shoot organogenesis in carnation and influence shoot hyperhydricity.

Keywords: *Dianthus caryophyllus*, hyperhydricity, *in vitro* culture, organogenesis.

I. INTRODUCTION

The carnation (*Dianthus caryophyllus* L.) is one of the leading commercial cut flowers worldwide and breeders attempt to create cultivars with new features. Although the high heterozygosity of this species severely restricts conventional breeding programs (Vainstein 2002), *in vitro* tissue culture techniques combined with genetic engineering can provide an alternative method to improve quality traits (Zuker et al. 1999; Casanova et al. 2003, 2004).

The growth and development rates of plant cultures *in vitro* are determined genetically, although they are limited by the physical and chemical microenvironment of the culture vessels. Most studies have been performed on the chemical environment (Fujiwara and Kozai 1995). Some environmental factors may also affect the quality of the plantlets, which could develop morphological and physiological anomalies (Ziv 1991), which are collectively referred to by the term hyperhydricity (Debergh et al. 1992). These disorders, often observed in carnation cultures (Mii et al. 1990; Ziv 1991), make the leaves glassy and translucent and do not allow the survival of micropropagated plants *ex vitro* (Ziv 1991; Kevers et al. 2004). Hyperhydric plants have a higher water content than normal (healthy) plants, lower chlorophyll levels and defective deposition of epicuticular waxes (Ziv 1991; Debergh et al. 1992; Kevers et al. 2004). However, the main cause of excessive water loss during acclimatization may be the malformed stomata, although their density in hyperhydric leaves is lower than that in leaves of normal plantlets (Ziv 1991; Majada et al. 2001; Kevers et al. 2004). Hyperhydricity, which is a hindrance to tissue culture, remains to be solved (Kevers et al. 2004).

Some requirements for shoot proliferation *in vitro*, such as excess nutrients, high concentration of plant-growth regulators or low light intensity, can contribute to plant malformation. However, the relative humidity (RH) of the culture atmosphere and the water potential of the medium are the main factors responsible for the hyperhydricity of plantlets grown *in vitro* (Ziv 1991; Fujiwara and Kozai 1995). Water potential is mainly regulated by the solutes (osmotic potential) and the gelling agents (matric potential) of the medium (Ziv 1991; Fujiwara and Kozai 1995). The higher the gelling agent concentration, the lower the water potential and, consequently, the lower the availability of water and dissolved substances (Smith and Spomer 1995).

Compared with the use of liquid medium, the addition of gelling agents like agar or gelrite gellan gum can circumvent the abnormal morphogenesis of plants (Ziv 1991; Debergh et al. 1992). However, the propagation rate (Debergh et al. 1981; Ziv et al. 1983; Mackay and Kitto 1988; Turner and Singha 1990; Yadav et al. 2003) and adventitious shoot organogenesis (Brown et al. 1979; Bornman and Vogelman 1984; Castro-Concha et al. 1990; Fisher et al. 1993) can be negatively affected, decreasing with increasing gelling agent concentration. Few studies have addressed the effect of gelling agents on the morphogenesis of adventitious organs (Fisher et al. 1993; Watad et al. 1996).

In culture vessels, the lower the RH, the fewer hyperhydric shoots are obtained (Ziv 1991; Fujiwara and Kozai 1995). RH can be reduced using dessicants (Ziv et al. 1983) or by ventilation (Debergh et al. 1992; Majada et al. 1997). Similarly to the effects of an increase in gelling agents, a decrease in the RH of the vessels also reduces the growth and propagation rate (Ziv et al. 1983; Sallanon and Maziere 1992; Majada et al. 1997) and, supposedly, adventitious morphogenesis, although to our knowledge there is no information about this effect.

It is widely accepted that hyperhydricity of cultured plants is mainly caused by high RH and high water availability in the vessel (Ziv 1991; Fujiwara and Kozai 1995). Here we studied the effect of decreased vessel RH and the

effect of agar concentration on adventitious shoot organogenesis from petal explants of carnation, cv. White Sim and cv. Early Sam, in a growth regulator-optimized media. We also analyzed their effect on the degree of hyperhydricity of the regenerated shoots and on the morphology and ultrastructure of their leaves. Hyperhydricity, which is usually considered a qualitative character, was quantified as the dry weight percentage of the shoots, and a putative relation with the number of layers and size of the leaf parenchyma cells was also examined.

II. MATERIALS AND METHODS

A. Plant material

Carnation plants, *Dianthus caryophyllus* L. cv. White Sim (Sim type) and cv. Early Sam (spray type), were grown in standard greenhouse conditions under a natural photoperiod at a latitude of 42° North (Barcelona, Spain). Young flower buds were removed, stored at 4°C for a maximum of 15 days and then used as the petal explant source.

B. Tissue culture

Flower buds were surface-sterilized for 5 s with 96% (v/v) ethanol. Petals were excised and basal parts were placed in MS basal medium (Murashige and Skoog 1962) with 2 mg l^{-1} glycine, 50 mg l^{-1} myo-inositol, 0.5 mg l^{-1} thiamine-HCl and 30 g l^{-1} sucrose (Gimelli et al. 1984). Medium for the culture of White Sim petals was supplemented with 0.5 μM 1-naphthaleneacetic acid (NAA) plus 0.5 μM thidiazuron or N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (TDZ) and medium for Early Sam with 5.0 μM NAA plus 5.0 μM TDZ, since they maximize the number of non-hyperhydric shoots from these explants (Casanova et al. 2004). Before autoclaving (121°C for 20 min), pH was adjusted to 5.8 and media were solidified with appropriate agar concentrations (High Gel Strength, Sigma), except the liquid medium.

Adventitious shoot regeneration, measured as the number of shoots per explant and the percentage of shoot-forming explants, was assessed after 30 days of culture by means of a stereomicroscope.

1. *Evaluation of the effect of tube closure on organogenesis of adventitious shoots and their hyperhydricity*

This experiment was performed with petals of White Sim and Early Sam cultivars, the flower buds having been removed in December. All media were solidified with 8 g l^{-1} agar (Casanova et al. 2004). Petal explants were cultured in 52-ml Pyrex tubes, containing 15 ml of medium (3-4 petals per tube), closed with aluminium caps. In each cultivar, half of the tubes were tightly sealed with paraffin film. Since caps were provided with an inner spring which prevented tight closure, the other half of the tubes were loosely closed, thereby allowing air exchange. For each treatment (cultivar and type of tube closure) we used 144 tubes. To minimize variability in the regeneration capacity of each flower bud, petals from the same bud were distributed between both tube closure groups. Cultures were kept in a growth chamber at 25.0±1.5°C in a 16-h photoperiod, using cool white fluorescent tubes (F72T12/CW/VHO 160W, Sylvania) supplemented with incandescent bulbs (Krypton 70W, Sylvania) at 65 μmol m^{-2} s^{-1}.

For each cultivar and type of tube closure, fresh weight (FW) and dry weight (DW) (60°C for 48 h) of all the regenerated shoots were measured. The percentage of DW [DWx100/FW] was used to quantify the degree of hyperhydricity, as reported elsewhere (Kevers et al. 1984; Turner and Singha 1990; Genkov et al. 1997).

RH and temperature were measured inside the two tube closure groups by capacitive humidity and temperature sensors (Vaisala HMP113Y, Finland). The sensors were inserted into caps, which replaced the original caps. Those that were sealed with paraffin film originally were also sealed in the same way in this case. The measurements began after several hours of stabilization. Once per week, several measurements were taken during the light and dark period, with a total of 56 readings during light and 16 during dark periods for each type of tube closure.

2. *Evaluation of the effect of agar concentration of the medium on organogenesis of adventitious shoots and their hyperhydricity*

This experiment was performed with petals of the White Sim cultivar, since it was the most regenerative, and the flower buds were removed in March. The medium was solidified with a range of agar concentrations (0, 2, 4, 6, 8, 10 and 12 g l^{-1}). Petal explants were cultured in 500-ml glass vessels with 100 ml of solid medium (30-35 petals per vessel, one vessel per treatment). The vessels contained 15 ml of liquid medium, following Fisher et al. (1993), in order to cover the bottom of the vessel but allow the explants contact with air. Vessels with liquid medium (15-17 petals per vessel, two vessels per treatment) were continuously agitated on an orbital shaker (100 rpm) and on days 15 and 21 of culture, 6 ml of medium was added. The experiment was performed in triplicate. To minimize variability, petals from the same flower bud were randomly distributed among media. These cultures were kept in a growth chamber at 25±1°C in a 16-h photoperiod using cool white fluorescent tubes (TLD 58W/33, Philips, France, 80 μmol m^{-2} s^{-1}).

For each agar treatment, the FW and DW (60°C for 48 h) of all the regenerated shoots were measured. The percentage of DW and the water content [(FW-DW)x100/FW] were used to quantify the degree of hy-

Cultivar	Growth regulators (μM)		Type of caps	Shoots per petal (no.)[a]	Shoot FW (mg)	Shoot DW (mg)	Shoot DW (%)
	NAA	TDZ					
White Sim	0.5	0.5	Tight	2.80 ± 0.18	13.36	0.92	6.75
			Loose	2.05 ± 0.18**	8.77	0.77	8.74
Early Sam	5.0	5.0	Tight	0.97 ± 0.11	3.03	0.26	8.69
			Loose	0.70 ± 0.08*	1.87	0.18	9.87

[a] Values are the mean ± SE. The total number of explants per treatment (type of tube closure) was 490-520 for White Sim and 460-470 for Early Sam. Within each cultivar, significant differences between treatments (one-way ANOVAs) are indicated by: (*) $p < 0.05$ and (**) $p < 0.01$.

TABLE I Adventitious shoot regeneration, and the fresh weight (FW), dry weight (DW) and the percentage of DW of the regenerated shoots, from petals of White Sim and Early Sam carnation cultivars (*Dianthus caryophyllus* L.) after 30 days cultured in MS media supplemented with NAA plus TDZ and solidified with 8 g l^{-1} agar, in tubes with tight or loose caps.

perhydricity.

C. Histological and ultrastructural analyses

Histological studies were performed on the leaves of shoots regenerated from White Sim petals cultured in media with a range of agar concentrations. Leaves, collected at the same time as regenerated shoots were quantified, were fixed in 2% (v/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 100 mM phosphate buffer, pH 7.4, under vacuum at 4°C for 1-2 days, postfixed in 1% (w/v) OsO_4 in the same buffer, dehydrated in an acetone series and embedded in Spurr's resin (Spurr 1969). Transversal thin sections (1 μm) were stained with 0.5% (w/v) methylene blue, observed under an Olympus BH2-UMA light microscope and recorded with a JVC TK1270 color video camera. Three leaf samples were analyzed per medium by quantifying the area of 55-60 parenchyma cells per sample with the "Imat" software, developed by the *Serveis Cientificotècnics* at the University of Barcelona. The number of parenchyma cell layers was also counted in four transects in each leaf sample, cut close to the central nerve. Because the palisade parenchyma was still not differentiated from the spongy parenchyma in these 30-day-old leaves, we counted the layers and measured the area of all the parenchyma cells between the two epidermal layers.

For ultrastructural analysis, ultrathin sections (50-60 nm) were cut from shoot leaves developed on media with 0, 8 and 12 g l^{-1} agar, stained with 2% (w/v) aqueous uranyl acetate for 30 min and lead citrate (Reynolds 1963) for 10 min, and observed with a transmission electron microscope JEOL 1010 operated at 80 kV.

D. Statistical analyses

Differences in shoot regeneration of the explants, for each cultivar, and in the RH inside the tubes between the two types of tube closure were tested using one-way ANOVAs. Differences in shoot regeneration between media with a range of solidification degrees, in the number of parenchyma cell layers and in the area of these cells between these growing media were also tested using one-way ANOVAs. Duncan's multiple range test was applied when one-way ANOVAs showed significant differences ($p < 0.05$). Correlations between the agar concentrations and all the variables were also analysed. All the statistical analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL.).

III. RESULTS

A. Effect of tube closure on organogenesis of adventitious shoots and their hyperhydricity

The adventitious shoot regeneration capacity of petals of the White Sim and Early Sam carnation cultivars, cultured in either tightly or loosely closed tubes, differed. The number of regenerated shoots was significantly higher in the tightly closed (non-ventilated) tubes, 37% higher in White Sim and 39% in Early Sam, compared with the loosely closed (ventilated) tubes (Table 1). The FW of the regenerated shoots, and their DW, were also higher in non-ventilated tubes in both cultivars (Table 1). In these tubes, shoots appeared normal, and almost none displayed hyperhydricity. However, shoots in the ventilated tubes looked dry. This was reflected in the percentage of shoot DW, which was higher in ventilated tubes in both cultivars (Table 1).

During the light period, with 26.2 (± 0.1) °C inside the tubes, the RH was 90.0 (± 0.5)% in ventilated tubes, and air exchange caused evaporation of water from the media. Non-ventilated tubes presented 94.3 (± 0.3) % of RH. In the dark period, with 23.6 (± 0.2) °C, the RH was 91.2 (± 1.4) % in the ventilated tubes and 97.8 (± 0.6) % in the non-ventilated ones. In both periods, the RH in non-ventilated tubes was significantly higher ($p = 0.000$). The RH outside the tubes was 20-40 %.

Agar concentration (g l^{-1})	Shoot-forming petals (%)	Hyperhydric shoots[a]	Shoot FW (mg)	Shoot DW (mg)	Shoot water content (%)
0	80.0	+ + +	57.20	2.92	94.89
2	81.4	+ + +	35.92	2.04	94.32
4	82.8	+ + +	33.10	1.77	94.65
6	75.8	+ + −	17.02	1.01	94.08
8	70.3	+ − −	6.80	0.46	93.25
10	71.1	− − −	3.63	0.28	92.18
12	66.5	− − −	1.80	0.15	91.41

[a]Proportion of hyperhydric shoots are indicated by: (+ + +) All of the shoots, (+ + −) 50%-100% of shoots, (+ − −) 0%-50% of shoots and (− − −) none of the shoots.

TABLE II Percentage of shoot-forming carnation petals (*Dianthus caryophyllus* L. cv. White Sim), the fresh weight (FW), dry weight (DW) and the water content of the regenerated shoots, and the proportion of hyperhydric ones, after 30 days of culture in MS media supplemented with 0.5 μM NAA plus 0.5 μM TDZ and solidified with a range of agar concentrations.

B. Effect of agar concentration of the medium on adventitious shoot organogenesis

Petal explants of White Sim carnation exhibited distinct organogenic potential when cultured in media with distinct degrees of solidification. The highest organogenic response was obtained in the medium solidified with 2 g l^{-1} agar, with 17.7 shoots per petal (Fig. 1). This number decreased progressively when the agar concentration was increased to 12 g l^{-1}, and 4.3 shoots per petal regenerated, which accounts for a 4-fold decrease in the shoot regeneration capacity. Moreover, excluding the results of the liquid medium, a linear and inverse correlation between the number of regenerated shoots per petal and the agar concentration in the medium ($R^2 = 0.98$, $p = 0.000$) was observed. In the liquid medium, the number of shoots per explant was similar to that in media with 8 or 10 g l^{-1} agar (Fig. 1). However, the percentage of shoot-forming petals in liquid medium (80.0 %) was similar to that in media with 2 and 4 g l^{-1} agar (Table 2), and decreased progressively until 12 g l^{-1} agar (66.5 %), presenting a linear and negative correlation with the agar concentration ($R^2 = 0.82$, $p = 0.005$), in this case including all the media.

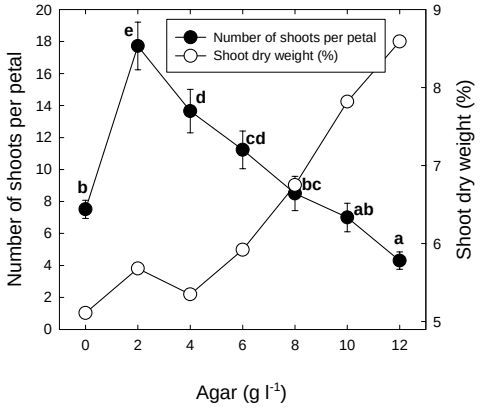

FIG. 1 Number of shoots, and their percentage of dry weight, regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30 days cultured in MS media supplemented with 0.5 μM NAA plus 0.5 μM TDZ and solidified with a range of agar concentrations. Shoot regeneration values are the mean ± SE. We used 90-105 petal explants per treatment (agar concentration in the medium). Distinct letters denote significant differences between media (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test.

C. Effect of agar concentration on the hyperhydricity of regenerated shoots

The FW and the DW of the adventitious shoots decreased with increasing agar concentration in the medium, and the highest values in the liquid medium and the lowest in 12 g l^{-1} agar were obtained (Table 2). The FW ($R^2 = 0.92$, $p = 0.001$) and DW of shoots ($R^2 = 0.95$, $p = 0.000$) correlated linearly and negatively with the agar concentration. Since shoot DW diminished in a lower proportion than the FW, the percentage of shoot DW increased (Fig. 1) and, thus, the water content decreased with higher agar concentrations (Table 2). In the liquid medium (5.1 % DW) the percentage of DW was similar to 2 and 4 g l^{-1} agar, from which it increased progressively until 12 g l^{-1} agar (8.6 % DW) (Fig. 1), showing a linear and positive correlation with the agar concentration ($R^2 = 0.89$, $p = 0.001$). The increase in the DW percentage reflected a decrease in the proportion of hyperhydric shoots. Morphological observations of adventitious shoots showed all of them to be hyperhydric from 0 to 4 g l^{-1} agar, whereas few shoots showed a normal appearance in 6 g l^{-1} agar, most of the shoots looked normal in 8 g l^{-1} agar and all of the shoots appeared normal and even looked dry in 10 and 12 g l^{-1} agar (Table 2).

Agar concentration (g l^{-1})	Parenchyma cell layers (no.)[a]	Area of parenchyma cells (μm^2)[b]
0	9.3 ± 0.9 b	968 ± 53 c
2	9.4 ± 0.3 b	934 ± 47 c
4	8.9 ± 0.3 ab	923 ± 71 c
6	8.3 ± 0.4 ab	456 ± 21 b
8	8.4 ± 0.3 ab	303 ± 20 a
10	7.8 ± 0.3 a	275 ± 27 a
12	7.7 ± 0.6 a	254 ± 13 a

[a]Twelve measurements per treatment (agar concentration).

[b]165-180 cells measured per treatment (agar concentration).

TABLE III Number of parenchyma cell layers and area of these cells of leaves of shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30 days cultured in MS media supplemented with 0.5 μM NAA plus 0.5 μM TDZ and solidified with a range of agar concentrations. Values represent the mean ± SE. Distinct letters denote significant differences between agar concentrations in the media (one-way ANOVA, $p < 0.05$) according to Duncan's multiple range test.

D. Effect of agar concentration on the histology of regenerated leaves

Leaf thickness of regenerated shoots decreased with increasing agar concentration in the medium (Fig. 2), and was related to the number of parechyma cell layers and the size of these cells (Table 3). The cell layers decreased progressively with increasing agar concentration (Table 3). The area of cells (Table 3), which was similar in 0, 2 and 4 g l^{-1} agar (Fig. 2A, B, C), decreased significantly in leaves grown in 6 g l^{-1} agar (Fig. 2D) and decreased again in leaves grown in 8 g l^{-1} agar, which had a similar size to those obtained in 10 and 12 g l^{-1} agar (Fig. 2E, F, G). The number of cell layers ($R^2 = 0.93$, $p = 0.000$) and the cell size ($R^2 = 0.87$, $p = 0.002$) also correlated linearly and negatively with the concentration of agar in the medium.

E. Effect of agar concentration on the ultrastructure of regenerated leaves

Observations were performed in parenchyma cells of large and hyperhydric leaves, grown in liquid medium, of morphologically normal leaves, grown in 8 g l^{-1} agar, and of small leaves with a dry appearance, grown in 12 g l^{-1} agar. The most marked changes at ultrastructural level were in the plastids (Fig. 3). In liquid medium, chloroplasts showed thylakoids organized in abundant grana and always contained small starch grains and very small plastoglobuli in the stroma (Fig. 3A). In 8 g l^{-1} agar, chloroplasts were smaller, with well-organized thylakoids and conspicuous plastoglobuli, and usually did not contain starch (Fig. 3B). Plastids developed in 12 g l^{-1} agar had large starch grains, few thylakoids and few plastoglobuli (Fig. 3C).

IV. DISCUSSION

The degree of solidification of the medium and the closure type of the culture tubes strongly affect the adventitious shoot regeneration capacity of carnation explants, and the water content of these shoots. In solid growth media, increasing the gelling agent concentration led to a progressive decrease in adventitious shoots per petal explant, as observed in explants of other species (Brown et al. 1979; Bornman and Vogelman 1984; Castro-Concha et al. 1990; Owens and Wozniak 1991). Similarly, the shoot propagation rate of cultured plantlets also decreases with increasing agar or gelrite concentration in various species (Debergh et al. 1981; Singha 1982; Ziv et al. 1983; Mackay and Kitto 1988; Pasqualetto et al. 1988; Turner and Singha 1990; Yadav et al. 2003), with the exception of pear, which shows increased shoot proliferation (Singha 1982; Turner and Singha 1990). The decrease in the regeneration capacity of explants could be due to a decrease in the amount of water available in the medium (Smith and Spomer 1995). Differences in the regeneration capacity of White Sim petals between the first experiment (2.8 shoots per petal) and the second (8.5 shoots per petal), both performed in 8 g l^{-1} agar, can be attributed to seasonal differences in the organogenic potential of tissues, higher in spring than in winter. However, the effect of experimental conditions cannot be excluded.

In the liquid medium, although the percentage of shoot-forming petals was as expected, the number of regenerated shoots from those petals was lower than expected. Although the vessels were shaken, this observation may be the result of anoxia. Similar results were found in carnation stems, with higher regeneration in solid than in liquid medium (Watad et al. 1996), but not in carnation petals, which were grown in a smaller volume of medium than those in the present study (Fisher et al. 1993).

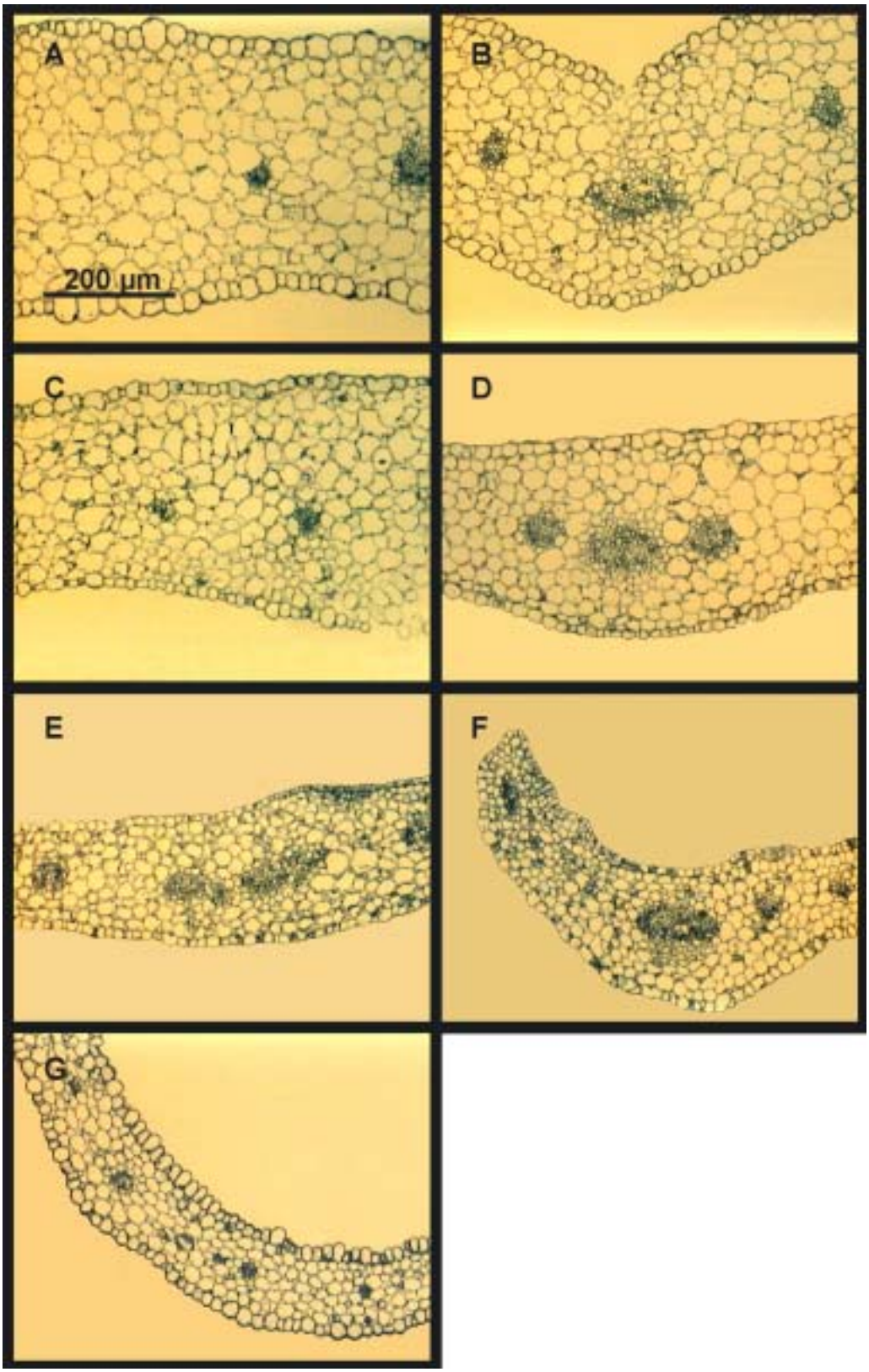

FIG. 2 Transversal sections of leaves of shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30 days cultured in MS media supplemented with 0.5 μM NAA plus 0.5 μM TDZ and solidified with (A) 0 g l^{-1}, (B) 2 g l^{-1}, (C) 4 g l^{-1}, (D) 6 g l^{-1}, (E) 8 g l^{-1}, (F) 10 g l^{-1} and (G) 12 g l^{-1} of agar.

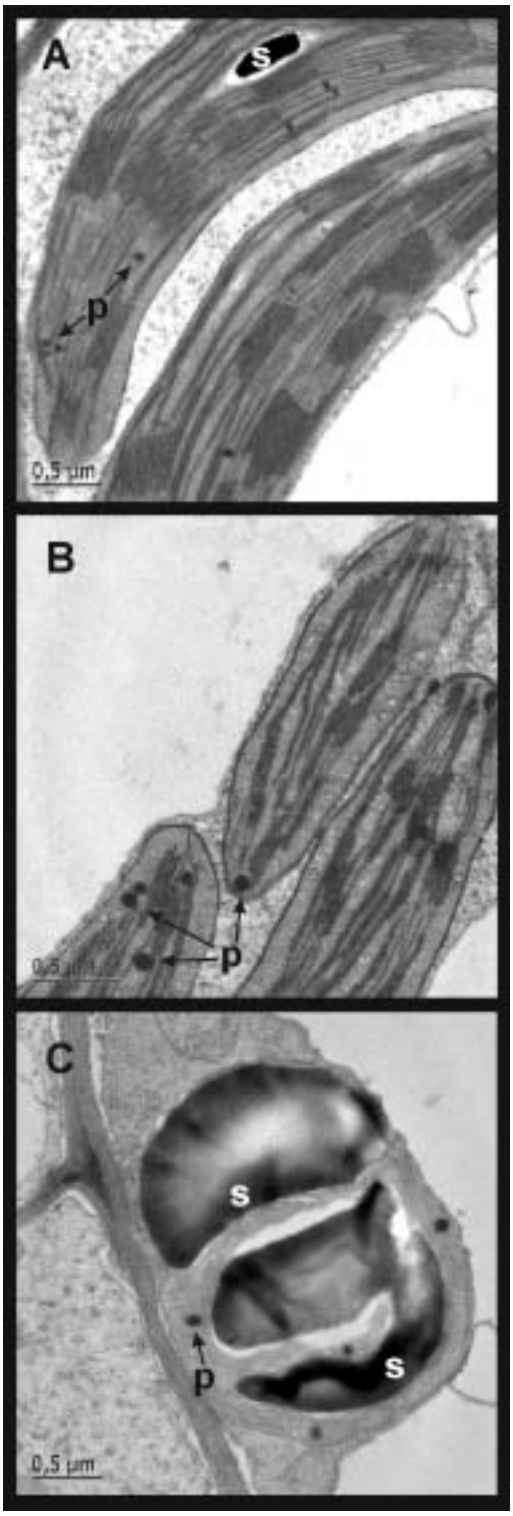

FIG. 3 Electron micrographs of representative plastids of leaves of shoots regenerated from carnation petals (*Dianthus caryophyllus* L. cv. White Sim) after 30 days cultured in MS media supplemented with 0.5 μM NAA plus 0.5 μM TDZ and solidified with (A) 0 g l^{-1}, (B) 8 g l^{-1}, (C) 12 g l^{-1} of agar. (s), starch grains; (p), plastoglobuli.

The features of adventitious carnation shoots, such as weight, water content, leaf parenchyma cell layers and cell size, correlate negatively with the agar concentration in the medium. Shoots obtained in liquid medium were the largest and most hyperhydric, since they had the highest water content and the lowest percentage of DW, and showed the highest number of leaf parenchyma cell layers and the largest cells. Shoots grown in 2 and 4 g l^{-1} agar had similar hyperhydricity and cell size, although their weight decreased. The large cells of hyperhydric tissues might undergo enhanced growth, called hypertrophy, which causes an abnormal volume of tissues (Werker and Leshem 1987), the result of a defective lignification of the cell wall, and thus allowing greater water uptake (Gaspar et al. 1987; Olmos et al. 1997). Ultrastructural studies of these cells showed well-developed chloroplasts with small starch grains, as reported in hyperhydric leaves of carnation and pepper plantlets, although in the latter large starch grains were found (Ziv et al. 1991; Olmos and Hellín 1998; Fontes et al. 1999).

The decrease in water content and the parallel increase in the percentage of DW of regenerated shoots, with increasing agar concentration from 4 to 8 g l^{-1}, reflect a lesser degree of shoot hyperhydricity. This observation is consistent with findings in several species, although in most of those studies the reduction in hyperhydricity was described macroscopically without any quantitative measure (Debergh et al. 1981; Ziv et al. 1983; Bornman and Vogelman 1984; Mackay and Kitto 1988; Pasqualetto et al. 1988; Castro-Concha et al. 1990; Turner and Singha 1990; Yadav et al. 2003). The hyperhydricity also correlated with shoot weight and the size of the leaf cells, since all of these variables decreased from 4 to 8 g l^{-1} agar, with most shoots showing a normal appearance on the latter. Accordingly, the mesophyll cellular area of carnation hyperhydric leaves was larger than that of normal leaves (Olmos and Hellín 1998; Majada et al. 2000).

From 8 to 12 g l^{-1} agar, there was a decrease in the weight and the water content of the shoots, and in the number of leaf cell layers. Carnation leaves developed in 12 g l^{-1} agar, the driest, show large starch grains, which could have grown due to immobilization of photoassimilates as a result of water stress associated with this medium. In agreement, starch accumulation was observed in carnation plantlets grown in ventilated vessels (Majada et al. 2000).

The lower organogenic capacity of carnation explants cultured in ventilated tubes, with lower RH, is analogous to the reduced growth or micropropagation rate in carnation and rose obtained by decreasing the RH of vessels with solidified medium (Ziv et al. 1983; Sallanon and Maziere 1992; Majada et al. 1997), but no data on the effect of RH on adventitious morphogenesis could be found. The percentage of DW of shoots in ventilated tubes, with initially 8 g l^{-1} agar, corresponds to that of shoots obtained in 12 g l^{-1} agar (Table 1, Fig. 1). Indeed, in our experiment tube ventilation led to a progressive increase in agar concentration in the medium, because of water

evaporation, as reported with ventilated vessels (Majada et al. 1997). On the other hand, Ziv et al. (1983) reported a decrease in RH in culture vessels with increasing agar concentrations. Therefore, the reduction in morphogenesis and hyperhydricity should be attributed to both an increase in gelling agent concentration and a decrease in RH, since these two factors cannot be clearly discriminated. However, one advantage of vessel ventilation is the development of plantlet photoautotrophy, and therefore an improved capacity to survive *ex vitro* (Kozai et al. 1995; Serret et al. 1997, Majada et al. 2001).

In conclusion, in addition to influencing the hyperhydricity of regenerated shoots, water availability and RH in the culture vessels affect the adventitious organogenic capacity of cultured carnation tissues. Moreover, our studies show that shoot hyperhydricity can be measured not only by water content, but also by weight, the number of cell layers of their leaves and the size of these cells. Although it might be different in other cultivars, the number of healthy White Sim carnation shoots is maximum at 8 g l^{-1} agar. However, the agar concentration, or the type of vessel closure, should be selected depending on whether the aim of the culture is to micropropagate and further acclimatize the plants *ex vitro* or to maximize the number of shoots (i.e. to obtain transgenic plants in a transformation process), since in a second cultivation cycle new healthy plants can be recovered by controlling the conditions and thereby overcome hyperhydricity (Ziv et al. 1983; Jain et al. 2001; Yadav et al. 2003).

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References

Bornman CH, Vogelmann TC (1984) Effect of rigidity of gel medium on benziladenine-induced adventitious bud formation and vitrification *in vitro* in *Picea abies*. Physiol Plant 61:505-512

Brown DCW, Leung DWM, Thorpe TA (1979) Osmotic requirement for shoot formation in tobacco callus. Physiol Plant 46:36-41

Casanova E, Valdés AE, Fernández B, Moysset L, Trillas MI (2004) Levels and immunolocalization of endogenous cytokinins in thidiazuron-induced shoot organogenesis in carnation. J Plant Physiol 161:95-104

Casanova E, Zuker A, Trillas MI, Moysset L, Vainstein A (2003) The *rolC* gene in carnation exhibits cytokinin- and auxin-like activities. Sci Hort 97:321-331

Castro-Concha L, Loyola-Vargas VM, Chan JL, Robert ML (1990) Glutamate dehydrogenase activity in normal and vitrified plants of *Agave tequilana* Weber propagated *in vitro*. Plant Cell Tiss Org Cult 22:147-151

Debergh P, Aitken-Christie J, Cohen D, Grout B, von Arnold S, Zimmerman R, Ziv M (1992) Reconsideration of the term 'vitrification' as used in micropropagation. Plant Cell Tiss Org Cult 30:135-140

Debergh P, Harbaoui Y, Lemeur R (1981) Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol Plant 53:181-187

Fisher M, Ziv M, Vainstein A (1993) An efficient method for adventitious shoot regeneration from cultured carnation petals. Sci Hort 53:231-237

Fontes MA, Otoni WC, Carolino SMB, Brommonschenkel SH, Fontes EPB, Fári M, Louro RP (1999) Hyperhydricity in pepper plants regenerated *in vitro*: involvement of BiP (Binding Protein) and ultrastructural aspects. Plant Cell Rep 19:81-87

Fujiwara K , Kozai T (1995) Physical microenvironment and its effects. In: Aitken-Christie J, Kozai T, Smith ML (eds) Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 319-369

Gaspar T, Kevers C, Debergh P, Maene L, Paques M, Boxus P (1987) Vitrification: morphological, physiological, and ecological aspects. In: Bonga JM, Durzan DJ (eds) Cell and Tissue Culture in Forestry. Vol.1. General Principles and Biotechnology. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp 152-166

Genkov T, Tsoneva P, Ivanova I (1997) Effect of cytokinins on photosynthetic pigments and chlorophyllase activity in *in vitro* cultures of axillary buds of *Dianthus caryophyllus* L. J Plant Growth Regul 16:169-172

Gimelli F, Ginatta G, Venturo R, Positano S, Buiatti M (1984) Plantlet regeneration from petals and floral induction *in vitro* in the Mediterranean carnation (*Dianthus caryophyllus* L.). Riv Ortoflorofrutt It 68:107-121

Jain A, Kantia A, Kothari SL (2001) De novo differentiation of shoot buds from leaf-callus of *Dianthus caryophyllus* L. and control of hyperhydricity. Sci Hort 87:319-326

Kevers C, Coumans M, Coumans-Gillès M-F, Gaspar T (1984) Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. Physiol Plant 61:69-74

Kevers C, Frank T, Strasser RJ, Dommes J, Gaspar T (2004) Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. Plant Cell Tiss Org Cult 77:181-191

Kozai T, Kitaya Y, Fujiwara K, Adelberg J (1995) Environmental control for large scale production of *in vitro* plantlets. In: Terzi M, Cella R, Falavigna A (eds) Current Issues in Plant Molecular and Cellular Biology. Kluwer Academic Publishers, Dordrecht, The Nether-

lands, pp 947-955

Mackay WA, Kitto SL (1988) Factors affecting *in vitro* shoot proliferation of French tarragon. J Amer Soc Hort Sci 113:282-287

Majada JP, Fal MA, Sánchez-Tamés R (1997) The effect of ventilation rate on proliferation and hyperhydricity of *Dianthus caryophyllus* L. *in vitro* Cell Dev Biol 33:62-69

Majada JP, Tadeo F, Fal MA, Sánchez-Tamés R (2000) Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell Tiss Org Cult 63:207-214

Majada JP, Sierra MI, Sánchez-Tamés R (2001) Air exchange rate affects the *in vitro* developed leaf cuticle of carnation. Sci Hort 87:121-130

Mii M, Buiatti M, Gimelli F (1990) Carnation. In: Ammirato PV, Evans DR, Sharp WR, Vajaj YPS (eds) Handbook of Plant Cell Culture. McGraw-Hill, New York, USA, pp 284-318

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Olmos E, Hellín E (1998) Ultrastructural differences of hyperhydric and normal leaves from regenerated carnation plants. Sci Hort 75:91-101

Olmos E, Piqueras A, Martínez-Solano JR, Hellín E (1997) The subcellular localization of peroxidase and the implication of oxidative stress in hyperhydrated leaves of regenerated carnation plants. Plant Sci 130:97-105

Owens LD, Wozniak CA (1991) Measurement and effects of gel matric potential and expressibility on production of morphogenic callus by cultured sugarbeet leaf discs. Plant Cell Tiss Org Cult 26:127-133

Pasqualetto P-L, Zimmerman R, Fordham I (1988) The influence of cation and gelling agent concentration on vitrification of apple cultivars *in vitro*. Plant Cell Tiss Org Cult 14:31-40

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17:208-212

Sallanon H, Maziere Y (1992) Influence of growth room and vessel humidity on the *in vitro* development of rose plants. Plant Cell Tiss Org Cult 30:121-125

Serret MD, Trillas MI, Matas J, Araus JL (1997) The effect of different closure types, light, and sucrose concentrations on carbon isotope composition and growth of *Gardenia jasminoides* plantlets during micropropagation and subsequent acclimation *ex vitro*. Plant Cell Tiss Org Cult 47:217-230

Singha S (1982) Influence of agar concentration on *in vitro* shoot proliferation of *Malus* sp. 'Almey' and *Pyrus communis* 'Seckel'. J Amer Soc Hort Sci 107:657-660

Smith MAL, Spomer LA (1995) Vessels, gels, liquid media, and support systems. In: Aitken-Christie J, Kozai T, Smith ML (eds) Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 371-404

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electronmicroscopy. J Ultrasctruct Res 26:31-43

Turner SR, Singha S (1990) Vitrification of crabapple, pear, and geum on gellan gum-solidified culture medium. HortSci 25:1648-1650

Vainstein A (ed) (2002) Breeding For Ornamentals: Classical and Molecular Approaches. Kluwer Academic Publishers, Dordrecht, The Netherlands

Watad AA, Ahroni A, Zuker A, Shejtman H, Nissim A, Vainstein A (1996) Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. Sci Hort 65:313-320

Werker E, Leshem B (1987) Structural changes during vitrification of carnation plantlets. Ann Bot 59:377-385

Yadav MK, Gaur AK, Garg GK (2003) Development of suitable protocol to overcome hyperhydricity in carnation during micropropagation. Plant Cell Tiss Org Cult 72:153-156

Ziv M (1991) Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh PC, Zimmerman R (eds) Micropropagation. Technology and Application. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 45-69

Ziv M, Meir G, Halevy AH (1983) Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. Plant Cell Tiss Org Cult 2:55-65

Zuker A, Ahroni A, Tzfira T, Ben-Meir H, Vainstein A (1999) Wounding by bombardment yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Mol Breed 5:367-375

CAPÍTOL 4

Efecte de citocinina i d'auxina del gen *rolC* en el clavell

Efecte de citocinina i d'auxina del gen *rolC* en el clavell

Resum

La sobreexpressió del gen *rolC* del plasmidi Ri d'*Agrobacterium rhizogenes* en plantes transgèniques n'altera el seu desenvolupament. S'han realitzat molt pocs estudis de la formació de tiges i rels adventícies en plantes transformades amb *rolC*. En aquest estudi hem avaluat un possible efecte de citocinina i d'auxina en l'organogènesi de tiges i rels a partir de pètals i fulles de quatre línies de clavell (*Dianthus caryophyllus* L. cv. White Sim) transgèniques amb el gen *rolC*. El gen *rolC* ha incrementat la regeneració de tiges tot augmentant el nombre de tiges per explant regeneratiu o el nombre d'explants formadors de tiges. En el medi amb només auxina, s'ha obtingut un increment en la regeneració de rels, degut a l'augment en el percentatge d'explants formadors de rels. Els nostres resultats mostren que el gen *rolC* manifesta activitats tant de citocinina com d'auxina en explants de clavells transgènics.

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The *rolC* gene in carnation exhibits cytokinin- and auxin-like activities

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Abstract

The overexpression of the *rolC* gene of the Ri plasmid of *Agrobacterium rhizogenes* in transgenic plants alters their development. Few studies have been performed on adventitious shoot or root formation in *rolC*-transformed plants. In this study we evaluated a possible cytokinin-like and auxin-like effect on shoot and root regeneration from petals and leaves of four lines of *rolC*-transgenic carnation plants (*Dianthus caryophyllus* L. cv. White Sim). *rolC* was found to enhance shoot regeneration, by increasing either the number of shoots per regenerative explant, or the number of shoot-forming explants. Remarkable root regeneration, in a medium with only auxin, was obtained from *rolC* explants, due to the increased percentage of root-forming explants. Our results show that *rolC* exhibits both cytokinin-like and auxin-like activities in *rolC*-transgenic carnation tissues.

Keywords: *Agrobacterium rhizogenes*; *Dianthus caryophyllus*; Leaf explants; Morphogenesis; Petal explants; *rol* genes

1. Introduction

The soil bacterium *Agrobacterium rhizogenes* causes hairy root disease in dicotyledonous plants. Four genes in the T-DNA segment (*rolA*, *rolB*, *rolC* and *rolD*) are responsible for the morphogenic effects of the bacterium on plants (White et al., 1985). *rolC* is essential to the

Abbreviations: BA, N^6-benzyladenine; CK, cytokinin; NAA, naphthaleneacetic acid; TDZ, thidiazuron or *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea

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establishment of hairy root disease, and its overexpression in transgenic plants causes pleiotropic developmental alterations (Faiss et al., 1996). The phenotypic alterations of 35S-*rolC*-transgenic tobacco plants consist of reduced apical dominance leading to increased branching, dwarfed plants with short internodes and male sterility (Schmülling et al., 1988; Nilsson et al., 1993). Such alterations have also been reported for other *rolC*-transformed species; viz. the potato (Fladung, 1990; Schmülling et al., 1993), belladonna (Kurioka et al., 1992), hybrid aspen (Nilsson et al., 1996) and the carnation (Ovadis et al., 1999; Zuker et al., 2001). Furthermore, *rolC* gene leads to better rooting ability in transformed plants (Ovadis et al., 1999; Zuker et al., 2001).

Few authors have studied morphogenesis in plant tissues transformed with *rol* genes. As happens in plants with hairy root disease, *rol* genes stimulate root formation in tobacco and tomato leaves (Cardarelli et al., 1987b; Spena et al., 1987; Spano et al., 1988; van Altvorst et al., 1992), since they induce an increased sensitivity to auxins (Cardarelli et al., 1987a; Spano et al., 1988; van Altvorst et al., 1992). In contrast, Tepfer (1984) found a direct plantlet formation from the same explants of tobacco after inoculation with *A. rhizogenes*. To our knowledge adventitious shoot formation from *rolC* plant tissues has never been studied, while rhizogenesis from *rolC* tissues has only been studied in tobacco and kalanchoe leaves (Spena et al., 1987).

Estruch et al. (1991a,b) showed that *rolC* codes for a cytosolic β-glucosidase that releases free forms of CK from their inactive glucosidic conjugates. Moreover, *rolC*-induced phenotypes also suggested that *rolC* induces CK-like activity (Schmülling et al., 1988; Walden et al., 1993). As the effect of CKs on adventitious shoot regeneration from several explants is widely known, our aim was to determine whether any CK-like effect could be observed on shoot regeneration from explants of *rolC*-transgenic carnation plants (*Dianthus caryophyllus* L.). It has also been proposed that *rolC* induces auxin-like activity (Spena et al., 1987; Ovadis et al., 1999; Zuker et al., 2001). Since auxins promote rhizogenesis in plant tissues, we intended to determine whether there was any auxin-like effect on root regeneration from explants of *rolC*-transgenic carnation plants. Thus, we regulated the plant growth regulator balance of the media in order to obtain mainly shoots or roots. We used NAA as auxin and TDZ or BA as cytokinins. TDZ, a substituted phenylurea with higher CK-like effect than BA (Malik and Saxena, 1992), has been reported to enhance shoot-regeneration frequency for various species (Hutteman and Preece, 1993). In carnation, comparative studies have shown TDZ to be the most effective cytokinin for adventitious shoot regeneration from petal explants (Nakano et al., 1994). The selection of the TDZ concentrations were based on our previous work and on Frey and Janick (1991).

Therefore, several *rolC*-transgenic carnation lines and, as a control, a *uidA*-transformed line of cv. White Sim were compared on the basis of their ability to induce adventitious shoot and root regeneration from leaf and petal explants, in media with several plant growth regulators.

2. Material and methods

2.1. Plant material

Control and *rolC*-transgenic carnation plants (*D. caryophyllus* L. cv. White Sim) were grown in standard greenhouse conditions. In our study, we used four *rolC*-transgenic

lines, *rolC*1[13(5)], *rolC*2[4(3)], *rolC*3[II(5)] and *rolC*4[6(19)], selected phenotypically, and whose transgenic nature was confirmed previously (Ovadis et al., 1999; Zuker et al., 2001). In these plants, *rolC* was driven by the CaMV-35S constitutive promoter. Control plants were a transgenic line containing the *uidA* gene, coding for β-glucuronidase, driven by a mannopine synthetase promoter. All the transgenic plants, including control, contained the *nptII* selection gene, coding for neomycin phosphotransferase II, also driven by a 35S promoter. The transformed carnation plants containing the *uidA* gene, without the *rolC*, were phenotypically and morphologically indistinguishable from the non-transformed plants (Zuker et al., 1999, 2001). The *rolC*-transgenic carnation plants exhibit increased axillary budbreak, generate more cuttings with better rooting ability and produce three times more flowering stems than control plants (Zuker et al., 2001).

Cuttings from vegetative shoots were harvested and used as the leaf explant source and, once the plants reached the flowering stage, flower buds were removed and used as the petal explant source. Both sources of explants were stored at 4 °C, and could be used for a maximum of 1 month.

2.2. Tissue culture

Cuttings were rinsed in 70% (v/v) ethanol, then sterilized for 8 m in 1.2% (w/v) sodium hypochlorite and rinsed three times for 10 m in sterile water. Leaves were removed as was suggested by van Altvorst et al. (1994), and placed in Petri dishes (90 mm diameter) with 25 ml of solid culture medium (10–12 explants per dish). We used MS basal medium (Murashige and Skoog, 1962) with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine–HCl and 0.1 mg l^{-1} thiamine–HCl, 30 g l^{-1} sucrose and 0.5 g l^{-1} casein enzymatic hydrolysate. Young flower buds (11–14 mm diameter) were harvested and surface-sterilized for 5 s with 96% (v/v) ethanol. Petals were excised and placed in 100 ml glass culture vessels with 3 ml of liquid medium for the first experiment, in Petri dishes (90 mm diameter) with 25 ml of solid medium for the second and the third experiments (25–30 explants per vessel or dish). The MS basal medium with 30 g l^{-1} sucrose, 2 mg l^{-1} glycine, 50 mg l^{-1} myo-inositol and 0.5 mg l^{-1} thiamine–HCl (Gimelli et al., 1984) was used in all of the experiments on adventitious shoot and root regeneration from petal explants. Appropriate plant growth regulators and 8 g l^{-1} agar oxoid (Sigma) (if necessary) were added to the media, and the pH adjusted to 5.8, before autoclaving at 121 °C for 20 m.

2.2.1. rolC effect on shoot and root regeneration from leaf and petal explants

Leaves from the control and all the *rolC* lines (110 leaf explants from each of them), and petals from the control and the *rolC*1 lines were used (160 and 260 petal explants, respectively). For the leaves, the medium was supplemented with 0.5 μM NAA and 4.4 μM BA (Ovadis et al., 1999), and solidified with 8 g l^{-1} agar oxoid (Sigma). The medium for petals was supplemented with 10.7 μM NAA and 2.2 μM BA (Gimelli et al., 1984), but, as suggested by Fisher et al. (1993), without agar.

2.2.2. Evaluation of a CK-like effect of rolC on shoot regeneration from petal explants

Petals (70 petal explants) from the control and both the *rolC*1 and *rolC*3 lines were used. The media were supplemented with 0.5 μM NAA plus 0.05, 0.25 or 0.5 μM TDZ (Frey and Janick, 1991), and solidified with 8 g l^{-1} agar oxoid (Sigma).

2.2.3. Evaluation of an auxin-like effect of rolC on root regeneration from petal explants

Petals (150 petal explants) from the control and the *rolC*3 lines were used. The media were supplemented with 0.5 μM NAA plus 0.0, 0.005, 0.025 or 0.05 μM TDZ, and solidified with 8 g l^{-1} agar oxoid (Sigma).

2.2.4. Culture conditions

All the cultures were kept in a growth room at 25 ± 1 °C in a 16 h photoperiod using cool white fluorescent lights (60 μmol m^{-2} s^{-1}). The vessels with liquid media were continuously agitated on an orbital shaker (100 rpm). To minimise the variability caused by the heterogeneous regeneration capacity intrinsic to each flower bud, in the second and the third experiment petals from the same flower bud were distributed to all the media. All experiments were performed in triplicate.

Shoot and root regeneration were assessed using a stereomicroscope after 30 days in culture. The results are reported as the number of shoots/roots per explant and per regenerative explant, and also as the percentage of shoot/root-forming explants.

2.3. Statistical analysis

For the first experiment, differences between lines were tested using one-way ANOVAs. Duncan's multiple range test was applied if one-way ANOVA showed significant differences. For the second and the third experiment, two-way ANOVAs (with interaction) were applied with lines and media as fixed factors. Differences within each factor were tested with the Duncan's multiple range test if interaction was not significant. When interaction was found, Duncan's multiple range test was used to look for differences between media within each line. All the statistical analyses were performed using SPSS 10.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Effect on shoot and root regeneration from carnation explants by rolC

In this first experiment, the number of shoots per regenerative leaf in the *rolC*1 and *rolC*4 transgenic lines was the same as in the control, *uidA*-transgenic plants, and in the *rolC*2 and *rolC*3 transgenic lines it was lower (Table 1). The percentage of shoot-forming leaves was higher in all the *rolC* lines except in the *rolC*4 line. The general effect of these two variables was reflected in the number of shoots per leaf, which was twice higher in the *rolC*1 line. The number of roots per regenerative leaf was higher in the *rolC*2 and *rolC*3 lines, whereas the percentage of root-forming leaves was higher in all the *rolC* lines except in the *rolC*4

Table 1
Adventitious shoot and root regeneration from leaves in control (*uidA*-transgenic), and *rolC*1, *rolC*2, *rolC*3 and *rolC*4 transgenic carnation lines in MS solid medium supplemented with 0.5 μM NAA + 4.4 μM BA[a,b]

	Control	*rolC*1	*rolC*2	*rolC*3	*rolC*4
Shoot per leaf (no.)	2.6 ± 0.5 a	5.3 ± 0.8 b	2.5 ± 0.4 a	3.1 ± 0.4 a	3.2 ± 0.5 a
Shoots per regenerative leaf (no.)	9.2 ± 1.2 c	9.6 ± 1.0 c	6.0 ± 0.7 a	6.4 ± 0.7 ab	8.8 ± 1.0 bc
Shoot-forming leaves (%)	27.8 ± 5.4 a	54.2 ± 5.5 c	42.8 ± 4.6 bc	45.7 ± 5.3 bc	37.4 ± 3.5 ab
Roots per leaf (no.)	0.1 ± 0.0 a	0.4 ± 0.1 bc	0.6 ± 0.2 c	0.5 ± 0.1 bc	0.2 ± 0.1 ab
Roots per regenerative leaf (no.)	1.1 ± 0.1 a	1.9 ± 0.2 ab	3.0 ± 0.5 b	2.5 ± 0.4 b	1.9 ± 0.3 ab
Root-forming leaves (%)	8.0 ± 1.8 a	19.3 ± 3.6 bc	23.3 ± 2.6 c	19.8 ± 4.0 bc	12.7 ± 3.3 ab

[a] Regeneration efficiencies were evaluated after 30 days in culture.
[b] Values represent the mean ± S.E.. Different letters show significant differences between lines ($P < 0.05$) according to the Duncan's multiple range test, when one-way ANOVAs are also significant ($P < 0.05$).

line, as also occurred in the percentage of shoot-forming leaves. Thus, the number of roots per leaf was higher in all the *rolC* lines, except in the *rolC*4 line (Table 1). In terms of petals, both the number of shoots per petal and the number of shoots per regenerative petal in the *rolC*1 transgenic line were higher than in the control, whereas no differences were found in the percentage of shoot-forming petals (Table 2). The number of roots per petal was the same for the control and the *rolC*1 lines, since both lines showed similar numbers of roots per regenerative petal and a similar percentage of root-forming petals (Table 2). Thus, both leaf and petal *rolC*-transgenic carnation explants showed an improved shoot regeneration, whereas an increase in root regeneration was observed in only *rolC* leaves.

3.2. CK-like effect of rolC on shoot regeneration from petal explants

In the second experiment, in the medium devoid of plant growth regulators, control line did not regenerate shoots or roots. Moreover, neither of *rolC*-transgenic lines, *rolC*1 and *rolC*3, regenerated any shoot or root (Table 3). In all the assayed plant material of the

Table 2
Adventitious shoot and root regeneration from petals in control (*uidA*-transgenic), and *rolC*1 transgenic carnation lines in MS liquid medium supplemented with 10.7 μM NAA + 2.2 μM BA[a,b]

	Control	*rolC*1
Shoots per petal (no.)	3.9 ± 0.2	5.1 ± 0.2***
Shoots per regenerative petal (no.)	4.6 ± 0.2	5.7 ± 0.2***
Shoot-forming petals (%)	84.4 ± 4.6	90.4 ± 3.4
Roots per petal (no.)	0.6 ± 0.1	0.6 ± 0.1
Roots per regenerative petal (no.)	2.1 ± 0.2	2.0 ± 0.2
Root-forming petals (%)	26.9 ± 8.3	29.5 ± 5.4

[a] Regeneration efficiencies were evaluated after 30 days in culture.
[b] Values represent the mean ± S.E..
*** Significant differences between lines (one-way ANOVAs), with $P < 0.001$.

Table 3
Adventitious shoot regeneration from petals in control (*uidA*-transgenic), and *rolC*1 and *rolC*3 transgenic carnation lines in MS solid media at several TDZ concentrations supplemented with 0.5 μM NAA[a,b]

Lines		Media[c]			Two-way ANOVA	
		0.05 μM TDZ	0.25 μM TDZ	0.5 μM TDZ	Lines	Media
Shoots per petal (no.)						
Control	a	2.3 ± 0.4	3.9 ± 0.5	5.1 ± 0.6	*	***
*rolC*3	b	3.3 ± 0.6	4.9 ± 0.6	6.1 ± 0.7		
*rolC*1	b	3.3 ± 0.6	5.4 ± 0.7	5.8 ± 0.8		
		a	b	b		
Shoots per regenerative petal (no.)						
Control	a	4.4 ± 0.5	5.5 ± 0.6	6.3 ± 0.6	***	*
*rolC*3	b	5.7 ± 0.9	6.7 ± 0.6	7.4 ± 0.7		
*rolC*1	c	7.7 ± 0.9	8.6 ± 0.8	8.8 ± 0.9		
		a	ab	b		
Shoot-forming petals (%)						
Control	a	54.2 ± 8.7	71.8 ± 8.9	81.4 ± 7.4	n.s.[d]	***
*rolC*3	a	58.9 ± 5.1	71.7 ± 7.7	82.2 ± 4.0		
*rolC*1	a	42.6 ± 9.8	63.1 ± 7.4	66.3 ± 9.5		
		a	b	b		

[a] Regeneration efficiencies were evaluated after 30 days in culture.
[b] Values represent the mean ± S.E.. Two-way ANOVA interactions were not significant. Within each line or medium, different letters show significant differences ($P < 0.05$) according to the Duncan's multiple range test.
[c] In the medium with no plant growth regulators none of the lines regenerated shoots or roots.
[d] No significant differences (two-way ANOVAs), with $P > 0.05$.
[*] Significant differences (two-way ANOVAs), with $P < 0.05$.
[***] Significant differences (two-way ANOVAs), with $P < 0.001$.

second experiment, higher TDZ concentrations in the media induced an increase in the number of shoots per regenerative petal (Table 3). The shoots per regenerative petal was higher in *rolC* lines compared to the control line, especially in the *rolC*1 line. The percentage of shoot-forming petals was similar in all three lines, although the *rolC*1 line had lower values, and increased from 0.05 to 0.25 μM TDZ. The number of shoots per petal increased from 0.05 to 0.25 μM TDZ and was higher in the *rolC* lines than in the control line. The number of shoots per petal of both *rolC* lines in the medium with 0.05 μM TDZ was similar to that of the control line in the medium with 0.25 μM TDZ. Moreover, the number of shoots per petal of the *rolC* lines in the medium with 0.25 μM TDZ was similar to that of the control line in the medium with 0.5 μM TDZ (Table 3). Thus, *rolC* lines showed an enhanced CK-like activity, resulting in a shoot regeneration from transgenic petals higher than that from control petals. Very few roots were obtained in this second experiment (data not shown).

3.3. Auxin-like effect of rolC on root regeneration from petal explants

In the third experiment, lower TDZ concentrations were used (compared to the second one) in order to induce roots. The number of shoots per regenerative petal increased on

Table 4
Adventitious shoot and root regeneration from petals of control (*uidA*-transgenic), and *rolC*3 transgenic carnation lines in MS solid media at several TDZ concentrations supplemented with 0.5 μM NAA[a,b]

Lines	Media				Two-way ANOVA		
	0.0 μM TDZ	0.005 μM TDZ	0.025 μM TDZ	0.05 μM TDZ	L	M	I
Shoots per petal (no.)							
Control a	0.1 ± 0.1	1.6 ± 0.3	2.9 ± 0.4	3.9 ± 0.5	*	***	n.s.[c]
*rolC*3 b	0.1 ± 0.0	1.5 ± 0.2	4.5 ± 0.4	4.3 ± 0.4			
	a	b	c	c			
Shoots per regenerative petal (no.)							
Control a	3.0 ± 0.7	4.8 ± 0.7	6.6 ± 0.6	7.1 ± 0.7	n.s.	***	n.s.
*rolC*3 a	1.5 ± 1.9	4.1 ± 0.4	7.8 ± 0.5	6.4 ± 0.4			
	a	b	c	c			
Shoot-forming petals (%)							
Control a	5.0 ± 2.2	33.3 ± 4.9	44.2 ± 4.6	55.2 ± 4.4	***	***	n.s.
*rolC*3 b	5.3 ± 1.9	39.1 ± 4.3	57.9 ± 4.5	68.7 ± 4.3			
	a	b	c	d			
Roots per petal (no.)							
Control	0.01 ± 0.01	0.09 ± 0.04	0.05 ± 0.03	0.03 ± 0.02	*	*	***
	a	b	ab	ab			
*rolC*3	0.18 ± 0.05	0.08 ± 0.03	0.05 ± 0.02	0.02 ± 0.01			
	B	A	A	A			
Roots per regenerative petal (no.)							
Control a	1.00 ± 0.00	2.00 ± 0.53	1.75 ± 0.48	1.25 ± 0.25	n.s.	n.s.	n.s.
*rolC*3 a	1.32 ± 0.19	1.20 ± 0.13	1.17 ± 0.17	1.00 ± 0.00			
	a	a	a	a			
Root-forming petals (%)							
Control	0.83 ± 0.83	4.22 ± 1.59	2.83 ± 1.35	2.83 ± 1.65	**	**	***
	a	a	a	a			
*rolC*3	14.14 ± 3.00	6.33 ± 2.09	4.67 ± 1.84	1.89 ± 1.06			
	B	A	A	A			

[a] Regeneration efficiencies were evaluated after 30 days in culture.
[b] Values represent the mean ± S.E.. Within each line (L) or medium (M), different letters show significant differences ($P < 0.05$) according to Duncan's multiple range test. When two-way ANOVAs are also significant, and there is no interaction (I) between lines and media. When interaction was found, Duncan's multiple range test was used to show differences ($P < 0.05$) between media within control line (small letters) and within *rolC* line (capital letters).
[c] No significant differences (two-way ANOVAs), with $P > 0.05$.
[*] Significant differences (two-way ANOVAs), with $P < 0.05$.
[**] Significant differences (two-way ANOVAs), with $P < 0.01$.
[***] Significant differences (two-way ANOVAs), with $P < 0.001$.

media containing 0.0–0.025 μM TDZ, and there were no differences between *rolC*3 and the control line (Table 4). Higher TDZ concentrations in the media increased the percentage of shoot-forming petals. This percentage was higher in the *rolC* line. The number of shoots per petal increased on media containing 0.0–0.025 μM TDZ and was also higher in the *rolC*3 line than to the control line. The number of roots per regenerative petal was not

affected either by the TDZ concentration in the media or by the presence of the *rolC* transgene. However, both media and lines affected the percentage of root-forming petals, which showed a difference in behaviour between control and *rolC* lines in the medium containing 0.0 μM TDZ (which contained only NAA). Whereas the control line recorded approximately the same percentage of root-forming petals in all media, the *rolC* line recorded a higher percentage in the medium containing only the auxin NAA. Tendencies similar to that shown in the percentage of root-forming petals were observed in the number of roots per petal pertaining to the medium containing only NAA, that was 18 times higher in the *rolC* line, which indicated an auxin-like effect of *rolC* on petal explants (Table 4).

In general, *rolC* roots, obtained from transgenic petal and leaf explants, were more branched than the control ones, even though the amount of branching was not quantitatively recorded.

4. Discussion

In these studies, the *rolC* transgene is shown to lead to improved shoot regeneration in carnation petal and leaf explants, due to either a higher percentage of shoot-forming explants or an increased number of shoots per each regenerative explant. Thus, explants of studied *rolC*-transgenic lines showed a number of shoots per explant higher than that of control explants. This enhanced CK-like activity shown by our *rolC* carnation explants is consistent with the hypothesis that several *rolC*-induced phenotypes, such as the reduction of apical dominance, or the stimulation of cell division and the callus growth from *rolC*-tobacco protoplasts in the absence of exogenous applied CKs, are suggestive of CK activity (Schmülling et al., 1988; Estruch et al., 1991a; Walden et al., 1993; Zuker et al., 2001), although root growth stimulation is observed (Zuker et al., 2001). Moreover, it was found that *rolC* codes for a β-glucosidase able to release free forms of CK from their inactive glucosides in vitro (Estruch et al., 1991a), but when free CKs and their glucosides were quantified in *35S-rolC*-transgenic plants, it was shown that RolC did not hydrolyse CK glucosides (Nilsson et al., 1993, 1996; Faiss et al., 1996). Thus, changes in CK levels, as well as in auxin or gibberellin levels, may be a secondary effect of the gene on hormone metabolism (Schmülling et al., 1993; Faiss et al., 1996; Nilsson et al., 1996). Furthermore, according with our results, *rolC*-transgenic carnation plants, growing in the greenhouse, showed an enhancement in lateral shoot development, that would also reflect the CK-like effect of *rolC*, leading to a yield of stem cuttings up to 48% higher than in the controls (Zuker et al., 2001).

A higher rate of root regeneration than in the control line was obtained from leaves of *rolC* lines. Our results are in agreement with the greater rooting ability of *rolC*-transgenic carnation plants (Ovadis et al., 1999; Zuker et al., 2001) and the enhanced growth capacity of *rolC*-transformed tobacco roots in in vitro cultures (Schmülling et al., 1988). Moreover, in the presence of auxin, and no CKs, the *rolC* carnation petals presented a much higher root regeneration than the control ones, up to 18 times more, indicating an auxin-like effect of *rolC* on carnation tissues. Our observations agree with those on *rolABC*-transformed tobacco and tomato leaves, which had been suggested to have an increase in auxin sensitivity (Spena et al., 1987; Spano et al., 1988; van Altvorst et al., 1992). Moreover, both

rolB and *rolC*-transgenic tobacco protoplasts showed increased auxin sensitivity, judging from their transmembrane potential and division frequency in response to auxins, although in *rolB* cells this was stronger than in *rolC* cells (Maurel et al., 1991, 1994; Walden et al., 1993). Thus, our results indicate an increase in auxin-like activity of *rolC* tissues, which might be caused by an increase in auxin sensitivity. Differences in branching of roots were also obtained by Schmülling et al. (1988) in in vitro cultures of tobacco roots, the *rolC* ones being more branched than those transformed with *rolA* or *rolB*.

Our control and the *rolC* petal explants required plant growth regulators in the media to regenerate shoots, whereas tobacco leaves inoculated with *A. rhizogenes* could form adventitious shoots without plant growth regulators (Tepfer, 1984). In contrast, root explants of *rolBC*-transformed aspen plants, using no plant growth regulators, showed lower shoot regeneration than non-transformed explants (Tzfira et al., 1998), which might be due to the interaction between *rolB* and *rolC*. *rolB* has an auxin-like effect, since it increases the sensitivity of cells to auxins (Hamill, 1993; Nilsson and Olsson, 1997). On the other hand, none of the lines in the medium with no plant growth regulators showed rhizogenesis. In contrast, Spena et al. (1987) obtained roots from *rolC*-transformed tobacco leaves, but not from *rolC* kalanchoe leaves, in a medium without plant growth regulators. Thus, an effect of the genotype, probably together with an effect of explant, interacting with the *rolC* effect, may account for the differences in shoot and root regeneration among several species and explants.

The four *rolC*-transgenic lines studied exhibited differences in shoot and root regeneration. Thus, the *rolC*1 line exhibited the most marked expression of *rolC*-related traits, mainly for shoot but also for root regeneration, whereas *rolC*4 line was the closest to the control line. Intermediate behaviour was observed for *rolC*2 and *rolC*3 lines, since they clearly showed the auxin-like effect of *rolC* on regenerating roots, but showed the CK-like effect less markedly. Zuker et al. (2001) showed that the *rolC*1 line had two copies of the gene, whereas the *rolC*2 and the *rolC*3 lines had just one. Different locations of the gene in the genome of each *rolC* line due to the random integration of the T-DNA mediated by *Agrobacterium tumefaciens* may also account for such dissimilarities.

In conclusion, *rolC* is unable to regenerate shoots or roots from transgenic carnation petal explants in a medium without plant growth regulators. However, we show that *rolC* has a CK-mimetic action, since it enhances the shoot regeneration of transgenic carnation explants in the presence of plant growth regulators. Moreover, the enhanced root regeneration of *rolC* carnation explants, mainly in medium with only auxin, indicates an auxin-like effect of the *rolC* transgene, which might increase the auxin sensitivity of transgenic carnation tissues. Nevertheless, the *rolC* mode of action in plants is still not clear. Therefore, further studies of the expression of the *rolC* gene in transgenic plants are required to clarify the specific function of *rolC* and to understand the biological effects of this gene and *rol* genes in general.

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References

Cardarelli, M., Spano, L., Mariotti, D., Mauro, L., van Sluys, M.A., Costantino, P., 1987a. The role of auxin in hairy root induction. Mol. Gen. Genet. 208, 457–463.

Cardarelli, M., Mariotti, D., Pomponi, M., Spano, L., Capone, I., Costantino, P., 1987b. *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. Mol. Gen. Genet. 209, 475–480.

Estruch, J.J., Chriqui, D., Grossmann, K., Schell, J., Spena, A., 1991a. The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates. EMBO J. 10, 2889–2895.

Estruch, J.J., Parets-Soler, A., Schmülling, T., Spena, A., 1991b. Cytosolic localization in transgenic plants of the *rolC* peptide from *Agrobacterium rhizogenes*. Plant Mol. Biol. 17, 547–550.

Faiss, M., Strand, M., Redig, P., Dolezal, K., Hanus, J., van Onckelen, H., Schmülling, T., 1996. Chemically induced expression of the *rolC*-encoded beta-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: rolC does not hydrolyze endogenous cytokinin glucosides *in planta*. Plant J. 10, 33–46.

Fisher, M., Ziv, M., Vainstein, A., 1993. An efficient method for adventitious shoot regeneration from cultured carnation petals. Sci. Hort. 53, 231–237.

Fladung, M., 1990. Transformation of diploid and tetraploid potato clones with the *rolC* gene of *Agrobacterium rhizogenes* and characterization of transgenic plants. Plant Breed. 109, 295–304.

Frey, L., Janick, J., 1991. Organogenesis in carnation. J. Am. Soc. Hort. Sci. 116, 1108–1112.

Gimelli, F., Ginatta, G., Venturo, R., Positano, S., Buiatti, M., 1984. Plantlet regeneration from petals and floral induction in vitro in the Mediterranean carnation (*Dianthus caryophyllus* L.). Riv. Ortoflorofrutt. It. 68, 107–121.

Hamill, J.D., 1993. Alterations in auxin and cytokinin metabolism of higher plants due to expression of specific genes from pathogenic bacteria: a review. Aust. J. Plant Physiol. 20, 405–423.

Hutteman, C.A., Preece, E.J., 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33, 105–119.

Kurioka, Y., Suzuki, Y., Kamada, H., Harada, H., 1992. Promotion of flowering and morphological alterations in *Atropa belladonna* transformed with a CaMV 35S-*rolC* chimeric gene of the Ri plasmid. Plant Cell Rep. 12, 1–6.

Malik, K.A., Saxena, P.K., 1992. Regeneration in *Phaseolus vulgaris* L.: high-frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. Planta 186, 384–389.

Maurel, C., Barbier-Brygoo, H., Spena, A., Tempé, J., Guern, J., 1991. Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. Plant Physiol. 97, 212–216.

Maurel, C., Leblanc, N., Barbier-Brygoo, H., Perrot-Rechenmann, C., Bouvier-Durand, M., Guern, J., 1994. Alterations of auxin perception in *rolB*-transformed tobacco protoplasts. Plant Physiol. 105, 1209–1215.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Nakano, M., Hoshino, Y., Mii, M., 1994. Adventitious shoot regeneration from cultured petal explants of carnation. Plant Cell Tiss. Org. Cult. 36, 15–19.

Nilsson, O., Olsson, O., 1997. Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol. Plant. 100, 463–473.

Nilsson, O., Moritz, T., Imbault, N., Sandberg, G., Olsson, O., 1993. Hormonal characterization of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* TL-DNA. Plant Physiol. 102, 363–371.

Nilsson, O., Moritz, T., Sundberg, B., Sandberg, G., Olsson, O., 1996. Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fasciation. Plant Physiol. 112, 493–502.

Ovadis, M., Zuker, A., Tzfira, T., Ahroni, A., Shklarman, E., Scovel, G., Itzhaki, H., Ben-Meir, H., Vainstein, A., 1999. Generation of transgenic carnation plants with novel characteristics by combining microprojectile bombardment with *Agrobacterium tumefaciens* transformation. In: Altman, A., Izhar, S., Ziv, M. (Eds.), Plant Biotechnology and In Vitro Biology in the 21st Century. Kluwer Academic Publishers, The Netherlands, pp. 189–192.

Schmülling, T., Schell, J., Spena, A., 1988. Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J. 7, 2621–2629.

Schmülling, T., Fladung, M., Grossmann, K., Schell, J., 1993. Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. Plant J. 3, 371–382.

Spano, L., Mariotti, D., Cardarelli, M., Branca, C., Costantino, P., 1988. Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. Plant Physiol. 87, 479–483.

Spena, A., Schmülling, T., Koncz, C., Schell, J., 1987. Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants. EMBO J. 6, 3891–3899.

Tepfer, D., 1984. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: sexual transmission of the transformed genotype and phenotype. Cell 37, 959–967.

Tzfira, T., Vinocur, B., Altman, A., Vainstein, A., 1998. *rol*-transgenic *Populus tremula*: root developement, root-borne bud regeneration and in vitro propagation efficiency. Trees 12, 464–471.

van Altvorst, A.C., Bino, R.J., van Dijk, A.J., Lamers, A.M.J., Lindhout, W.H., van der Mark, F., Dons, J.J.M., 1992. Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. Plant Sci. 83, 77–85.

van Altvorst, A.C., Koehorst, H.J.J., Bruinsma, T., Dons, J.J.M., 1994. Improvement of adventitious shoot formation from carnation leaf explants. Plant Cell Tiss. Org. Cult. 37, 87–90.

Walden, R., Cazaja, I., Schmuelling, T., Schell, J., 1993. *Rol* genes alter hormonal requirements for protoplast growth and modify the expression of an auxin responsive promoter. Plant Cell Rep. 12, 551–554.

White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P., Nester, E.W., 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J. Bacteriol. 164, 33–44.

Zuker, A., Ahroni, A., Tzfira, T., Ben-Meir, H., Vainstein, A., 1999. Wounding by bombardment yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Mol. Breed. 5, 367–375.

Zuker, A., Tzfira, T., Scovel, G., Ovadis, M., Shklarman, E., Itzhaki, H., Vainstein, A., 2001. *rolC*-transgenic carnation with improved agronomic traits: quantitative and qualitative analyses of greenhouse-grown plants. J. Am. Soc. Hort. Sci. 126, 13–18.

CAPÍTOL 5

Plantes de clavell transgèniques amb *rolC*: organogènesi adventícia i nivells d'auxines i citocinines endògenes

Plantes de clavell transgèniques amb *rolC*: organogènesi adventícia i nivells d'auxines i citocinines endògenes

Resum

La sobreexpressió del gen *rolC* d'*Agrobacterium rhizogenes* en plantes n'altera el seu creixement i desenvolupament. Nosaltres hem estudiat si l'efecte de citocinina (CK) i d'auxina del *rolC* en l'increment en la regeneració de tiges i rels en explants de tres línies de clavell (*Dianthus caryophyllus* L. cv. White Sim) transgèniques amb el gen *rolC* està relacionat amb alteracions en els nivells de l'auxina endògena àcid indol-3-acètic (IAA) o de les CKs tipus isoprenoide. El gen *rolC* ha provocat un increment en l'organogènesi de tiges i rels en explants de pètals i fulles transgènics, amb diferències entre les línies. Les CKs totals o la relació CK/auxina no s'han correlacionat amb l'organogènesi de les línies transgèniques. Tanmateix, els nivells del ribòsid de la zeatina han incrementat fins a 3 vegades en fulles *rolC* i els nivells d'N^6-isopenteniladenina, fins a 7 vegades en pètals *rolC*. La immunolocalització d'aquesta última CK ha mostrat marcatge en el citoplasma, però principalment en el nucli, tant de pètals *rolC* com de pètals no transgènics. L'increment en l'organogènesi adventícia de tiges es podria relacionar amb els nivells elevats de CKs específiques, encara que aquests nivells poden ser la causa d'un efecte indirecte del *rolC* en el metabolisme hormonal. Com que els nivells d'IAA no estan afectats en plantes transgèniques, proposem que l'increment en la rizogènesi seria degut a un augment en la sensibilitat a les auxines. Tanmateix, calen més estudis per aclarir la funció concreta de la proteïna RolC en les plantes transgèniques.

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rolC-transgenic carnation plants: adventitious organogenesis and levels of endogenous auxin and cytokinins

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Abstract

Overexpression of the *rolC* gene of *Agrobacterium rhizogenes* in plants alters growth and development. We studied whether the cytokinin (CK)-like and auxin-like effect of *rolC* on enhanced shoot and root regeneration in explants of three lines of *rolC*-transgenic carnation plants (*Dianthus caryophyllus* L. cv. White Sim) was related to altered levels of the endogenous auxin indole-3-acetic acid (IAA) or isoprenoid-CKs. *rolC* led to improved shoot and root organogenesis in transgenic petal and leaf explants and differences were observed between the lines. Total CKs and the CK/auxin ratio were not correlated with the organogenesis of these lines. However, zeatin riboside levels were up to 3-fold higher in *rolC* leaves and N^6-isopentenyladenine levels up to 7-fold higher in transgenic petals. Immunolocalization of the latter showed cytoplasmic but mainly nuclear labelling in *rolC* and non-transgenic petals. The enhanced adventitious shoot organogenesis could be correlated with higher specific CK levels, although they might be an indirect effect of *rolC* on hormone metabolism. Since levels of IAA remained unaffected in transgenic explants, we propose that an increase in the auxin sensitivity in *rolC* carnation tissues is responsible for improved rhizogenesis. However, more studies are needed for clarification of the precise function of the RolC protein in transgenic plants.

Keywords: *Agrobacterium rhizogenes*; *Dianthus caryophyllus*; Endogenous hormones; In vitro culture; Organogenesis; *rol* genes

1. Introduction

The carnation (*Dianthus caryophyllus* L.), one of the top-selling commercial cut-flower crops worldwide, is an important target for the breeding of new varieties with improved agronomic traits. The high heterozygosity of this flower restricts traditional breeding programs, therefore genetic engineering can provide an alternative method for crop improvement [1]. Genes from *Agrobacterium rhizogenes*, a soil bacterium that causes hairy root disease in susceptible dicotyledonous plants, have been used to alter plant morphology in several species. Specifically, *rolA*, *rolB*, *rolC* and *rolD* genes, within the T-DNA segment of the Ri plasmid, are responsible for inducing the morphogenic effects of the bacterium [2,3]. *rolC* is required for hairy root disease, and its overexpression in transgenic plants alters growth and development [4]. This gene has been used to transform tobacco [5,6], potato [7,8], belladonna [9], hybrid aspen [10,11], petunia [12], chrysanthemum [13] and also carnation [14]. These 35S-*rolC*-transgenic plants show common developmental alterations, including male sterility, dwarfed plants with short internodes, reduced apical dominance that leads to increased axillary bud breakage and, in some cases, advanced flowering and increased number of flowers. More-

Abbreviations: BA, N^6-benzyladenine; CK, cytokinin; DHZ, dihydrozeatin; DHZR, dihydrozeatin riboside; IAA, indole-3-acetic acid; iP, N^6-isopentenyladenine; iPR, N^6-isopentenyladenosine; NAA, 1-naphthaleneacetic acid; TDZ, *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea; Z, zeatin; ZR, zeatin riboside

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over, *rolC* leads to improved rooting of cuttings in two fruit trees [15,16] and in carnation [14]. *rolC*-transgenic carnations, particularly, in addition to showing increased axillary budbreak, thus generating more cuttings, also produce three times more flowering stems than controls [14].

The fact that the *rolC* gene product can induce plant dwarfing or reduce apical dominance is suggestive of a cytokinin (CK) activity of this gene [5]. However, the RolC protein, which has β-glucosidase activity, releases free forms of CKs from their inactive glucosidic conjugates in vitro [17], but not in vivo [4,10]. Moreover, data on the levels of CKs in *rolC* plants are not consistent, since they decrease, increase or remain stable depending not only on the species but also on the tissues analyzed [2,18]. In addition to the CK-like effect of *rolC*, improved root formation indicates that this gene exerts auxin-like activity [14]. However, this activity has mainly been associated with the *rolABC* combination, and specifically with *rolB* [2,18]. The natural endogenous auxin indole-3-acetic acid (IAA) does not increase in *rolC* tobacco, potato or hybrid aspen [6,8,10].

rol genes induce adventitious root formation in tobacco, kalanchoe and tomato leaves in vitro [19–21]. To our knowledge, adventitious shoot formation from *rolC* tissues has only been studied in carnation plants [22], while rhizogenesis has also been reported in tobacco and kalanchoe leaves [19]. In a previous study we reported the induction of CK-like and auxin-like activities by *rolC* during adventitious organogenesis in carnation explants [22].

Here we aimed to study whether the CK- and auxin-like effects of the *rolC* gene on shoot and root organogenesis in explants of transgenic carnation tissues of cv. White Sim could be related to altered levels of endogenous CKs or auxin. Therefore, we measured the levels of isoprenoid-CKs and IAA of leaf and petal explants of three *rolC* carnation lines, and the non-transformed line as a control, and looked for correlations with the organogenic potential of these explants. We also immunolocalized N^6-isopentenyladenine (iP), the most abundant CK in *rolC* petals, to compare its subcellular compartmentation in control and transgenic petals.

2. Materials and methods

2.1. Plant material

Carnation plants (*D. caryophyllus* L. cv. White Sim), control and *rolC*-transgenic, were grown in standard greenhouse conditions, under a natural photoperiod in a latitude of 42°N (Barcelona, Spain). We used non-transformed plants as controls and three *rolC*-transgenic lines, *rolC*1, *rolC*2 (Fig. 1) and *rolC*4, selected phenotypically and whose transgenic nature was confirmed previously [14]. In these transgenic plants, *rolC* was driven by the CaMV-35S constitutive promoter. The non-transgenic plants were phenotypically and morphologically indistinguishable from the transformed plants with the *uidA* gene and without *rolC* [14].

Fig. 1. Morphological alterations in aerial and subterranean parts of *rolC*-transgenic carnation plants. Non-transformed carnation (left), *rolC*2- (middle) and *rolC*1-transgenic carnations (right).

Cuttings from vegetative shoots were harvested in June–July and used as a source of leaf explants. Young flower buds were removed in December–January, since the plants flower throughout the year, albeit with varying intensity, and were used as the petal explant source. All sources of explants were stored at 4 °C, and were used for a maximum of 15 days.

2.2. Tissue culture

Cuttings were rinsed in 70% (v/v) ethanol, then surface-sterilized for 8 min in 1.2% (w/v) sodium hypochlorite and rinsed in sterile water three times for 10 min. Leaves, 10–14 per cutting, were removed from stems following the method described by van Altvorst et al. [23], and basal parts were placed in Murashige and Skoog (MS) medium with 2 mg l^{-1} glycine, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine-HCl, 0.1 mg l^{-1} thiamine-HCl and 30 g l^{-1} sucrose [24]. Flower buds (11–14 mm diameter) were surface-sterilized for 5 s

with 96% (v/v) ethanol. Petals were excised and basal parts were placed in MS basal medium [24] with 2 mg l^{-1} glycine, 50 mg l^{-1} myo-inositol, 0.5 mg l^{-1} thiamine-HCl and 30 g l^{-1} sucrose [25]. Media for leaves and petals were supplemented with several plant growth regulators in order to obtain shoot and root organogenesis. Before autoclaving (121 °C for 20 min), pH was adjusted to 5.8 and media were solidified with 8 g l^{-1} agar (High Gel Strength, Sigma).

2.2.1. Evaluation of the effect of rolC on shoot and root organogenesis in leaf and petal explants

Shoot and root regeneration in control and *rolC*-transgenic petal explants was evaluated by supplementing media with 1-naphthaleneacetic acid (NAA) (0.5 μM) plus *N*-phenyl-*N*′-1,2,3-thiadiazol-5-ylurea (TDZ) (0.0, 0.005, 0.05 or 0.5 μM); higher TDZ concentrations produce highly vitrified regenerated shoots [26]. We used TDZ, a substituted phenylurea with high CK-like activity, since comparative studies have shown TDZ to be the most effective CK for adventitious shoot regeneration from carnation petal explants [27]. Plant growth regulator-free medium was not assessed in petals, since it does not yield organogenesis in these explants [22,26]. For control and *rolC*-transgenic leaf explants, we added NAA (0.5 μM) plus N^6-benzyladenine (BA) (0.0, 0.5, 2.5 or 5.0 μM) or TDZ (0.5 μM), or no plant growth regulators. In this case, BA was chosen as the main CK because of the high vitrification caused by TDZ in leaves [26].

Shoot and root regeneration was assessed after 30 days of culture by counting the number of shoots and roots produced per explant, using a stereomicroscope.

2.2.2. Culture conditions

Leaf and petal explants were cultured in 500 ml glass vessels containing 100 ml of solid medium (25–28 leaves and 50–55 petals per vessel). Each experiment, one vessel per treatment, was performed in triplicate. To minimize the variability in the regeneration capacity of cuttings and flower buds, leaves from the same cutting and petals from the same flower bud were randomly distributed among all media. All the cultures were kept in a growth chamber at 25 ± 1 °C in a 16 h photoperiod using cool white fluorescent tubes (TLD 58W/33, Philips, France, 80 μmol m^{-2} s^{-1}).

2.3. Measurement of phytohormone levels

Fresh leaves and petals of control and *rolC*-transgenic plants, specifically the bases of these organs (10 mm from leaves and 5 mm from petals) since they are the regenerative part, were collected in September to determine the levels of endogenous phytohormones. Tissues were frozen in liquid nitrogen and lyophilized before measuring IAA and the main CKs: iP, zeatin (Z), dihydrozeatin (DHZ), and their 9-ribosides (iPR, ZR and DHZR). Extraction and purification of phytohormones were performed according to Fernández et al. [28], by using 100 mg of dry weight per sample. A combination of liquid and solid phase extractions with organic solvents and immunoaffinity chromatography techniques were used to obtain two fractions: an aqueous fraction that contained the IAA and a methanolic fraction with the CKs. The six CKs were separated by reverse-phase high performance liquid chromatography (HPLC) on a Kromasil 100 C18–5 μm (150 mm × 4.6 mm) column (Teknokroma), and kinetin was used as an internal standard to corroborate CK retention times. The mobile phase was acetonitrile and triethylammonium acetate 40 mM, at pH 7, with a linear gradient from 5 to 20% acetonitrile (v/v) over 40 min, and 1.5 ml fractions were collected. CKs were quantified by competitive enzyme-linked immunosorbent assays (ELISA) using three polyclonal rabbit antibodies (anti-ZR to quantify Z and ZR, anti-DHZR to quantify DHZ and DHZR and anti-iPR to quantify iP and iPR), obtained following Fernández et al. [28]. IAA samples were methylated before quantification by ELISA using monoclonal antibodies against the methyl ester of IAA (Agdia®). Radiolabelled standards, [8-^{14}C]BA (2.0 GBq $mmol^{-1}$) and 3-[5(n)-^{3}H]IAA (999 GBq $mmol^{-1}$) (Amersham), were added to each sample at the beginning of the analyses to determine purification losses. For each treatment (explant and plant line) we analyzed three samples. Abbreviations of CKs are named according to Kamínek et al. [29].

2.4. Immunolocalization of cytokinins

The iP was immunocytochemically labeled in the bases of fresh petals of control and *rolC*1 plants, the latter being the *rolC* tissue with the highest iP levels. Petals were collected at the same time as for phytohormone quantification and were prepared following Casanova et al. [26], with modifications to adapt the method to ultrathin sections. Tissues were fixed in 4% (v/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, under vacuum at 4 °C for 4–6 days, washed in this buffer, dehydrated in an acetone series and embedded in Spurr's [30] resin. Ultrathin transversal sections (50–60 nm) on gold grids were etched for 5 min in 10% (v/v) hydrogen peroxide, rinsed with distilled water, incubated in the block solution (0.1 M phosphate buffer saline (PBS), pH 7.4, with 1% (w/v) bovine serum albumin (BSA) and 0.05% (v/v) Tween-20) at room temperature for 15 min, and then incubated in the primary antibody anti-iPR diluted in the block solution, in a moist chamber overnight at 4 °C. This polyclonal antibody (OlChemIm Ltd., Czech Republic) was used at a concentration of 0.498 μg/μl. After washing the sections in the block solution, they were incubated with Protein A conjugated to colloidal gold of 10 nm diluted 1:70 in 0.1 M PBS, pH 7.4, with 5% BSA and 0.05% Tween-20, at room temperature for 1 h, washed with 0.1 M PBS, pH 7.4, and rinsed with distilled water. Gold-stained sections were submitted to a silver-enhancement reaction for 1 min (British BioCell International kit, UK). After washing with distilled water, sections were counterstained with 2% (w/v) aqueous uranyl acetate for 15 min and lead citrate

[31] for 3 min. The grids were observed with a transmission electron microscope JEOL 1010 operated at 80 kV, and silver particles were visualized as black spots because of their high electron density.

Controls for the specificity of the immunolabelling were obtained by replacing the antibodies by PBS-BSA. Some cellular compartments, such as the cell wall and the vacuole, lacked labelling and thus served as internal controls. The antibody anti-iPR recognized the free base iP, the riboside, the riboside-5′-monophosphate and the 9-glucoside, with minimal cross-reaction between CK types. Since free bases of CKs have been shown to bind to cell proteins in the presence of aldehydes, used as fixatives [32,33], our labelling indicated iP, the free-base form of iP-type CKs.

2.5. Statistical analyses

Differences in the shoot and root regeneration between media and plant lines for each explant were tested using two-way ANOVAs. Differences within each factor (medium and line) were tested with Duncan's multiple range test when two-way ANOVA showed significant differences ($P < 0.05$). When interaction was found, Duncan's multiple range test was also used to identify differences between lines within each medium. Differences in the concentration of hormones between lines were tested for each explant using one-way ANOVAs. Duncan's multiple range test was applied when the one-way ANOVA showed significant differences ($P < 0.05$). All the statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, Illinois).

3. Results

3.1. Effect of rolC on shoot and root organogenesis in carnation leaf and petal explants

The number of adventitious shoots regenerated from leaf explants was essentially the same in all the lines when cultured in the absence of plant growth regulators (Table 1). In media with 0.5 μM NAA and increasing BA concentrations up to 5.0 μM, shoot regeneration from leaves increased progressively in all the lines, and the medium with 0.5 μM NAA plus 0.5 μM TDZ produced the highest number of shoots. Leaves of the non-transformed (control) line were the least regenerative, while *rolC*1 regenerated the highest number of shoots per leaf. The interaction found between lines and media showed that differences in shoot regeneration between lines were more remarkable in media with 0.5 μM NAA plus 0.5 or 2.5 μM BA, and mainly in medium with 0.5 μM NAA plus 0.5 μM TDZ. Thus, in the latter medium, shoot regeneration from *rolC*1 leaves was almost 3-fold higher than in controls, whereas in *rolC*2 and *rolC*4 leaves it was 2-fold higher.

The highest root regeneration from leaves was obtained in 0.5 μM NAA medium for all the lines, and decreased with increasing concentrations of BA (Table 1). The media with 0.5 μM TDZ and with 5.0 μM BA, in addition to 0.5 μM NAA, yielded the least number of roots for all the lines. Considering all the media, root regeneration in *rolC*1 leaves was the same as control, whereas *rolC*2 and *rolC*4 leaves showed higher regeneration. Differences

Table 1
Adventitious shoot and root regeneration from leaves of control (non-transformed), and *rolC*1-, *rolC*2- and *rolC*4-transgenic carnation lines (*D. caryophyllus* L. cv. White Sim) after 30 days of culture in MS solid media at a range of NAA, BA and TDZ concentrations

Media (μM)				Lines				Two-way ANOVA[a]		
NAA	BA	TDZ		Control	*rolC*1	*rolC*2	*rolC*4	L	M	I
Shoots per leaf (no.)[b]										
0.0	0.0	0.0	A	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	***	***	***
0.5	0.0	0.0	A	0.5 ± 0.2	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.2			
0.5	0.5	0.0	A	0.5 ± 0.2	2.1 ± 0.7	1.3 ± 0.5	1.3 ± 0.5			
0.5	2.5	0.0	B	2.1 ± 0.5	4.6 ± 0.9	2.8 ± 0.5	3.7 ± 0.8			
0.5	5.0	0.0	B	3.6 ± 0.6	4.3 ± 0.7	4.1 ± 0.8	2.8 ± 0.6			
0.5	0.0	0.5	C	4.3 ± 0.7	12.4 ± 1.6	8.8 ± 1.2	8.9 ± 1.3			
				A	C	B	B			
Roots per leaf (no.)[b]										
0.0	0.0	0.0	B	0.60 ± 0.14	0.31 ± 0.11	0.56 ± 0.17	0.88 ± 0.17	***	***	*
0.5	0.0	0.0	D	2.75 ± 0.34	2.54 ± 0.31	3.01 ± 0.37	3.21 ± 0.37			
0.5	0.5	0.0	C	0.30 ± 0.10	0.72 ± 0.17	1.95 ± 0.32	1.16 ± 0.26			
0.5	2.5	0.0	AB	0.13 ± 0.08	0.09 ± 0.05	0.63 ± 0.15	0.51 ± 0.15			
0.5	5.0	0.0	A	0.01 ± 0.01	0.07 ± 0.04	0.10 ± 0.06	0.11 ± 0.05			
0.5	0.0	0.5	A	0.03 ± 0.02	0.13 ± 0.06	0.57 ± 0.13	0.40 ± 0.13			
				A	A	B	B			

[a] Significant differences (two-way ANOVAs) in lines (L) and media (M), and interactions between these two factors (I) are indicated by single and triple asterisks. Within each line or medium, distinct letters show significant differences ($P < 0.05$) according to Duncan's multiple range test.
[b] Values represent the mean ± S.E. We used 75–85 leaf explants per treatment (line × medium).
* $P < 0.05$.
*** $P < 0.001$.

Table 2
Adventitious shoot and root regeneration from petals of control (non-transformed), and *rolC*1-, *rolC*2- and *rolC*4-transgenic carnation lines (*D. caryophyllus* L. cv. White Sim) after 30 days of culture in MS solid media at a range of NAA and TDZ concentrations

Media (μM)			Lines				Two-way ANOVA[a]		
NAA	TDZ		Control	*rolC*1	*rolC*2	*rolC*4	L	M	I
Shoots per petal (no.)[b]									
0.5	0.0	A	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	***	***	***
0.5	0.005	B	1.9 ± 0.3	5.4 ± 0.6	2.7 ± 0.4	2.2 ± 0.4			
0.5	0.05	C	2.9 ± 0.4	7.8 ± 0.7	5.3 ± 0.7	5.1 ± 0.6			
0.5	0.5	D	5.9 ± 0.5	12.5 ± 0.7	8.5 ± 0.7	9.1 ± 0.8			
			A	C	B	B			
Roots per petal (no.)[b]									
0.5	0.0	AB	0.00 ± 0.00	0.03 ± 0.02	0.07 ± 0.02	0.04 ± 0.02	***	*	n.s.
0.5	0.005	B	0.01 ± 0.01	0.09 ± 0.04	0.07 ± 0.03	0.01 ± 0.01			
0.5	0.05	B	0.01 ± 0.01	0.08 ± 0.03	0.05 ± 0.02	0.02 ± 0.01			
0.5	0.5	A	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.00 ± 0.00			
			A	B	B	B			

[a] Significant differences (two-way ANOVAs) in lines (L) and media (M), and interactions between these two factors (I) are indicated by single, triple asterisks and (n.s.) $P > 0.05$. Within each line or medium, distinct letters show significant differences ($P < 0.05$) according to Duncan's multiple range test.
[b] Values represent the mean ± S.E. We used 150–165 petal explants per treatment (line × medium).
* $P < 0.05$.
*** $P < 0.001$.

in root regeneration between lines were more notable in media with 0.5 μM NAA plus 0.5 μM BA, 2.5 μM BA or 0.5 μM TDZ. The largest differences were observed in the latter, as the number of roots was 19-fold and 13-fold greater in *rolC*2 and *rolC*4 leaves, respectively, than in controls.

In all the lines, the number of shoots per petal explant increased progressively in media with 0.5 μM NAA and increasing TDZ concentrations from 0.0 to 0.5 μM (Table 2). In *rolC*2 and *rolC*4 petals, a higher number of shoots than the controls was recorded, and the *rolC*1 petals showed the highest shoot regeneration, similarly to leaf explants. The interaction between lines and media showed that in media with 0.5 μM NAA plus 0.05 or 0.5 μM TDZ, the differences between lines were greater than in the other media. Thus, the higher the TDZ concentration in the medium, the clearer the effect of *rolC* on increasing shoot organogenesis. In the medium with 0.5 μM NAA plus 0.5 μM TDZ, the number of shoots produced per petal was double in *rolC*1, and 1.5-fold in the *rolC*2 and *rolC*4 lines, compared with controls.

Root regeneration from petals was maximum in the medium that contained 0.005 or 0.05 μM TDZ, and the number of roots decreased with higher TDZ concentrations (0.5 μM) (Table 2). All the *rolC* petals produced a higher number of roots than the controls. This root regeneration was much lower than that in leaf explants.

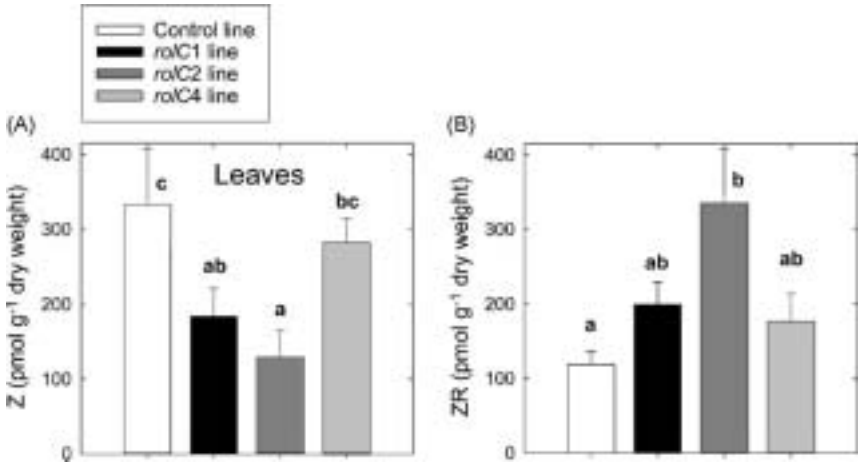

Fig. 2. Content of (A) Z and (B) ZR (pmol g^{-1} dry weight) in fresh carnation leaf bases (*D. caryophyllus* L. cv. White Sim) of the control (non-transformed) line and the *rolC*1-, *rolC*2- and *rolC*4-transgenic lines. Values represent the mean ± S.E. Distinct letters denote significant differences (one-way ANOVA, $P < 0.05$) according to Duncan's multiple range test.

3.2. Effect of rolC on levels of endogenous CKs and IAA in carnation leaves and petals

The most abundant endogenous CK in control leaves was Z (332 ± 75 pmol g^{-1} dry weight), followed by DHZR (251 ± 107 pmol g^{-1}), DHZ (167 ± 67 pmol g^{-1}) and ZR (119 ± 18 pmol g^{-1}). The amounts of iP-type CKs, iP (16 ± 4 pmol g^{-1}) and iPR (4 ± 1 pmol g^{-1}), were the lowest. The total amount of CKs, sum of iP, Z, DHZ and their ribosides, in *rolC*2 leaves was similar to that in controls, while *rolC*1 and *rolC*4 leaves showed slightly lower amounts. Differences in the levels of specific CKs were detected in all the lines (Fig. 2). All the transgenic lines presented lower amounts of Z than the control, although only in *rolC*1 and *rolC*2 they were significantly lower, 2- and 2.5-fold, respectively (Fig. 2A). In contrast, the levels of ZR in *rolC* leaves were greater than in controls, but only *rolC*2 leaves were significantly higher, 3-fold, than controls (Fig. 2B). Z and ZR levels of the studied lines were not correlated with their root or shoot regeneration. The content of iP-type and DHZ-type CKs in transgenic leaves was similar to that in controls.

In control petals, ZR (225 ± 40 pmol g^{-1} dry weight) was the most abundant CK, followed by DHZR (174 ± 30 pmol g^{-1}), iP (135 ± 18 pmol g^{-1}), DHZ (111 ± 15 pmol g^{-1}) and Z (102 ± 32 pmol g^{-1}) with similar levels, and iPR (46 ± 6 pmol g^{-1}), which was also the least abundant (Fig. 3). The total amount of CKs in *rolC*2 petals was similar to that in controls, whereas in *rolC*1 and *rolC*4 petals it was almost 2- and 1.5-fold higher, respectively. Moreover, we also detected differences in the levels of specific CKs (Fig. 3). The most abundant CK in the transgenic petals was iP (Fig. 3A, note the distinct scale). In *rolC*1, *rolC*2 and *rolC*4 petals, iP levels were 7-, 4.5- and 3.5-fold

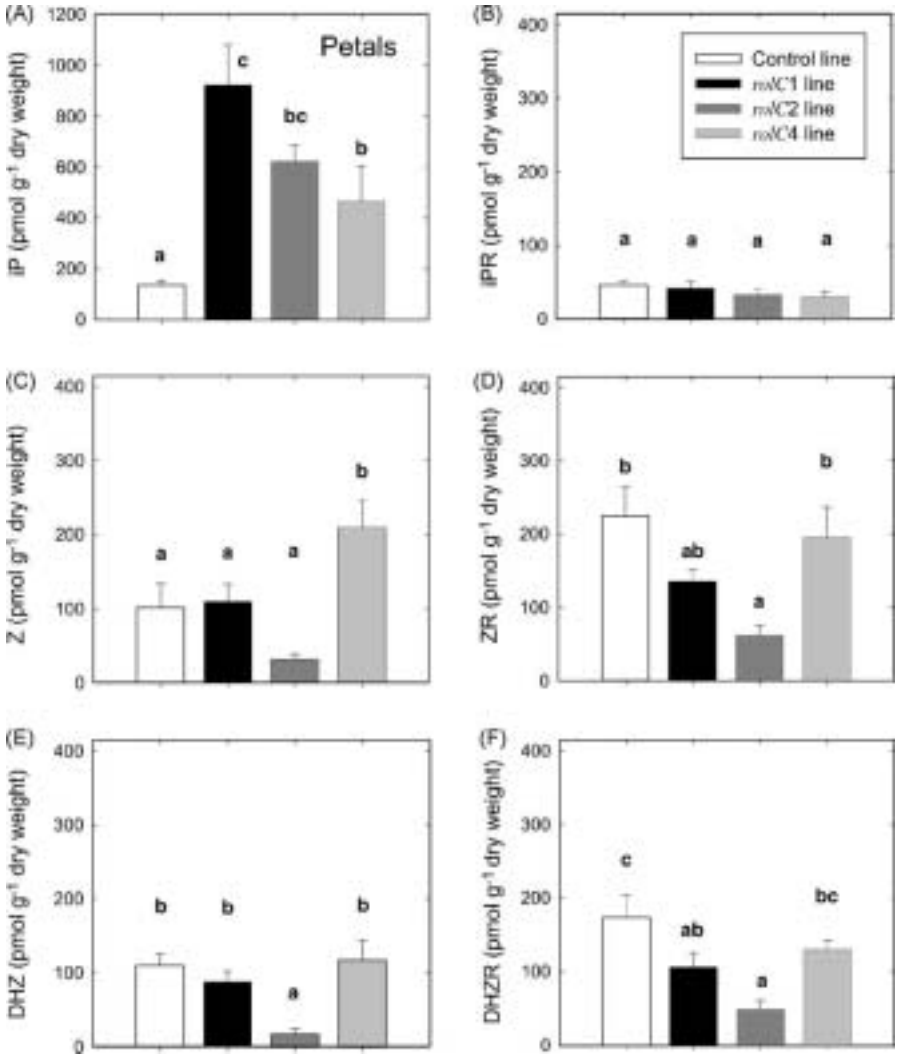

Fig. 3. Content of: (A) iP; (C) Z; (E) DHZ; and their respective ribosides (B) iPR; (D) ZR; (F) DHZR (pmol g^{-1} dry weight) in fresh carnation petal bases (*D. caryophyllus* L. cv. White Sim) of the control (non-transformed) line and the *rolC*1-, *rolC*2- and *rolC*4-transgenic lines. Values represent the mean ± S.E. Distinct letters denote significant differences (one-way ANOVA, $P < 0.05$) according to Duncan's multiple range test. Note the distinct scale for the iP content.

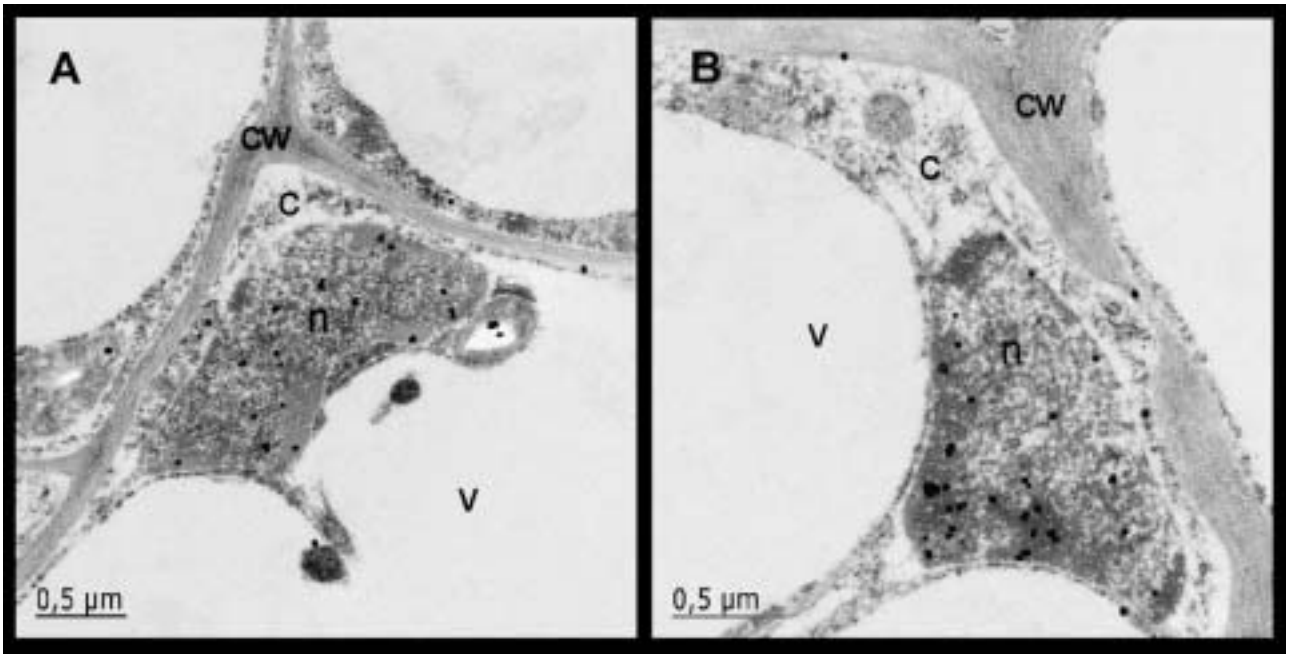

Fig. 4. Immunolocalization of iP in ultrathin sections of the basal region of fresh carnation petals (*D. caryophyllus* L. cv. White Sim), using protein A-gold and silver-enhancement method (Section 2). (A) Transversal section of a non-transformed petal; (B) transversal section of a *rolC*1-transgenic petal: (c) cytoplasm; (cw) cell wall; (n) nucleus; (v) vacuole.

higher than those in controls, respectively. The content of this CK in the three *rolC* lines and the control line correlated linearly with shoot regeneration, both in media with 0.05 μM TDZ ($R^2 = 0.97$, $P = 0.014$) and with 0.5 μM TDZ ($R^2 = 0.91$, $P = 0.047$). However, the iPR content of the three lines were similar to that in control petals (Fig. 3B). The Z content was 2-fold higher in *rolC*4 petals, while in the other transgenic petals it was the same as in the controls (Fig. 3C). The levels of ZR in *rolC*1 and *rolC*2 petals were lower than in the controls, being significantly lower, 3.5-fold, in the latter, whereas in *rolC*4 they were similar to that in controls (Fig. 3D). All the lines had similar amounts of DHZ, except *rolC*2 petals, which showed 6.5-fold lower levels than those in controls (Fig. 3E). All the transgenic lines had lower levels of DHZR than the controls, but only *rolC*1 and *rolC*2 had significantly lower amounts, 1.5- and 3.5-fold, respectively (Fig. 3F).

The levels of IAA in the leaves of *rolC*1 (23.0 ± 4.4 nmol g^{-1} dry weight), *rolC*2 (18.9 ± 1.2 nmol g^{-1}) and *rolC*4 (16.9 ± 1.7 nmol g^{-1}) were the same as control (18.0 ± 2.1 nmol g^{-1}). Similarly, IAA content of petals of *rolC*1 (21.6 ± 1.2 nmol g^{-1}), *rolC*2 (26.4 ± 1.5 nmol g^{-1}) and *rolC*4 (22.2 ± 1.4 nmol g^{-1}) was the same as control (26.8 ± 3.8 nmol g^{-1}). Control petals, thus, had slightly higher levels of IAA than control leaves. Since levels of IAA were similar between control and transgenic lines, the ratio of total CKs/IAA showed a similar pattern to that of the total CKs, being slightly lower in *rolC*1 and *rolC*4 leaves than in control leaves and up to twice that of controls in *rolC*1 and *rolC*4 petals. Similar CKs/IAA values as those in controls were found in *rolC*2 explants.

3.3. Immunolocalization of iP in rolC1-transgenic petals

Immunolocalization of iP, the most abundant CK in *rolC* petals, was performed in *rolC*1 petals since they had the highest iP levels among the transgenic lines. The iP distribution, both in control and *rolC*1 petals, was studied at cytological level, by electron microscope, since at the histological level, using a light microscope, hardly any label was obtained. At subcellular level, immunoreactivity, indicated by the silver particles, was observed in control (Fig. 4A) and transgenic petal bases (Fig. 4B), with no differences between them. Labelling of iP was intense in the nucleus but much weaker in the cytoplasm. The control sections, with the primary antibody omitted, showed no silver marks (data not shown).

4. Discussion

Here we show that *rolC* enhances adventitious shoot and root regeneration in carnation petals and leaves. Our results show that this gene exerts a dual effect; it displays not only a CK-like action but also an auxin-like effect, thus extending and confirming our previous results [22]. The CK-like activity of *rolC* was also manifested through decreased apical dominance, with the consequent improved development of lateral shoots, in *rolC* carnation plants [14] and in most *rolC*-transgenic plants of other species [5,7,10]. In agreement with the auxin-like effect of *rolC*, not only transgenic carnation plants [14], but also *rolC* trifoliate orange and

Japanese persimmon [15,16] showed improved rooting ability.

The distinct *rolC* lines in our study displayed a strong relationship between the degree of adventitious organogenesis and their phenotypes. The *rolC*1 line was the most caulogenic in vitro, whereas *rolC*2 and *rolC*4 showed intermediate behavior between *rolC*1 and non-transgenic (control) lines. Lateral shoot production in vivo was also highest in *rolC*1, intermediate in *rolC*2 and *rolC*4 and lowest in control plants [14]. In terms of roots, all the transgenic lines presented a similar adventitious rhizogenesis, that was higher than control. The rooting capacity of the *rolC*-transgenic cuttings was also higher than control [14]. The phenotypic differences between *rolC*-transgenic carnation lines might be explained by distinct degree of expression of the gene, as reported in other species transformed with *rolC* [9,10,12,34]. In addition to the line-dependent effects, the *rolC* transgene also interacts with the genetic background of species, which is seen in root regeneration from leaf explants cultured in medium without growth regulators. While in carnation adventitious roots regenerated from both *rolC* and control leaves, in tobacco adventitious roots regenerated only from *rolC* but not from control leaves, and in kalanchoe such roots did not regenerate from either control or *rolC* leaves [19].

The precise mode of action of *rolC* in transgenic plants remains unclear. However, the phenotypes observed are indicative of a biochemical effect of this gene on endogenous hormones, through either altering their metabolism or the sensitivity to them. The overall CK content in our *rolC* carnation tissues and the CKs/IAA ratio, which shows a similar pattern, are not related to the adventitious organogenesis observed, since they increased, decreased or showed no changes compared with levels in the control line. Indeed, depending on the species and, more specifically, organ studied, the total amounts of CKs in *rolC* plants were higher [8,10,13], lower [6,11] or similar [4,8] to that in control plants. In our case with *rolC* carnation organs the levels of specific CKs appear to be more crucial than their total amounts for the adventitious organogenesis.

rolC carnation leaves contained less Z and up to 3-fold more ZR than the controls, but these CK changes were not correlated with the root or shoot regeneration capacity of these explants. However, since Z-type CKs, Z, its riboside and its riboside-5′-monophosphate, have been widely associated with cell division in several systems [35], the higher ZR content in *rolC* leaves could account for the improved shoot organogenesis observed. These high levels of ZR are consistent with quantifications in leaves of *rolC* potato and shoot apices of *rolC* hybrid aspen and *rolC* chrysanthemum, the latter during flower formation [8,10,13], although the content of this CK in young leaves of *rolC* tobacco was lower than controls [6].

In *rolC* carnation petals, the most relevant change in CKs was the higher levels of iP, which were up to 7-fold. Moreover, the content of iP correlated with the adventitious shoot organogenesis in petals of all the lines. This high iP content is consistent with the significant increase in this CK during TDZ-induced shoot organogenesis in non-transgenic carnation petals [26], thus indicating that iP is the endogenous CK that makes the greatest contribution to caulogenesis in these explants. The effect of iP could be due to its conversion by means of hydroxylation reactions to Z [36], the latter being one of the most active forms of CK. A higher iP content has not been reported in any other *rolC* tissue, including our *rolC* carnation leaves, in which iP levels were similar to those in controls. However, higher levels of iPR than controls were found in *rolC* potato tissues [8] whereas much lower levels of iPR were reported in *rolC* tobacco leaves [6]. Accumulation of iP-type CKs has also been found in young tissues of lettuce plants that overexpressed *KNAT1*, a *knotted1*-like homeobox gene involved in the maintenance of meristematic activity. These transgenic plants, analogously to our *rolC* carnations, display a phenotype which resembles that induced by overproduction of CKs [37].

The in situ localization of iP, at tissue level, showed both non-transgenic and *rolC* carnation petals to be poorly immunoreactive, as expected, since most of their cells were not in division at the stage analyzed. Accordingly, only dividing cells of adventitious bud primordia from carnation petals presented detectable CK accumulation [26]. Our results, thus, agrees with the important role attributed to CKs in cell division [35]. Although histological distribution of CKs has been reported in several species [26,32,33,38–40], including carnations, their cytological localization has not been studied in detail. Our results of immunolocalization of iP at subcellular level showed this CK to be faintly distributed in the cytoplasm and mainly compartmentalized in the nucleus, in both *rolC* and control petals. Therefore, using this qualitative technique we did not detect differences in the iP compartmentation between lines. According to our results, iP-type CKs were immunolocated in the nucleus, and also in the cytoplasm, in cells of developing embryos of *Dactylis glomerata* [38] and *Tilia cordata* [39], and also in tomato shoot and root apices [32]. In contrast, in tobacco apices iP was only cytoplasmic and perinuclear [33]. Overall, since carnation petal cells did not present meristematic activity, the nuclear localization of iP could implicate this CK in the regulation of gene expression, as described for CKs [41].

Our *rolC*-transgenic carnation tissues presented no differences in the endogenous IAA levels with respect to controls, which is in agreement with *rolC* tobacco and potato plants [6,8]. IAA levels in *rolC* hybrid aspen and chrysanthemum were even lower than controls [10,13]. Thus, our results indicate that tissues with *rolC*, with a higher rate of root regeneration, present an increase in auxin sensitivity, as proposed for the *rolABC* combination [20,21] and particularly for *rolB* [2,18]. Accordingly, measurements of the transmembrane potential of *rolC* tobacco protoplasts in response to auxins showed them to be more sensitive to auxins than the wild type, although in *rolB* protoplasts this sensitivity was higher [42].

Overall, our results show that the activity of RolC induces an increase in specific CKs that is line- and organ-dependent, which would enhance shoot organogenesis in *rolC* carnation explants. The specific CK alterations in *rolC* carnation tissues appeared to be more decisive than overall CK levels for adventitious organ regeneration. In petals there was a correlation between shoot organogenic potential and the levels of iP. However, this effect might be indirect, since, with the constitutive expression of the transgene, a direct effect of RolC should cause similar results in any organ. Other authors also suggested an indirect effect of RolC, although in their case no clear correlations between the CK levels and the phenotypic features of the transgenic plants were found [8,10]. In addition, since IAA levels were unaffected by the *rolC* expression, an increase in auxin sensitivity may explain the improved adventitious rhizogenesis. This study sheds light on the amounts of endogenous hormones in a new *rolC*-transgenic species, carnation, including the first immunolocalization of a CK in a *rolC* tissue. However, more studies are needed to clarify the precise function of the RolC protein in transgenic plants.

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References

[1] A. Vainstein (Ed.), Breeding For Ornamentals: Classical and Molecular Approaches, Kluwer Academic Publishers, Dordrecht, The Netherlands, 2002.

[2] A.D. Meyer, J. Tempé, P. Costantino, Hairy root: a molecular overview, Functional analysis of *Agrobacterium rhizogenes* T-DNA genes, in: G. Stacey, N.T. Keen (Eds.), Plant-Microbe Interactions, APS Press, St. Paul, MI, USA, 2000, pp. 93–139.

[3] M.C. Christey, Use of Ri-mediated transformation for production of transgenic plants, In Vitro Cell. Dev. Biol. 37 (2001) 687–700.

[4] M. Faiss, M. Strnad, P. Redig, K. Dolezal, J. Hanus, H. van Onckelen, T. Schmülling, Chemically induced expression of the *rolC*-encoded β-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: *rolC* does not hydrolyze endogenous cytokinin glucosides in planta, Plant J. 10 (1996) 33–46.

[5] T. Schmülling, J. Schell, A. Spena, Single genes from *Agrobacterium rhizogenes* influence plant development, EMBO J. 7 (1988) 2621–2629.

[6] O. Nilsson, T. Moritz, N. Imbault, G. Sandberg, O. Olsson, Hormonal characterization of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* TL-DNA, Plant Physiol. 102 (1993) 363–371.

[7] M. Fladung, Transformation of diploid and tetraploid potato clones with the *rolC* gene of *Agrobacterium rhizogenes* and characterization of transgenic plants, Plant Breed. 104 (1990) 295–304.

[8] T. Schmülling, M. Fladung, K. Grossmann, J. Schell, Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA, Plant J. 3 (1993) 371–382.

[9] Y. Kurioka, Y. Suzuki, H. Kamada, H. Harada, Promotion of flowering and morphological alterations in *Atropa belladonna* transformed with a CaMV 35S-*rolC* chimeric gene of the Ri plasmid, Plant Cell Rep. 12 (1992) 1–6.

[10] O. Nilsson, T. Moritz, B. Sundberg, G. Sandberg, O. Olsson, Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fasciation, Plant Physiol. 112 (1996) 493–502.

[11] M. Fladung, K. Grossmann, M.R. Ahuja, Alterations in hormonal and developmental characteristics in transgenic *Populus* conditioned by the *rolC* gene from *Agrobacterium rhizogenes*, J. Plant Physiol. 150 (1997) 420–427.

[12] C. Winefield, D. Lewis, S. Arathoon, S. Deroles, Alterations of *Petunia* plant form through the introduction of the *rolC* gene from *Agrobacterium rhizogenes*, Mol. Breed. 5 (1999) 543–551.

[13] T.Y. Mitiouchkina, S.V. Dolgov, Modification of chrysanthemum flower and plant architecture by *rolC* gene from *Agrobacterium rhizogenes* introduction, in: Proceedings of the 19th International Symposium on Improvement of Ornamental Plants, Acta Hort. 508 (2000) 163–169.

[14] A. Zuker, T. Tzfira, G. Scovel, M. Ovadis, E. Shklarman, H. Itzhaki, A. Vainstein, *rolC*-transgenic carnation with improved agronomic traits: quantitative and qualitative analyses of greenhouse-grown plants, J. Am. Soc. Hort. Sci. 126 (2001) 13–18.

[15] J. Kaneyoshi, S. Kobayashi, Characteristics of transgenic trifoliate orange (*Poncirus trifoliata* Raf.) possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid, J. Jpn. Soc. Hort. Sci. 68 (1999) 734–738.

[16] Y. Koshita, Y. Nakamura, S. Kobayashi, K. Morinaga, Introduction of the *rolC* gene into the genome of the Japanese persimmon causes dwarfism, J. Jpn. Soc. Hort. Sci. 71 (2002) 529–531.

[17] J.J. Estruch, D. Chriqui, K. Grossmann, J. Schell, A. Spena, The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates, EMBO J. 10 (1991) 2889–2895.

[18] O. Nilsson, O. Olsson, Getting to the root: the role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots, Physiol. Plant. 100 (1997) 463–473.

[19] A. Spena, T. Schmülling, C. Koncz, J. Schell, Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants, EMBO J. 6 (1987) 3891–3899.

[20] L. Spanò, D. Mariotti, M. Cardarelli, C. Branca, P. Costantino, Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA, Plant Physiol. 87 (1988) 479–483.

[21] A.C. van Altvorst, R.J. Bino, A.J. van Dijk, A.M.J. Lamers, W.H. Lindhout, F. van der Mark, J.J.M. Dons, Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development, Plant Sci. 83 (1992) 77–85.

[22] E. Casanova, A. Zuker, M.I. Trillas, L. Moysset, A. Vainstein, The *rolC* gene in carnation exhibits cytokinin- and auxin-like activities, Sci. Hort. 97 (2003) 321–331.

[23] A.C. van Altvorst, H.J.J. Koehorst, T. Bruinsma, J.J.M. Dons, Improvement of adventitious shoot formation from carnation leaf explants, Plant Cell Tiss. Org. Cult. 37 (1994) 87–90.

[24] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.

[25] F. Gimelli, G. Ginatta, R. Venturo, S. Positano, M. Buiatti, Plantlet regeneration from petals and floral induction in vitro in the Mediterranean carnation (*Dianthus caryophyllus* L.), Riv. Ortoflorofrutt. It. 68 (1984) 107–121.

[26] E. Casanova, A.E. Valdés, B. Fernández, L. Moysset, M.I. Trillas, Levels and immunolocalization of endogenous cytokinins in

thidiazuron-induced shoot organogenesis in carnation, J. Plant Physiol. 161 (2004) 95–104.
[27] M. Nakano, Y. Hoshino, M. Mii, Adventitious shoot regeneration from cultured petal explants of carnation, Plant Cell Tiss. Org. Cult. 36 (1994) 15–19.
[28] B. Fernández, M.L. Centeno, I. Feito, R. Sánchez-Tamés, A. Rodríguez, Simultaneous analysis of cytokinins, auxins and abscisic acid by combined immunoaffinity chromatography, high performance liquid chromatography and immunoassay, Phytochem. Anal. 6 (1995) 49–54.
[29] M. Kamínek, A. Brezinová, A. Gaudinová, V. Motyka, R. Vanková, E. Zazímalová, Purine cytokinins: a proposal of abbreviations, Plant Growth Regul. 32 (2000) 253–256.
[30] A.R. Spurr, A low viscosity epoxy resin embedding medium for electronmicroscopy, J. Ultrastruct. Res. 26 (1969) 31–43.
[31] E.S. Reynolds, The use of lead citrate at high pH as an electron opaque stain in electron microscopy, J. Cell Biol. 17 (1963) 208–212.
[32] L. Sossountzov, R. Maldiney, B. Sotta, I. Sabbagh, Y. Habricot, M. Bonnet, E. Miginiac, Immunocytochemical localization of cytokinins in Craigella tomato and a sideshootless mutant, Planta 175 (1988) 291–304.
[33] W. Dewitte, A. Chiappetta, A. Azmi, E. Witters, M. Strnad, J. Rembur, M. Noin, D. Chriqui, H.A. van Onckelen, Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation, Plant Physiol. 119 (1999) 111–121.
[34] A. Giovannini, M. Zottini, G. Morreale, A. Spena, A. Allavena, Ornamental traits modification by *rol* genes in *Osteospermum ecklonis* transformed with *Agrobacterium tumefaciens*, In Vitro Cell. Dev. Biol. 35 (1999) 70–75.
[35] D. Francis, D.A. Sorrell, The interface between the cell cycle and plant growth regulators: a mini review, Plant Growth Regul. 33 (2001) 1–12.
[36] C.-M. Chen, Cytokinin biosynthesis and interconversion, Physiol. Plant. 101 (1997) 665–673.
[37] G. Frugis, D. Giannino, G. Mele, C. Nicolodi, A. Chiappetta, M.B. Bitonti, A.N. Innocenti, W. Dewitte, H. van Onckelen, D. Mariotti, Overexpression of *KNAT1* in lettuce shifts leaf determinate growth to a shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins, Plant Physiol. 126 (2001) 1370–1380.
[38] I. Ivanova, I.T. Todorov, L. Atanassova, W. Dewitte, H.A. van Onckelen, Co-localization of cytokinins with proteins related to cell proliferation in developing somatic embryos of *Dactylis glomerata* L, J. Exp. Bot. 45 (1994) 1009–1017.
[39] A. Kärkönen, L.K. Simola, Localization of cytokinins in somatic and zygotic embryos of *Tilia cordata* using immunocytochemistry, Physiol. Plant. 105 (1999) 356–366.
[40] A. Jacqmard, N. Detry, W. Dewitte, H.A. van Onckelen, G. Bernier, In situ localisation of cytokinins in the shoot apical meristem of *Sinapis alba* at floral transition, Planta 214 (2002) 970–973.
[41] T. Schmülling, S. Schäfer, G.A. Romanov, Cytokinins as regulators of the gene expression, Physiol. Plant. 100 (1997) 505–519.
[42] C. Maurel, H. Barbier-Brygoo, A. Spena, J. Tempé, J. Guern, Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*, Plant Physiol. 97 (1991) 212–216.

Immunoblot analysis of the RolC protein in the *rolC*1-, *rolC*2- and *rolC*4-transgenic carnation lines

Introduction

The *rolC*-transgenic carnation lines, obtained in 1997, presented differences both in their adventitious organogenesis (Casanova et al. 2004) and in their phenotypes (Zuker et al. 2001). The adventitious shoot regeneration was highest from petal and leaf explants of the *rolC*1 carnation line, intermediate from petal and leaf explants of *rolC*2 and *rolC*4 and lowest from non-transgenic explants (Casanova et al. 2004). Similarly, *rolC*1 carnation plants displayed the highest number of lateral shoots, whereas *rolC*2 and *rolC*4 showed behaviour midway between that of *rolC*1 and that of non-transgenic plants (Zuker et al. 2001). In terms of roots, however, all the transgenic lines presented a similar adventitious rhizogenesis and a similar rooting capacity in their cuttings, both being higher than in the control (Zuker et al. 2001, Casanova et al. 2004). The fact that the *rolC*1 line has two copies of the transgene while the *rolC*2 line has one copy (Zuker et al. 2001) (there are no studies on *rolC*4) or that there are distinct locations for the transgene in each genome could account for the phenotypic differences between *rolC*-transgenic carnation lines. These differences could also be due to the different expression of the *rolC* gene in each line, as reported for *rolC* plants of other species (Kurioka et al. 1992, Nilsson et al. 1996, Winefield et al. 1999, Giovannini et al. 1999). Therefore, our aim was to assess whether there is any relationship between the levels of the RolC protein and the adventitious organogenesis in each *rolC*-transformed carnation line. We should note that the immunoblot analysis of the RolC protein was performed in January 2004, whereas the experiments on adventitious organogenesis in leaf and petal explants were carried out in November 1998-January 2002.

Material and Methods

Plant material

Carnation plants (*Dianthus caryophyllus* L., cv. White Sim), non-transformed (control) and *rolC*1-, *rolC*2- and *rolC*4-transgenic lines, were grown from cuttings in a greenhouse of the *Servei de Camps Experimentals* of the University of Barcelona, over a natural photoperiod, in 4.7 L-pots (19 cm in diameter, 18 cm high) with peat:perlite (2:1, v/v), irrigated daily with Hoagland nutrient solution (Hoagland and Arnon 1938). The cuttings were obtained in 1999 from the laboratory of Dr. Vainstein of the Department of Horticulture, Faculty of Agricultural, Food and Environmental Quality Sciences at The Hebrew University of Jerusalem (Rehovot, Israel). When the experiments on adventitious organogenesis were finished (January 2002), plants were eliminated but very small cuttings were saved and cultured

in the greenhouse. These plants were kept small, by removing the apical shoot and most of the lateral shoots, and provided the plant material to perform the protein extraction (January 2004).

In conjunction with this, plantlets of the non-transformed and the *rolC*-transgenic lines, obtained by adventitious shoot organogenesis from leaves in the year 2000, were grown *in vitro* in 500-ml vessels, with 100 ml of MS medium (Murashige and Skoog 1962) containing 30 g l^{-1} sucrose, without plant growth regulators, and solidified with 8 g l^{-1} agar (High Gel Strength, Sigma). These plantlets were subcultured bimonthly. The cultures were maintained in a growth chamber at 25±1ºC in a 16-h photoperiod using cool white fluorescent tubes (TLD 58W/33, Philips, France, 80 μmol m^{-2} s^{-1}).

Protein extraction

Proteins were extracted from 1 g of fresh leaves from the control and the *rolC*-transgenic plants. Proteins from leaves from the *rolC*1 *in vitro*-grown plantlets were also extracted. The entire extraction process was performed at 4ºC. Collected leaves were frozen with liquid nitrogen and, immediately, they were powdered in a mortar and homogenized in 2 ml of the extraction buffer, consisting of 20 mM Tris-HCl, pH 7.5, containing 5 mM EGTA, 2 mM EDTA, 20% (v/v) glycerol, 40 mM η-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 2.5 μg ml^{-1} of each protease inhibitor (leupeptin, aprotinin and pepstatin). The homogenates were centrifuged at 17,000 *g* at 4ºC for 30 min and the supernatants were further centrifuged at 100,000 *g* at 4ºC for 1 h. Since the RolC protein was reported to be soluble (Estruch et al. 1991), the resulting supernatants were stored at –20ºC and used as protein extracts for further analysis. Soluble protein concentration was determined by the Bradford (1976) method by using bovine serum albumin (BSA) as standard.

Immunoblot analysis of the RolC protein

Protein extracts (35 μg per sample) were resolved on 13.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels (Laemmli 1970), during 15 min at 100 V plus 30 min at 30 mA. We used molecular mass markers of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa. For immunoblotting, proteins were electroblotted to nitrocellulose membranes (0.45 μm pore size), during 2 h at 100 V. To verify the transfer efficiency, nitrocellulose membranes were reversibly stained with Red Ponceau (0.5 %). Membranes were blocked with PBS-T buffer (20 mM phosphate buffer saline (PBS), pH 7.2, and 0.05% (v/v) Tween-20) containing 5% (w/v) non-fat dry milk, at room temperature for 2 h, and then incubated with the primary antibody diluted 1:500 in the block solution, at room temperature for 1 h. After washing the membranes in PBS-T (3 x 5 min) and PBS (1 x 5 min), they were incubated with goat anti-rabbit antibody conjugated to alkaline phosphatase (A-3687, Sigma) diluted 1:2000 in the block solution at room temperature for 1 h. Then the membranes were washed as described above and developed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (BCIP/NBT) (Sigma) as a substrate according to the manufacturer's instructions. The polyclonal antibody against the 20.1 kDa polypeptide encoded by the *rolC* gene was a gift from M.T. Piñol (Faculty of

Pharmacy, University of Barcelona, Spain), obtained as described by Palazón et al. (1998). Controls for the specificity of immunolabelling were obtained by omitting the primary antibody.

The deposits of the alkaline phosphatase reaction were scanned and then quantified using the IMAT software, developed by the *Serveis Cientificotècnics* of the University of Barcelona. Since each lane of the gel was charged with the same amount of protein, the reactivity intensity of the immunoblot analysis provided a relative measurement of the *rolC* gene product.

Results

Immunoblot analysis of the RolC protein

The concentration of total soluble proteins obtained from leaf tissues for each carnation line was 2.8 μg μl^{-1} for the non-transformed line, and 2.1, 2.9 and 3.3 μg μl^{-1} for the *rolC*1, *rolC*2 and *rolC*4, respectively. The results of the immunoblot analysis (Fig. 1) showed that in *rolC*2 and *rolC*4 lines a polypeptide of approximately 20 kDa reacted to the anti-RolC antibody with an intensity of 1.041×10^9 and 1.048×10^9 arbitrary units mg^{-1} total protein, respectively. No immunoreaction was observed in the lanes corresponding to the *rolC*1 or to the non-transformed lines (Fig. 1). The experiment was repeated using the proteins extracted from leaves of *in vitro* plantlets of the *rolC*1 line with the same results. The controls omitting the antibody did not show any immunoreaction.

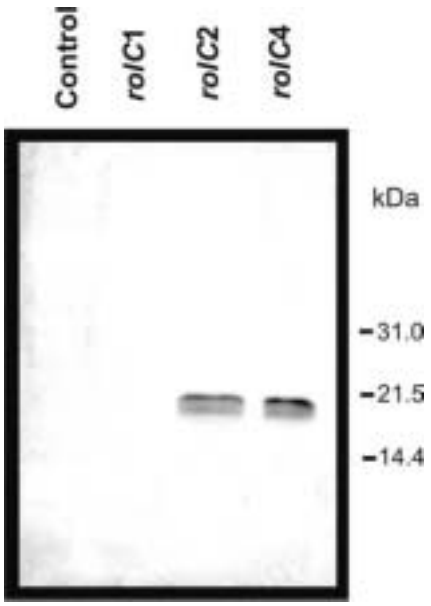

Fig. 1. Immunoblot analysis of RolC isolated from leaves of non-transformed and *rolC*1-, *rolC*2- and *rolC*4-transgenic carnation plants (*Dianthus caryophyllus* L. cv. White Sim). Aliquots of total soluble protein extract (35 μg per lane) from each carnation line were analysed by SDS-polyacrylamide gel electrophoresis (PAGE), electroblotted to nitrocellulose and immunodetected with 1:500 dilution of the antiserum with the anti-RolC polyclonal antibody. Molecular mass markers (kDa) are indicated on the right.

Discussion

The *rolC*-transgenic carnation lines were expected to present distinct or the same levels of the RolC protein, depending on whether the adventitious organogenesis (Casanova et al. 2004) and the *rolC* phenotypes (Zuker et al. 2001) were different or similar between transgenic lines. According with our hypothesis, we found the *rolC*2 and *rolC*4 lines to show a strong similar labelling in the 20.1 kDa band, corresponding to the RolC protein (Slightom et al. 1986), which would be related to their similar phenotype and similar degree of adventitious organogenesis. This relation was also reported in *rolC*-transgenic plants of *Osteospermum* (Giovannini et al. 1999), in which the different *rolC* phenotypes of the transgenic lines could be related to the distinct levels of the RolC protein in the transformed tissues. In another system, the amounts of nicotine, an alkaloid synthesized by *rolC* transgenic roots of tobacco, also correlated with the levels of the RolC protein in these organs (Palazón et al. 1998). In other species, such as belladonna (Kurioka et al. 1992), the hybrid aspen (Nilsson et al. 1996) and the petunia (Winefield et al. 1999), although the RolC protein levels were not analyzed, a positive correlation was found between the phenotype and the levels of the *rolC* transcript.

The *rolC*1 carnation line was expected to be the line with the highest levels of the RolC protein due to its phenotype, although the *rolC*1 and *rolC*2 lines of the carnation (the *rolC*4 was not studied) showed the same levels of the *rolC* mRNA (Zuker et al. 2001), the Northern analysis being made immediately after the plants were obtained. However, this *rolC*1 line did not present any labelling, as occurs with the non-transformed line, in the immunoblot analysis. The absence of the RolC protein in the *rolC*1 line could be explained by the silencing of the *rolC* transgene in this line, a frequent phenomenon in transgenic plants, in which transgenes can become silent after a long phase of expression (Fagard and Vaucheret 2000). The fact that the immunoblot analysis was performed two years after the last experiments on adventitious organogenesis, and seven years after obtaining the *rolC*-transgenic carnation plants studied is in agreement with this explanation. The putative gene silencing of our transgenic plants could be a post-transcriptional gene silencing (or RNA silencing). In our *rolC*1 line, the gene silencing would lead to the degradation of homologous RNA from sense transgenes (*trans*-inactivation), in a not very well known mechanism that shares many characteristics of virus-induced gene silencing (Fagard and Vaucheret 2000, Susi et al. 2004), due to the two copies of the *rolC* transgene in that line (Zuker et al. 2001). The post-transcriptional gene silencing mediated by sense transgenes is observed mainly in strongly transcribed transgenes (Fagard and Vaucheret 2000, Vaucheret et al. 2001), as would take place in our *rolC* carnations, in which the transgene is driven by the constitutive 35S promoter. On the other hand, we cannot exclude the possibility that gene silencing could be the result of impairment of gene transcription, through the methylation of the coding sequences of the transgene or through chromatin condensation around the point of insertion (Fagard and Vaucheret 2000, Vaucheret and Fagard 2001, Bender 2004). Whatever the silencing mechanism may be, it has occurred both in plants grown in the greenhouse and also in plantlets grown *in vitro*.

More studies would be necessary to check whether the *rolC* phenotype and the great regeneration capacity of the *rolC*1 line have been lost and, thus, corroborating the hypothesis of gene silencing in this carnation line. Then, molecular analyses should be done to determine whether the putative gene silencing is at a transcriptional or post-transcriptional level.

References

Bender J (2004) Chromatin-based silencing mechanisms. Curr. Opin. Plant Biol. 7:521-526

Bradford MM (1976) A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254

Casanova E, Valdés AE, Zuker A, Fernández B, Vainstein A, Trillas MI, Moysset L (2004) *rolC*-transgenic carnation plants: adventitious organogenesis and levels of endogenous auxin and cytokinins. Plant Sci. 167:551-560

Estruch JJ, Parets-Soler A, Schmülling T, Spena A (1991) Cytosolic localization in transgenic plants of the *rolC* peptide from *Agrobacterium rhizogenes*. Plant Mol. Biol. 17:547-550

Fagard M, Vaucheret H (2000) (Trans)gene silencing in plants: How many mechanisms? Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:167-194

Giovannini A, Zottini M, Morreale G, Spena A, Allavena A (1999) Ornamental traits modification by *rol* genes in *Osteospermum ecklonis* transformed with *Agrobacterium tumefaciens*. In Vitro Cell. Dev. Biol. 35:70-75

Hoagland DR, Arnon DI (1938) The water-culture method for growing plants without soil. Univ. Calif. Agric. Exp. St. Circ. No 347

Kurioka Y, Suzuki Y, Kamada H, Harada H (1992) Promotion of flowering and morphological alterations in *Atropa belladonna* transformed with a CaMV 35S-*rolC* chimeric gene of the Ri plasmid. Plant Cell Rep. 12:1-6

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497

Nilsson O, Moritz T, Sundberg B, Sandberg G, Olsson O (1996) Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fasciation. Plant Physiol. 112:493-502

Palazón J, Cusidó RM, Roig C, Piñol MT (1998) Expression of the *rolC* gene and nicotine production in transgenic roots and their regenerated plants. Plant Cell Rep. 17:384-390

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J. Biol. Chem. 261:108-121

Susi P, Hohkuri M, Wahlroos T, Kilby NJ (2004) Characteristics of RNA silencing in plants: similarities and differences across kingdoms. Plant Mol. Biol. 54:157-174

Vaucheret H, Béclin C, Fagard M (2001) Post-transcriptional gene silencing in plants. J. Cell Sci. 114:3083-3091

Vaucheret H, Fagard M (2001) Transcriptional gene silencing in plants: targets, inducers and regulators. Trends Genet. 17:29-35

Winefield C, Lewis D, Arathoon S, Deroles S (1999) Alterations of *Petunia* plant form through the introduction of the *rolC* gene from *Agrobacterium rhizogenes*. Mol. Breed. 5:543-551

Zuker A, Tzfira T, Scovel G, Ovadis M, Shklarman E, Itzhaki H, Vainstein A (2001) *rolC*-transgenic carnation with improved agronomic traits: Quantitative and qualitative analyses of greenhouse-grown plants. J. Amer. Soc. Hort. Sci. 126:13-18

CAPÍTOL 6

Influència dels gens *rol* en la floricultura

Influència dels gens *rolC* en la floricultura

Resum

Tradicionalment, s'han introduït nous trets en plantes ornamentals a través de la hibridació clàssica. Tanmateix, actualment l'enginyeria genètica permet introduir alteracions específiques d'una sola característica en varietats que ja tinguin moltes característiques valuoses. Es poden millorar o obtenir noves varietats de plantes ornamentals amb flor actuant en els trets florals, com el color, la forma o la fragància de la flor, en la vida de la base en espècies de flor tallada, i en la capacitat d'arrelament o en la morfologia global de la planta. La sobreexpressió dels gens *rol* del plasmidi Ri d'*Agrobacterium rhizogenes* en plantes n'altera alguns dels processos de desenvolupament n'afecta la seva arquitectura. Tant les plantes transformades amb l'*A. rhizogenes* com amb els gens *rol* mostren el fenotip de les rels velloses, encara que es troben diferències concretes entre espècies i entre línies transgèniques. En general, aquestes plantes mostren un fenotip nanitzat, la dominància apical reduïda, les fulles petites i arrugades, més capacitat d'arrelament, la floració alterada i la fertilitat reduïda. Entre els gens *rol*, anomenats *rolA*, *B*, *C* i *D*, el *rolC* ha estat el més estudiat, ja que els seus efectes són els més avantatjosos pel que fa a la millora de trets ornamentals i horticulturals. A més del nanisme i l'increment en les tiges laterals que provoquen un fenotip compacte, les plantes *rolC* mostren més flors i més petites, i una floració avançada; sorprenentment, aquestes plantes poden tenir una capacitat d'arrelament millorada i pràcticament no mostren trets desavantatjosos. El gen *rolD*, el menys estudiat entre els gens *rol*, ofereix aplicacions prometedores ja que promou la floració. Encara que les funcions bioquímiques dels gens *rol* continuen sense aclarir-se, són eines útils per la millora de flors ornamentals, ja que la seva expressió en plantes transgèniques genera molts trets beneficiosos.

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Research review paper

Influence of *rol* genes in floriculture

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Abstract

Traditionally, new traits have been introduced into ornamental plants through classical breeding. However, genetic engineering now enables specific alterations of single traits in already successful varieties. New or improved varieties of floricultural crops can be obtained by acting on floral traits, such as color, shape or fragrance, on vase life in cut-flower species, and on rooting potential or overall plant morphology. Overexpression of the *rol* genes of the Ri plasmid of *Agrobacterium rhizogenes* in plants alters several of the plant's developmental processes and affects their architecture. Both *A. rhizogenes*- and *rol*-transgenic plants display the "hairy-root phenotype", although specific differences are found between species and between transgenic lines. In general, these plants show a dwarfed phenotype, reduced apical dominance, smaller, wrinkled leaves, increased rooting, altered flowering and reduced fertility. Among the *rol* genes, termed *rolA*, *B*, *C* and *D*, *rolC* has been the most widely studied because its effects are the most advantageous in terms of improving ornamental and horticultural traits. In addition to the dwarfness and the increase in lateral shoots that lead to a bushy phenotype, *rolC*-plants display more, smaller flowers, and advanced flowering; surprisingly, these plants may have better rooting capacity and they show almost no undesirable traits. *rolD*, the least studied among the *rol* genes, offers promising applications due to its promotion of flowering.

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Although the biochemical functions of *rol* genes remain poorly understood, they are useful tools for improving ornamental flowers, as their expression in transgenic plants yields many beneficial traits.

Keywords: *Agrobacterium rhizogenes*; Hairy-root disease; Ornamental plants; Rhizogenesis; Transformation; *rol* genes

Contents

1. Introduction

New ornamental plant varieties are continuously being created by breeders in response to consumer demand for new products. Altered plant morphology and colors, better flower fragrance, and longer vase life in the case of cut flowers, are some of the desirable novel traits. Growers also look for plants with improved agronomic traits, such as increased production yield and resistance to plant pathogens or herbicides.

Traditionally, classical breeding has been used to introduce new traits and create new varieties in many species, including ornamentals. However, it is a tedious process, based on crosses between related species or within the same species, and on the selection of offspring with promising characteristics. The selected offspring, in many cases, are maintained through vegetative propagation to ensure the production of genetically identical plants. With classical breeding, the available gene pool for new traits is limited to the genetic background of the parents. In addition, the high heterozygosity in many valuable floricultural crops, such as rose, chrysanthemum and carnation, and the limited knowledge of their genetic make-up, hamper advances in breeding (Vainstein, 2002). Moreover, many varieties of ornamental plants are sterile.

Recent developments in genetic engineering, with its tools for the transfer of foreign genes into plants, together with progress in gene identification and isolation, have enabled specific alterations of single traits in already successful varieties. These techniques allow the available gene pool to be extended, since not only can genes from other species be delivered to plants but genes from other kingdoms, such as bacterial or even viral genes, can also be used. Moreover, native genes can be overexpressed or suppressed. Thus, in plant breeding, the selection of phenotypes by classical breeding is being replaced, at least in part, by the selection of genes, either directly or indirectly (Koornneef and Stam, 2001). In recent years, increases in both the efficiency of transformation procedures and the regeneration of transgenic plants have enabled the improvement of ornamental varieties by molecular breeding (Zuker et al., 1998). Even several monocotyledonous (plants with a single seed leaf) flowering plants, whose genetic transformation is limited by their low susceptibility to *Agrobacterium* infection, have been transformed, using microprojectile bombardment as an alternative method (Deroles et al., 2002). However, plant biotechnology will not—in the foreseeable future—replace plant breeding, even though the latter is empirical rather than based on an understanding of physiological and molecular processes; for the time being, both methods will continue to be used to improve horticultural and floricultural plants (Schell et al., 1998; Vainstein, 2002; Morandini and Salamini, 2003).

2. Transformation in floricultural crops

Plant breeders are continually striving to improve many aspects of crop production. These include increased crop yield, better nutritional value, more efficient use of water, improved post-harvest crop quality and resistance to pests. Many genes related to these

parameters have already been isolated. Better rooting of cuttings, as well as of the whole plant, is another desirable trait from the agronomic point of view. Genes of *Agrobacterium rhizogenes* are presently being used for this purpose, because transgenic plants carrying its T-DNA display increased rooting ability. An example of this is an apple rootstock variety, whose regenerated shoots rooted easily after infection with this bacterium, in contrast to non-transformed shoots (Pawlicki-Jullian et al., 2002). Direct infection of cuttings with *A. rhizogenes* is another way of improving adventitious root formation, as reported in jujube (Hatta et al., 1996). The commonly obtained lower plant weight in *A. rhizogenes*-transformed plants indicates that their genes are unsuitable for increasing crop yield in some plant systems (van der Salm et al., 1996). However, the altered phenotype can be advantageous in some ornamental and horticultural crops (Christey, 1997, 2001).

The increasing economic importance of ornamentals worldwide suggests a bright future for ornamental plant breeding. Ornamental crops include flowering herbaceous plants, shrubs and trees. Floriculture specifically includes most herbaceous ornamental plant species, such as cut flowers, pot plants and bedding plants. The most important cut flowers worldwide are rose, carnation, chrysanthemum and gladiolus, but many others, such as tulip, freesia, gerbera, orchid, lily, alstroemeria, anthurium, gypsophila and aster are also significant in certain countries. In terms of flowering pot plants, important species are African violet, kalanchoe, azalea, pointsettia and cyclamen, whereas the most cultivated bedding-plant species are geranium, pansy, fuchsia, petunia, impatiens and begonia (Horn, 2002).

The plant cell, tissue or organ culture of these species and their regeneration are essential for providing the material and systems for their genetic manipulation, and this is therefore the first requirement of genetic engineering. At present, although some plant species are recalcitrant to in vitro culture and furthermore, genotypes may sometimes be unstable in vitro giving rise to somaclonal variation (Cassells, 2002), these systems are quite well established in many plant species, including ornamentals (Zuker et al., 1998). Moreover, transformation methods are no longer a limiting step in the introduction of new traits. Since 1987, when petunia was reported as the first transformed flower (Meyer et al., 1987), there has been extensive research on the genetic transformation of different flowering plant species, and many ornamental species have now been successfully transformed, including those which are most important commercially. To date, more than 30 ornamental species have been transformed, including anthurium, begonia, carnation, chrysanthemum, cyclamen, datura, daylily, gentian, gerbera, gladiolus, hyacinth, iris, lily, lisianthus, orchid, pelargonium, petunia, poinsettia, rose, snapdragon and torenia (Deroles et al., 2002).

Many useful genes for ornamental plant breeding have now been isolated. These include genes which affect flowering and flower architecture, genes related to the biosynthesis of several flower pigments, genes from the biosynthetic pathway of flower scents, and those related to the vase life of cut flowers. Moreover, the possibility of using molecular techniques to transfer genes, not only within and between plant species, but also from other kingdoms, has greatly increased the resources available to plant breeders. An example of this is transformation with oncogenes originating from *Agrobacterium tumefaciens* or *A. rhizogenes*, an example from the latter being the *rol* genes, which have proven highly useful for improving certain agronomic traits and even for producing novel

plant morphologies. The present article provides a detailed review of the increasing importance of *rol* genes in floricultural crops.

2.1. Flower development

The control of flowering and flower development has long attracted the interest of growers. An intricate network of signaling pathways, that regulate flowering in response to endogenous and environmental signals, converge on the activation of flowering-time genes, which regulate floral-identity genes. Those, in turn, control the expression of floral-organ-identity genes, resulting in the development of flower organs (reviewed by Mouradov et al., 2002; Sung et al., 2003; Zik and Irish, 2003). In *Arabidopsis*, four different pathways (photoperiodic, vernalization, autonomous and gibberellin) control flowering time and they are integrated at the point of transcriptional regulation of the gene *LFY* and two flowering-time genes, *FT* and *SOC1*, which upregulate floral-meristem-identity genes (Mouradov et al., 2002). Orthologous genes involved in the flowering transition have not been isolated in ornamental species. The identity of the floral meristems is promoted by interactions between *LFY* and three MADS-box transcription factors, *AP1*, *CAL* and *FUL*. These genes repress *TFL1*, which maintains the apical meristem (growing point at tip of stem) of the inflorescence (arrangement of flowers on a floral stem) in an indeterminate state, with an unlimited growth. Orthologues of *LFY*, *AP1* and *TLF1* (*FLO*, *SQUA* and *CEN*), as well as their co-regulator *UFO*, have been isolated in the ornamental species *Antirrhinum* (snapdragon). Interestingly, *tfl1*/*cen* mutants exhibit determinate inflorescences, with a limited growth (Ma, 1998; Banfield and Brady, 2000; Sung et al., 2003).

Flower structure, as well as color and fragrance, are critical factors attracting pollinators in nature, and attracting consumers in the flower market. The wide diversity in flower forms is due to changes in their four organ types, whose identity is controlled by homeotic genes. The "ABC model" classifies the floral-organ-identity genes as: type A, which control the organ identity of the sepals and petals, type B, of the petals and stamens, and type C, of stamens and carpels (Coen and Meyerowitz, 1991). In *Arabidopsis*, *AP1* and *AP2* are A-function genes, *AP3* and *PI* are B-function genes and *AG* is the only known C-function gene (reviewed by Ma and dePamphilis, 2000; Vishnevetsky and Meyerowitz, 2002). Orthologues of ABC genes have been isolated in *Antirrhinum* (Ma and dePamphilis, 2000), petunia (van der Krol and Chua, 1993) and gerbera (Yu et al., 1999), and homeotic mutants of *Primula* are currently being studied (Webster and Gilmartin, 2003). In petunia, a D function has been described that is related to ovule development (Angenent and Colombo, 1996) and additional factors required in all whorls, *SEP* genes in *Arabidopsis*, have been classified as E-class genes (Soltis et al., 2002). Genes required for the establishment of floral-organ polarity and floral symmetry have been studied in *Antirrhinum* (Zik and Irish, 2003). Flowering plants transformed with homeotic genes generate new flower forms, both when they are ectopically expressed and when they are repressed. Further work on different species will provide new insight into the mechanisms of flowering and flower architecture and will also aid in the genetic engineering of non-model species.

2.2. Flower color

Flower color is one of the most important traits in ornamental plants in that it generates consumer interest. Anthocyanins, a class of flavonoids responsible for pink, red, violet and blue colors, may be altered by modulating the expression of genes in their biosynthetic pathway. The expression of maize dihydroflavonol-4-reductase in petunia and the consequent synthesis of pelargonidin generated a new petunia flower color in the first transgenic flower (Meyer et al., 1987). Introduction of the native gene chalcone synthase in its antisense or sense orientation led to a decrease in anthocyanins that lightened the original color in gerbera, rose and torenia, and modified the color to white in petunia, chrysanthemum, carnation and lisianthus (reviewed by Ben-Meir et al., 2002). Recently, the introduction of the antisense of flavanone 3-hydroxylase led to attenuation or complete loss of the orange-reddish color in carnations (Zuker et al., 2002) and the antisense of flavonol synthase produced magenta lisianthus from purple ones (Nielsen et al., 2002). Florigene produced violet carnations by introducing the absent flavonoid 3′ ,5′ -hydroxylase gene from petunia into white carnations (Fukui et al., 2003). Flavonoid metabolic engineering will continue to open up new perspectives in plant molecular breeding.

Carotenoids, a class of isoprenoids, are responsible for the yellow, orange and red colors displayed by many flowers and fruits. The genes for almost all of the enzymes in their biosynthetic pathway have been identified (reviewed by Cunningham and Gantt, 2002). Although no manipulation of flower color by engineering of the carotenoid pathway has yet been reported, it may soon prove to be a way of increasing the range of colors in a given species.

2.3. Flower fragrance

Over the years, classical breeding of ornamental plants to increase qualitative and quantitative traits has produced a concomitant reduction in flower fragrance, which should be solvable once the biosynthetic pathways of fragrance compounds are better characterized and their genes isolated. The volatile products are secondary metabolites such as terpenoids, phenylpropanoids and fatty acid derivatives (Knudsen et al., 1993). To date, only a few genes directly involved in fragrance production have been characterized. Studies with *Clarkia breweri* have enabled cloning of the linalool synthase (*lis*) gene, the gene encoding IEMT, which generates methyleugenol and isomethyleugenol, the gene encoding BEAT, which catalyzes the formation of benzylacetate, and the gene encoding SAMT, which generates methylsalicylate (reviewed by Dudareva, 2002). In snapdragon flowers, both the gene BAMT, which catalyzes the formation of methylbenzoate, and the genes (E)-β-ocimene and myrcene synthases have been isolated (Dudareva et al., 2000, 2003). Recently, transgenic carnations expressing the *lis* gene from *C. breweri* were reported to emit linalool, lacking in the studied cultivar, but it did not lead to olfactorally detectable changes in flower fragrance (Lavy et al., 2002). Moreover, the same *lis* construct in petunia did not generate detectable levels of linalool in transgenic flowers (Lücker et al., 2001). The development of plants with modified scent composition will increase the value of ornamentals, but a deeper knowledge of the overall process is still needed to successfully modify flower fragrances.

2.4. Vase life

Cut flowers must survive transport to the marketplace and also satisfy consumer demand for long vase life. Senescence in climacteric flowers is sensitive to ethylene (Thompson and Wang, 2002). We can modify their ethylene levels by introducing genes involved in ethylene biosynthesis, coding for ACC synthase or ACC oxidase, in antisense, or genes encoding enzymes that degrade the ethylene precursors SAM or ACC, such as SAM hydrolase or ACC deaminase. Additionally, ethylene's effects can be altered by reducing the plant's ability to perceive it (reviewed by Stearns and Glick, 2003). In carnation, the suppression of ACC oxidase activity was achieved by transforming plants with the gene in its antisense or sense orientation. Those flowers presented a marked delay in petal senescence, thus exhibiting increased longevity (Savin et al., 1995; Kosugi et al., 2002). To confer ethylene insensitivity to plants, the *Arabidopsis etr1-1* mutant gene, which encodes a mutated ethylene receptor, was successfully transferred into carnation and petunia plants, resulting in decreased ethylene perception and therefore increased vase life (Bovy et al., 1999; Gubrium et al., 2000). Various ethylene receptors are currently being identified, isolated and studied in ornamentals, such as rose and carnation (Müller et al., 2000; Shibuya et al., 2002). Much more information is needed to achieve commercial success with genetically engineered ornamentals with improved vase life, along with the post-harvest management implications.

2.5. Insect and disease resistance

Insects and diseases caused by fungal, viral and bacterial pathogens are responsible for major losses in the flower industry. Several available genes confer plant species with genetic resistance to pathogens (reviewed by Lorito et al., 2002). Since chitinases and glucanases are produced by plants in response to pathogen attack, their overexpression could improve resistance to fungi (Lorito et al., 2002). Resistance to a range of fungal pathogens was achieved in petunia plants expressing an endochitinase from a fungus (Esposito et al., 2000). Lower severity of blackspot in roses was also achieved with the expression of a chitinase transgene (Marchant et al., 1998). A typical approach to the control of fungi and bacteria is to transfer genes encoding toxins to plants, such as pathogenesis-related proteins (Lorito et al., 2002). Recent strategies for enhancing disease resistance to fungi and bacteria involve the use of avirulence and resistance genes to artificially induce programmed cell death and/or activate SAR in the plant (Martin et al., 2003). Although some of these genes have been introduced in plants, they have not yet been applied to ornamentals (Lorito et al., 2002). In terms of virus, a putative virus-resistant transgenic *Gladiolus* containing either the bean yellow mosaic virus coat protein gene or its antisense was developed (Kamo et al., 1997). The insecticidal crystal proteins encoded by the *cry* genes from *Bacillus thuringiensis* have been used directly to control insect pests for more than 30 years. Nevertheless, *cry*-transgenic plants have been engineered, as well as transgenic plants expressing inhibitors of the digestive enzymes amylase and protease (reviewed by Schuler et al., 1998). Although the production of genetically modified ornamentals resistant to pests and diseases is still in its early stages,

since most of this research has been performed on food crops or model plants, these methods could be applicable to ornamentals in the near future.

2.6. Plant morphology

Modification of plant architecture may be both an aesthetic requirement and an agronomically desirable trait. The flower and its green parts form a unit, and thus a specific vegetative form and size will contribute to the market success of that flower. Plant architecture might be modified by introducing genes related to hormone metabolism. These genes are better known in bacteria such as *Agrobacterium* than they are in plants (Gaudin et al., 1994), but research with the latter is gaining momentum (Kakimoto, 2001; Takei et al., 2001). *A. tumefaciens* and *A. rhizogenes* are pathogenic soil bacteria that have morphogenic effects on dicotyledonous plants (plants with two leaves in the embryo). *A. tumefaciens* induces unorganized tumors at the crown of plants, leading to crown-gall disease, and *A. rhizogenes* induces hairy-root disease, with root proliferation from the infection site, or the formation of tumors without root development (Gaudin et al., 1994; Binns and Costantino, 1998; Meyer et al., 2000). The *Agrobacterium* genes involved in tumorigenesis and rhizogenesis (root formation) via modification of plant-cell growth and developmental regulation are called oncogenes (Binns and Costantino, 1998). They are located in the Ti (tumor-inducing) plasmid in *A. tumefaciens* and the Ri (root-inducing) plasmid in *A. rhizogenes*, specifically in the T-DNA (DNA transferred to the plant host cells). Some of these genes are involved in auxin or cytokinin biosynthesis, leading to higher than normal levels of these endogenous hormones, while others, including some that have yet to be studied in detail, are involved in either hormone metabolism or the plant cell's response to these hormones (Binns and Costantino, 1998; Meyer et al., 2000).

2.6.1. ipt gene

In *A. tumefaciens*, there is an oncogene belonging to the cytokinin biosynthetic pathway, the *ipt* gene, which encodes the enzyme isopentenyl transferase and which is introduced into the plant genome by the bacterium. Its product, IPT, catalyzes the first step in cytokinin biosynthesis. No homologous genes have been found in *A. rhizogenes* (Binns and Costantino, 1998; Meyer et al., 2000). Introduction of the *ipt* gene under the constitutive promoter CaMV-35S in tobacco and cucumber induces high cytokinin levels, with the consequent induction of shoot organogenesis and inhibition of root formation in the transformed tissues (Smigocki and Owens, 1989). Transgenic tobacco plants with *ipt* under its own promoter are also highly branched and have tiny leaves as a result of apical dominance (predominance of growth of apical bud over lateral buds) release (Schmülling et al., 1989). Similarly, *ipt*-transgenic potato plants grown in vitro displayed a shortening of the internodes (parts of the stem between two consecutives nodes or leaves), reduced leaf surface, decreased apical dominance and less rooting than controls, as well as an increase in stolon and tuber formation (Macháčková et al., 1997; Sergeeva et al., 2000). Under inducible promoters, the *ipt* gene can influence morphogenic processes, such as delayed leaf senescence, which are under cytokinin control (Schmülling et al., 1989; Smart et al., 1991). The *ipt* gene under the control of a chalcone synthase promoter caused increased chlorophyll levels in tobacco leaves, increased stem thickness, and delayed

flowering onset and flower development (Wang et al., 1997). In *ipt*-transgenic *Artemisia annua* increased levels of artemisinin, an antimalarial drug, were reported (Geng et al., 2001). Among ornamentals, introduction into petunia of the *ipt* gene, linked to senescence-associated SAG promoters from *Arabidopsis*, was able to inhibit senescence of the lower leaves, although transgenic lines showed stronger symptoms of nutritional deficiency (Jandrew and Clark, 2001). Therefore, by controlling the expression of the *ipt* gene, using its own promoter, constitutive promoters or, preferably, inducible promoters, some interesting characteristics could be introduced into commercial plants.

2.6.2. iaa and aux genes

iaaM and *iaaH* are two genes of the auxin biosynthetic pathway that may also be integrated into the plant genome by *A. tumefaciens*. They convert tryptophan to the auxin indole-3-acetic acid (IAA) in two steps. Their homologues in *A. rhizogenes* are known as *aux1* and *aux2* (Binns and Costantino, 1998; Meyer et al., 2000). The introduction of *iaa* genes under their own promoter into tobacco produced normal phenotypes, with high levels of conjugated IAA (Budar et al., 1986; Sitbon et al., 1991). However, transgenic tobacco flowers showed larger pistils and severely reduced pollen and seed production (Sitbon et al., 1991). The introduction of both *iaa* genes under the constitutive promoter CaMV-35S altered the growth of tobacco plants, showing dwarfism, curly leaf growth, smaller leaves, adventitious root formation, increased apical dominance, less flowers and delayed flowering, as well as an overall delay in their development; they also exhibited elevated levels of both IAA and its conjugates (Sitbon et al., 1992a,b). In petunia, the introduction of CaMV-19S-*iaaM* led to strong apical dominance with very little branching, lance-shaped leaves with extreme curling and adventitious root formation, abnormalities that are consistent with overproduction of auxin and ethylene (Klee et al., 1987). Therefore, apart from better rooting, there would appear to be fewer applications for genes associated with auxin biosynthesis. However, the use of suitable tissue-specific promoters or promoters of different strength might improve the usefulness of these interesting genes, since IAA levels may be altered more precisely.

2.6.3. rol genes

In several species, whole plants can sometimes be regenerated from hairy roots which have been induced after infection by *A. rhizogenes*. These plants display a characteristic phenotype which includes reduced apical dominance in both stems and roots, shortened internodes, wrinkled and wider leaves, adventitious root production, altered flower morphology, late flowering and reduced pollen and seed production (Tepfer, 1984; Christey, 1997). These combined symptoms are termed hairy-root phenotype. There are four loci in the T_L-DNA (the left stretch of the T-DNA) of the agropine-type Ri plasmids of *A. rhizogenes* involved in this phenotype: *rolA*, *rolB*, *rolC* and *rolD* (root loci) or ORFs (open reading frames) 10, 11, 12 and 15, respectively (White et al., 1985; Slightom et al., 1986). The *rol* genes show no homology to genes of *A. tumefaciens* T-DNA (Meyer et al., 2000). The capacity of *rolA*, *B* and *C* genes to induce neoplastic roots (growing quicker than normal) in tissues is equivalent to that of the whole T_L-DNA of the Ri plasmid (Spanò et al., 1988). The *aux* genes, located in the T_R-DNA (the right stretch of the T-DNA) of the Ri plasmid, play an ancillary role in hairy-root induction (Cardarelli et al., 1987b). The T_R-

DNA is only found in agropine-type Ri plasmids of *A. rhizogenes*, since the strains of *A. rhizogenes* that produce other opines, such as mannopine, cucumopine or mikimopine, transfer a single T-DNA fragment, homologous to the agropine T_L-DNA but without the *rolD* gene (Meyer et al., 2000; Christey, 2001).

Transformation with *A. rhizogenes* or with its *rol* genes has been performed in several ornamental plants in order to change their architecture, including aerial and subterranean parts, and even to alter the process of flowering. Plant morphology may be modified for aesthetic or agronomical reasons.

3. Manipulation of plant characteristics using *A. rhizogenes*

Many species have been transformed using *A. rhizogenes* strains (Christey, 1997, 2001). Plant tissues infected with *A. rhizogenes* develop hairy roots, with the typical fast growth, high lateral branching and plagiotropism (growth away from the vertical line). From these roots, transgenic plants easily arise. The typical hairy-root phenotype described earlier can be observed in a number of species, although to varying degrees depending on the species or the clone within the same species, and may even differ between individuals obtained from the same root clone (Tepfer, 1984). The phenotype has also been reported to be inheritable, although in some cases lateral shoots of hairy-root plants revert to the normal phenotype without losing the T-DNA (Tepfer, 1984). Although some pleiotropic effects (multiple effects from a single cause) can partially restrict the practical application of such transformed plants (van der Salm et al., 1996), some of the hairy-root characteristics may be of agronomic importance and useful in improving crops. *A. rhizogenes* has been used to transform several ornamental species (Table 1).

Angelonia salicariifolia is a perennial plant cultivated for pot or garden uses. After the infection of leaf explants with mikimopine-type strains of *A. rhizogenes*, hairy roots were obtained (Koike et al., 2003). Adventitious shoots were regenerated from the hairy roots by transferring to an appropriate medium. These *A. rhizogenes*-transformed plants exhibited a dwarf phenotype with shorter internodes and smaller, but no wrinkled, leaves. Roots grew faster and more vigorously from the stem cuttings of transformed lines than from controls. The number, shape and size of the flowers were not altered, but pollen fertility was reduced in transgenic plants.

Black-eyed Susan or gloriosa daisy, *Rudbeckia hirta*, is a perennial ornamental plant, used as both a garden flower and a landscape plant, whose roots can control nematodes. Leaf segments of cv. Highway Yellow were infected with a mikimopine-type strain of *A. rhizogenes*, and adventitious shoots regenerated from the hairy roots (Daimon and Mii, 1995). The *A. rhizogenes*-transgenic plants showed wrinkled leaves, smaller flowers and abundant lateral branching of the roots. The increased root system, with more thiophenes (nematocidal compounds), might improve the nematocidal ability of this species.

One cupflower species, *Nierembergia scoparia*, a native of Argentina, has been recently introduced into Japan as an ornamental plant due to its pale blue flowers and long flowering period. Plants transformed with a mikimopine-type strain of *A. rhizogenes*, with the aim of producing dwarfed plants for pot and cover plants, showed short internodes and smaller leaves that were narrower and curly (Godo et al., 1997). Transgenic plants also

Table 1
Ornamental flower species transformed with *Agrobacterium rhizogenes*

Common name	Plant species	Inserted genes	Phenotype	References
Angelonia	*Angelonia salicariifolia*	T-DNA of *A. rhizogenes*	Dwarf phenotype with shorter internodes, smaller leaves, cuttings with more vigorous roots, unaltered number, size and shape of flowers, and reduced pollen fertility	Koike et al., 2003
Black-eyed Susan or gloriosa daisy	*Rudbeckia hirta*	T-DNA of *A. rhizogenes*	Wrinkled leaves, abundant lateral branching of roots and smaller flowers	Daimon and Mii, 1995
Cupflower	*Nierembergia scoparia*	T-DNA of *A. rhizogenes*	Dwarfness, short internodes, smaller, narrower and curly leaves, increased rooting and normal fertility	Godo et al., 1997
Datura	*Datura arborea Datura sanguinea*	T-DNA of *A. rhizogenes*	Reduced plant height, reduced internode length, increased internode number, increased number of leaves with reduced size, darker color and dentate or curly, increased ability of cuttings to root and smaller flowers when flowering was not inhibited	Giovannini et al., 1997
Gentian	*Gentiana* sp.	T-DNA of *A. rhizogenes*	Dwarfed plants, short internodes although longer in one species, reduced apical dominance, branched stems, smaller and elliptical, rolled or wrinkled leaves, increased rooting, increased number of flowers and, in one species, early flowering	Suginuma and Akihama, 1995; Hosokawa et al., 1997; Momcilovic et al., 1997
Lisianthus or prairie gentian	*Eustoma grandiflorum*	T-DNA of *A. rhizogenes*	Decreased plant height, increased internode number, decreased internode length, more lateral branches, small and wrinkled leaves, increased rooting ability, modified shape of the corolla, reduced fertility and, in some progeny, altered phyllotaxis or fasciation	Handa, 1992b; Handa et al., 1995; Giovannini et al., 1996
Madagascar periwinkle	*Catharanthus roseus*	T-DNA of *A. rhizogenes*	Shorter internodes, wrinkled leaves, prolific rooting and, in one cultivar, changed flower color	Choi et al., 2004
Scented geranium	*Pelargonium* sp.	T-DNA of *A. rhizogenes*	Shorter stature, more internodes, more lateral branches, more leaves, more dentate and with darker color, better rooting of cuttings, inhibition of flowering and increased concentration of essential oils	Pellegrineschi et al., 1994; Pellegrineschi and Davolio-Mariani, 1996
Sharp-pod morning glory	*Ipomoea trichocarpa*	T-DNA of *A. rhizogenes*	Reduced stem length, smaller and wrinkled leaves, abundant roots, smaller flowers with altered morphology, decreased number of flowers, sometimes delayed time of flowering and normal pollen fertility	Otani et al., 1996

(continued on next page)

Table 1 *(continued)*

Common name	Plant species	Inserted genes	Phenotype	References
Snapdragon	*Antirrhinum majus*	T-DNA of *A. rhizogenes*	Dwarfness, short internodes, decreased apical dominance with highly branched stems, smaller leaves, broad and short, either stimulation of root production or poor root system, either increased number of flowers or delayed flowering, smaller flowers and reduced fertility or even sterility	Handa, 1992a; Senior et al., 1995; Hoshino and Mii, 1998

produced many roots with vigorous growth, extensive branching and plagiotropic response. No differences were found in the number of flowers on transgenic vs. control plants. Pollen viability was retained, in contrast to the reduced fertility often observed in *A. rhizogenes*-transformed species (Christey, 1997, 2001; Meyer et al., 2000), and this was suggested to be due to the use of the mikimopine-type strain A13 instead of the more widely used agropine-type strains. Dwarfness was obtained in the progeny.

Datura is a genus containing species of ornamental shrubs and small trees, some of which have huge, trumpet-like, bright-colored flowers with a pleasant odor. When *D. arborea* and *D. sanguinea* leaf explants were co-cultivated with an agropine-type strain of *A. rhizogenes*, they produced typical hairy roots. Transgenic plants obtained from the hairy roots displayed a reduction in plant height and internode length, and an increase in internode number (Giovannini et al., 1997). The number of leaves increased, and they were smaller, darker, and dentate or curly, depending on the clones. The increased rooting ability of cuttings is particularly important agronomically. Flowers of *D. sanguinea* were smaller than the wild type, whereas flowering was inhibited in *D. arborea*.

The genus *Gentiana* contains herbaceous perennial plants of ornamental and medicinal importance, with flowers that are used for cut and pot flowers. *G. scabra* was infected with a mikimopine-type strain of *A. rhizogenes*, and transformed plantlets showed elongated internodes compared to the rosette phenotype of non-transformed plants, reduced apical dominance, with vigorous emergence of lateral branches at the base of the stems, wrinkled leaves and increased root growth with plagiotropic roots (Suginuma and Akihama, 1995). Surprisingly, transgenic plants exhibited early flowering and were the only *A. rhizogenes*-transformed ornamental species with this trait. Transformation of *G. triflora*×*G. scabra* with an agropine-type strain of *A. rhizogenes* using stem explants caused, to varying degrees, dwarfing, reduced apical dominance, highly branched stems with increased flowering, and smaller, elliptically shaped leaves (Hosokawa et al., 1997). Extensive branching and increased numbers of flowers in transgenic gentians could be very useful horticultural traits. Shoots of micropropagated *G. cruciata*, *G. purpurea*, *G. acaulis* and *G. lutea* were infected with two agropine-type strains of *A. rhizogenes* in order to establish root cultures, since active compounds used in the pharmaceutical industry are extracted from their roots (Momcilovic et al., 1997). Only the hairy roots of *G. cruciata* and *G. purpurea* gave rise to plants, which exhibited the typical phenotype of *A. rhizogenes*-

transformed plants, with short internodes, but with smaller and rolled leaves rather than the typical wrinkled ones.

Another Gentianaceae, lisianthus or prairie gentian, has become a popular cut flower. Thus, attempts have been made to develop new cultivars with novel traits, such as branching stems with multiple flowers, altered corolla shape or a dwarf phenotype for pot plants (Handa and Deroles, 2001). Inoculation of seedling stems of lisianthus, *Eustoma grandiflorum*, with a mikimopine-type strain of *A. rhizogenes* produced transgenic plants with the typical hairy-root phenotype, such as dwarfism, short internodes, small and wrinkled leaves, cup-shaped instead of bell-form corollas and reduced fertility, although some of them set viable seeds. In addition, some regenerants produced an abundant root system (Handa, 1992b). The progeny also exhibited dwarfness and, in addition, some displayed an increase in lateral branching, while others showed verticillate phyllotaxis (leaves arranged in verticils or whorls around the stem) or expressed fasciation (malformation that leads to a flat stem) relative to their parents (Handa et al., 1995). Giovannini et al. (1996) also transformed leaf fragments of lisianthus, but with an agropine-type strain of *A. rhizogenes*. Transgenic plants exhibited varying degrees of altered morphology, with increased rooting ability and more branched roots, slightly decreased plant height, increased internode number and decreased internode length. Interestingly, although the number of flowers was similar to that of control plants, the characteristic bell-shaped corolla was also modified into a cup-like corolla.

The Madagascar periwinkle is a common garden plant, which is also used to obtain drugs to treat certain types of leukemia. Several ornamental cultivars of *Catharanthus roseus* were transformed with an agropine-type strain of *A. rhizogenes* (Choi et al., 2004). Plants regenerated from hairy roots displayed prolific rooting and shorter internodes. Moreover, half of the plants exhibited wrinkled leaves, whereas the others appeared morphologically normal. Surprisingly, the red color in the proximal region of the petals of cv. Cooler Apricot was white in flowers of transformed plants.

Pelargoniums, the most popular potted plant on the market, are often chosen as garden plants as well. The lemon-scented geranium, *Pelargonium graveolens*, transformed with agropine-type strains of *A. rhizogenes*, had increased fragrance and improved ornamental characteristics: it is normally unattractive due to its long internodes and chaotic growth. Transformed plants were shorter, had more internodes and increased lateral branching, showed accelerated rooting of cuttings, and its roots were shorter and highly branched (Pellegrineschi et al., 1994). Transformants presented a greater number of leaves, which were darker, more dentate and resisted typical yellowing. Increased concentrations of essential oils, used to perfume cosmetics, may also be a beneficial trait. Inhibition of flowering was not a negative trait in this infrequently flowering species. Similar results were obtained with the other scented geranium species, *P. fragans*, *P. odoratissimus* and *P. quercifolia* (Pellegrineschi and Davolio-Mariani, 1996).

The sharp-pod morning glory or blue morning glory, *Ipomoea trichocarpa*, is a prospective ornamental crop, as other *Ipomoea* known as morning glories are already used as ornamentals. This species has been transformed with several strains of *A. rhizogenes* (Otani et al., 1996). The better-studied hairy-root-derived plants, obtained by using mikimopine-type strains, displayed one-third of the stem length of normal plants and smaller, wrinkled leaves. They had abundant roots, highly branched and with plagiotropic

growth. The flowers were always smaller and their morphology changed to star-shaped with recurved petals. In contrast, the flowers of plants transformed by the agropine-type strain, which were slightly smaller, showed a wide variation in morphology. Traits related to flowering were negatively affected: the number of flowers decreased several-fold and the time of flowering was delayed by several days, although the latter was not observed in plants transformed with the agropine strain. On the positive side, pollen fertility was not altered, and thus seeds were obtained by self-fertilization, similar to plants transformed by agropine and mannopine strains. These transgenic plants could therefore be useful as a gene source for the breeding of this species with novel traits.

The snapdragon, *Antirrhinum majus*, an important ornamental crop, has been transformed with *A. rhizogenes* and, very recently, with *A. tumefaciens*. The variety Floral Carpet was transformed with an agropine- and a mikimopine-type strain of *A. rhizogenes*, which led to stimulation of root production, dwarfness due to short internodes, decreased apical dominance with highly branched stems, smaller, elliptical leaves, an increased number of smaller flowers due to extensive branching, and reduced fertility or even sterility (Handa, 1992a; Hoshino and Mii, 1998). The variety Golden Monarch was transformed with an agropine-type strain of *A. rhizogenes*, and hairy-root-derived transformants showed the typical dwarf phenotype, with broad and short leaves, although they displayed a poor root system and delayed flowering (Senior et al., 1995).

Interestingly, transformation with *A. rhizogenes* has been reported to circumvent the need for vernalization to produce flowering in biennial species such as Belgian endive (*Cichorium intybus*) (Sun et al., 1991b; Limami et al., 1998). The effects of Ri T-DNA were similar to those observed with the application of gibberellic acid to non-vernalized juvenile endive plants. A biennial to annual switch was also observed in carrot, in which *rolC* was the primary promoter of annualism (Tepfer, 1984; Limami et al., 1998). Although annualism is an undesirable trait in these biennial crops, the results could be applied to biennial ornamental plants in order to stimulate their annual flowering without the vernalization period.

Agropine-type strains of *A. rhizogenes* are the most widely used strains for transformations, although mikimopine-type strains are becoming more popular, especially among Japanese researchers. Alterations in flowering time, mainly inhibition and delay, are usually obtained with agropine-type strains, although there is likely to be an effect of plant genotype. When checking several *A. rhizogenes* strains in the same genotype we found a few differences in the phenotypes, offering a greater likelihood of finding a suitable/desirable plant morphology (Otani et al., 1996). However, further studies are needed to determine whether the obtained differences were due to the strain used or to transformation event. In the latter case, the different phenotypes obtained might be due to differences in the number of integrated copies of T-DNA, in the insertion site of the T-DNA or even in the T-DNA extent (Tepfer, 1984). The *rol* genes, sometimes in concert with genes in the T_R-DNA, such as *aux* genes, in the case of agropine-type strains, are responsible for the phenotypic modifications observed in *A. rhizogenes*-transformed plants.

A. rhizogenes strains are not only used as genetic sources to create new genotypes and phenotypes. They also serve as transformation vectors and have both advantages and disadvantages relative to the typically used *A. tumefaciens*. As *A. rhizogenes* is able to co-transfer the T-DNA of a binary vector it can be used to produce transgenic plants with the

desired foreign gene via marker-free selection through easy regeneration from the hairy roots. In verticordia, an ornamental plant prized for its bright red flowers, transformation using *A. rhizogenes* with a binary vector was more efficient than using *A. tumefaciens* with the same binary vector (Stummer et al., 1995). However, the former generated transgenic plants that exhibited the altered morphology associated with the presence of Ri T-DNA. Since the insertion of Ri and vector T-DNA usually occur on different chromosomes during co-transformation, their segregation in the progeny allows the recovery of transgenic plants with a normal phenotype, as reported in some species, such as *Brassica oleracea* (Puddephat et al., 2001). Recent reports on the transformation of *Antirrhinum* used a *rol*-type multi-auto-transformation (MAT) vector system (Cui et al., 2000, 2001). This system uses the morphological changes caused by the oncogenes of *Agrobacterium* (*rol* or *ipt* genes) to select the transgenic regenerated plants. This vector excises the oncogenes of the regenerated plants and therefore transgenic plants which are morphologically normal and carry the gene of interest are produced. These marker-free transgenic plants are produced without the need for sexual crossing, and are thus particularly beneficial for woody species.

In sum, genetic transformation with *A. rhizogenes*, in addition to enabling the introduction of specific genes into the plant genome, can induce desired characteristics in ornamentals due to the expression of *rol* genes carried by the Ri T-DNA. All transformed plants show modifications in their morphological traits, such as reduced plant height due to shorter, more numerous internodes; reduced apical dominance with increased lateral branching; and leaves, which sometimes increase in number, of decreased size and modified shape—wrinkled or curly. Almost all the transformants display increased rooting potential, with shorter and more branched roots. In several species, flowering is delayed or inhibited and the number of flowers sometimes decreases. In a few others, flowering is stimulated, a phenomenon that was not previously described in tobacco (Tepfer, 1984). Flowers are usually of the same size, although they may be smaller, sometimes altered in shape and, in one case, modified in color. Fertility was reduced or even lost, although surprisingly, in some cases it was retained. Of particular interest is the dwarfed phenotype, which should be considered for creating new pot-plant species from bigger ones, whereas the increased rooting ability could be useful for all commercial plants. Furthermore, the capacity to produce more flowers or even to change flower shape, as revealed with some species, is a desirable modification for any ornamental plant. Most of these traits have been described as the hairy-root phenotype.

4. Effects of *rol* genes on plants in vitro

Plants carrying only the *rolA*, *rolB* and *rolC* genes are morphogenetically equivalent to those carrying the whole T_L-DNA (Spanò et al., 1988). Several studies on *rol* genes introduced singly or in combination into plants have been performed in order to distinguish their effects in hairy-root syndrome, which has been frequently studied in plant tissues. Adventitious rhizogenesis was induced in leaves of *rolABC*-transformed tobacco, kalanchoe and tomato plants (Cardarelli et al., 1987a; Spena et al., 1987; Spanò et al., 1988; van Altvorst et al., 1992), and the genes acted synergistically in promoting hairy

roots from tobacco and kalanchoe leaves, when compared with the effect of single genes (Spena et al., 1987). In roses, the presence of *rolA*, *B* and *C* also enhanced adventitious root formation in both leaflets and stem explants (van der Salm et al., 1997). *rol*-transformed plants or tissues have been suggested to undergo an increase in auxin sensitivity (Spena et al., 1987; Spanò et al., 1988; Shen et al., 1990; van Altvorst et al., 1992; van der Salm et al., 1997), but the mechanisms of action of *rol* genes within plants remain poorly understood (Meyer et al., 2000).

Among *rol* genes, *rolA* has been reported to induce root formation in tobacco leaves, but not in kalanchoe (Spena et al., 1987). Studies in the latter species showed that roots proliferating from leaf tumors induced by *A. rhizogenes* were thicker, more curled and more stunted than roots induced by a strain without *rolA* (White et al., 1985).

rolB has been found capable of inducing roots from transgenic tissues, such as tobacco stems (Cardarelli et al., 1987a), carrot discs (Capone et al., 1989) and tobacco and kalanchoe leaves (Spena et al., 1987), although in some cases auxin was required. Moreover, after infecting kalanchoe leaves with a strain of *rolB*-mutated *A. rhizogenes*, root initiation was inhibited compared with the abundant root formation in the wild-type strain, indicating a basic role for *rolB* in hairy-root induction (White et al., 1985). It has also been reported that *rolB*'s primary action in morphogenesis in vitro consists of strongly promoting the formation of meristems of all types of organs: roots, flowers and vegetative shoots (Altamura et al., 1994, 1998). Thus, *rolB* might not specifically promote root differentiation, but act in general as a meristem-inducing gene (Altamura, 2004).

Tobacco leaves transformed with *rolC* driven by the CaMV-35S promoter were able to generate roots which were more branched than *rolA*- or *rolB*-roots, but no such effect was found with kalanchoe leaves (Spena et al., 1987; Schmülling et al., 1988). When the latter were transformed with a *rolC*-mutated *A. rhizogenes* strain, root growth was retarded, suggesting that *rolC* might be involved in hairy-root elongation (White et al., 1985). The *rolC*-transgene in carnation, under the CaMV-35S promoter, led not only to improved root regeneration in carnation explants, which in *rolC*-petals was up to 18-fold higher than in controls, but also to increased adventitious shoot formation in both petal and leaf explants (Casanova et al., 2003, 2004). Leaflet explants of micropropagated *rolC*-carnation plants cultured in a medium with auxin displayed up to four times more roots than did control (*uidA*-transgenic) leaflets (Fig. 1).

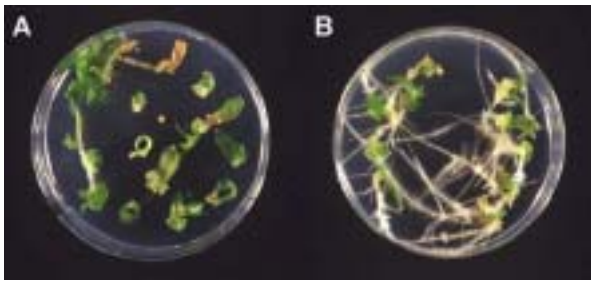

Fig. 1. Adventitious root regeneration from detached leaflets of in vitro-grown carnations cultured in MS medium supplemented with 0.5 μM of the auxin 1-naphthaleneacetic acid; (A) control (*uidA*-transgenic) leaves, (B) *rolC*-transgenic leaves.

Most of the studies on *rol* genes have been performed on *rolB* and *rolC*, and to a lesser extent on *rolA* and *rolD*. When kalanchoe tissues were infected using a *rolD*-mutated *A. rhizogenes* strain, root initiation still occurred, but subsequent root growth was severely retarded, suggesting a role for *rolD* in root elongation (White et al., 1985). Tobacco stems transformed with this gene were not able to produce roots, making it the only *rol* gene incapable of triggering root formation (Mauro et al., 1996). However, thin-cell-layer explants of floral branches of this transgenic tobacco displayed enhanced organogenesis of flowers which, moreover, appeared much earlier than in controls (Mauro et al., 1996; Altamura, 2004).

5. Effects of *rol* genes on plants in vivo

The first studies on *rol* genes were mostly performed on tissue culture, since basic research was the main objective. However, research studies on whole plants, most of them using tobacco as the model, began to show the putative applicability to agriculture. Many plants have now been successfully transformed with *rol* genes and show improved rooting or lateral shoot growth, a phenotype similar to that obtained by transformation with *A. rhizogenes*. Since *rol* genes seem to interfere somehow with hormone metabolism, flower development and fertility are also affected by these genes. Moreover, an indirect effect of *rol* genes is that they are able to modify secondary metabolism, and therefore they also may have applications in the pharmaceutical and cosmetics industries. Genes homologous to *rol* genes have only been found in *Nicotiana* plants, although no transcription was detected (Furner et al., 1986). It was hypothesized that these genes were an ancestral incorporation from *A. rhizogenes* into the plant genome.

5.1. Transgenic plants with rolA, B and C

Transgenic tobacco plants carrying the entire T_L-DNA (*rolA*, *rolB*, *rolC* and *rolD*) showed highly wrinkled and small leaves, reduced internode distances and plant height, and an increase in adventitious roots from stems. These plants also presented a loss of apical dominance with the consequent bushy appearance, their roots exhibited a plagiotropic response and flowers showed larger pistils (Sinkar et al., 1988). Similarly, introducing *rolA*, *B* and *C* into the same species also produced plants with hairy-root symptoms (Cardarelli et al., 1987a; Spena et al., 1987) and, moreover, with reduced flower size and seed production (Schmülling et al., 1988). An unexpectedly small root system was obtained in *rolABC*-tomato plants, which exhibited normal flower production and small flowers with decreased pollen viability (van Altvorst et al., 1992). Several ornamental plant species have also been transformed with a combination of the *rolA*, *B* and *C* genes (Table 2).

Various species of begonia are used as potted or garden plants. *Begonia tuberhybrida* has been transformed with the *rolA*, *B* and *C* genes (Kiyokawa et al., 1996). Regenerated plants displayed the typical phenotypes associated with *rol* genes, such as dwarfing, increased number of leaves which were dark green and wrinkled, and retarded or even inhibited flowering and wrinkled petals. There was variability in the *rol* phenotypes,

Table 2
Ornamental flower species transformed with the *rol* genes of *Agrobacterium rhizogenes*

Common name	Plant species	Inserted gene(s)	Phenotype	References
Begonia	*Begonia tuberhybrida*	*rolA+B+C*	Dwarfed plants, increased number of leaves, darker and wrinkled, delayed or inhibited flowering and wrinkled petals	Kiyokawa et al., 1996
Lily	*Lilium longiflorum*	*rolA+B+C*	Dwarfness, with decreased internode length, normal leaves with reduced size, increased rooting ability, smaller flowers, reduced number of flowers, early flowering in some lines and reduced pollen viability	Mercuri et al., 2003b
Limonium	*Limonium* sp.	*rolA+B+C*	Reduced plant size, with decreased internode length, reduced apical dominance, smaller and curly or wrinkled leaves, increased number of roots, early flowering, in some lines contemporaneous flowering yielding a high flower density, smaller flowers and reduced pollen viability	Mercuri et al., 2001, 2003a
Osteospermum	*Osteospermum ecklonis*	*rolA+B+C*	Erect plant growth habit, higher number of branches, shorter branches, increased rooting *in vitro*, earlier flowering, increased number of flowers, increased length of flower life, reduced flower size and reduced fertility	Giovannini et al., 1999b; Allavena et al., 2000
		rolA+B	Fairly erect plant habit, shorter branches, dark green leaves, abundant root system *in vitro*, slight advanced flowering, slight increase in the number of flowers	Giovannini et al., 1999b; Allavena et al., 2000
Rose	*Rosa hybrida*	*rolA+B+C*	Decreased shoot length, reduced apical dominance, smaller leaves, wrinkled and curly leaves in one transformant, and increased rooting capacity of cuttings. Increased releasing of lateral buds in untransformed scions grafted on *rolABC* rootstocks	van der Salm et al., 1997, 1998
		rolB	Reduction of lateral shoots (one single transformant)	van der Salm et al., 1996, 1997
Carnation	*Dianthus caryophyllus*	35S-*rolC*	Slightly dwarfed plants, with increased number of lateral shoots, better rooting ability of cuttings, increased number of flowering stems and smaller flowers in one line	Ovadis et al., 1999; Zuker et al., 2001
Chrysanthemum	*Chrysanthemum morifolium*	35S-*rolC*	Dwarfed plants with shorter internodes, increased branching, narrower and paler leaves, wider petals, more compact flower stems with more, but smaller flowers	Mitiouchkina and Dolgov, 2000
Osteospermum	*Osteospermum ecklonis*	35S-*rolC*	Erect plant habit, pale green leaves, promotion of flowering due to earlier flowering, increased number of flowers and longer flower life, smaller flowers and reduced fertility or even sterility	Giovannini et al., 1999b; Allavena et al., 2000

Table 2 *(continued)*

Common name	Plant species	Inserted gene(s)	Phenotype	References
Painted tongue	*Salpiglossis sinuata*	*rolC*	Reduced plant height, more branches, narrower leaves, smaller flowers and male sterility	Lee et al., 1996
Petunia	*Petunia axillaris*× *(P. axillaris*× *P. hybrida)*	35S-*rolC*	Dwarfness, due to the decrease in internode length, decreased apical dominance with increased branching, either reduced or increased leaf size, smaller flowers, advanced flowering, severe decrease in male and female fertility	Winefield et al., 1999
Regal pelargonium	*Pelargonium*× *domesticum*	35S-*rolC*	Dwarf phenotype, reduced leaf area, reduced petal area and flower size, increased number of flowers and early flowering	Boase et al., 2004
Rose	*Rosa hybrida*	*rolC*	Dwarf phenotype, increased lateral branching, wrinkled and chlorotic leaves, reduced root system, smaller flowers with reduced fertility and changes in color, and numerous thorns	Souq et al., 1996

classified as semi-dwarf and super-dwarf, which was not associated with the transgene copy number, and which may have been due to transgene inactivation or to the effect of the location of the inserted genes.

Lily is one of the three most important ornamental bulb crops. *Lilium longiflorum* cv. Snow Queen has recently been transformed with *rolA*, *B* and *C* (Mercuri et al., 2003b). The *rolABC* transformants displayed increased rooting ability with root branching. Some of the transgenic lilies exhibited a bushy and disorderly habit with six or seven stems, whereas others presented a desirable aspect with one or two stems and a severe reduction in internode length. With respect to leaves, all of them presented a normal shape, with no wrinkling or curling, and a reduced size. Flowers were also smaller, their number per stem was reduced, and pollen viability was greatly reduced. Although some transgenic plants flowered earlier, this was not a general trait. These new phenotypes can be considered innovative products.

Limonium is a bushy perennial species that is popular as a cut flower for both fresh and dry flower arrangements. Limonium plants (both the sterile hybrid *Limonium otolepis*×*L. latifolium* and the wild species *L. gmelinii*) have been transformed with *rolA*, *B* and *C*. Transgenic limonium plants displayed high phenotypic variety, and a range of compact growth habits termed super-compact, compact and semi-compact (Mercuri et al., 2001, 2003a). Transgenic plants also presented reduced apical dominance, decreased internode length, curly or wrinkled leaves with a reduced area, and an increased number of roots with respect to controls. Moreover, all the selected transgenic lines displayed early flowering and the compact type displayed contemporaneous flowering, a new trait in these species; a high flower density and compact inflorescence were thus obtained. In addition, the hybrid species had smaller flowers (Mercuri et al., 2001) and *L. gmelinii* species presented a reduced pollen viability and germination (Mercuri et al., 2003a). It was

suggested that the diversity in the *rol* phenotypes did not originate from the copy number of the insert, but rather from the positional effect of the inserts and the levels of the *rolABC* transcripts and encoded proteins. The super-compact and compact types could be used as pot plants and as small bouquets, respectively.

Osteospermum is a ground-cover ornamental, whose varieties are mainly interspecific hybrids. It is also used as a cut flower and has recently become popular as a pot plant. *Osteospermum ecklonis* has been transformed with *rol* genes (Giovannini et al., 1999b; Allavena et al., 2000). The introduction of *rolA* and *B* did not cause dwarfing. These transgenic plants displayed a fairly erect plant habit, shorter branches, dark green leaves, and a more profuse root system when grown in vitro. They displayed slightly earlier flowering, with a slight increase in the number of flowers, which were all fertile. When *rolA* and *rolB* were introduced together with *rolC*, they caused an erect plant growth habit, with a higher number of shorter branches and increased rooting in vitro. *The rolABC*-osteospermums also displayed earlier flowering, an increased number of flowers—up to twice the control, with increased length of flower life. Flower size was reduced, as was fertility. The combination of the bushy phenotype, the upright growth habit and the higher number of—albeit smaller—flower heads per plant gives the transgenic plants the appearance of a new ornamental.

Rose is one of the most important ornamental crops worldwide, used both in gardens and as cut flowers. In *Rosa hybrida* cv. Moneyway, used as a rose rootstock, the introduction of a combination of *rolA*, *B* and *C* resulted in a threefold increase in the rooting capacity of the cuttings (van der Salm et al., 1997). The transformants formed adventitious roots at their base, showed decreased shoot length, reduced apical dominance and smaller leaves to varying degrees, whereas one of them displayed wrinkled and curly leaves. Thus, in addition to the improved traits obtained with *rol* genes, some pleiotropic side effects may appear in the aerial parts of transformed plants (van der Salm et al., 1996). Interestingly, when a non-transformed rose scion (detached bud-containing shoot to be grafted onto another plant) was grafted onto a rootstock of *rolABC*-transformed rose, the release of lateral shoots from the base of the grafted scions was increased with respect to that of the scions grafted on control rootstocks. These results indicate that some root-to-shoot signal was transported from the rootstock to the scion (van der Salm et al., 1998). Since the development of lateral shoots is correlated with the final number of flowers, such genetically modified rootstocks will presumably lead to increased flower production. To avoid the transmission of undesirable pleiotropic effects, *rol* genes driven by tissue- or organ-specific promoters, enabling their more controlled expression, may be employed (van der Salm et al., 1996).

In summary, all the ornamental plants transformed with *rolA*, *B* and *C* have been obtained by introducing the T-DNA restriction fragment EcoRI 15 excised from the agropine-type Ri plasmids pRi1855 or pRiA4 of *A. rhizogenes*. Thus, *rol* genes were driven by their own promoters. The *rolABC*-transgenic plants displayed reduced plant size, with decreased internode length, and reduced apical dominance with more branching. The leaves were smaller and sometimes wrinkled or curly. The rooting capacity was improved for both the whole plants and cuttings. In general, flowers showed a normal size and shape, although sometimes they were smaller. The number of flowers sometimes increased and flowering was often advanced, traits that have not been reported in *rolABC*-tobacco plants

(Schmülling et al., 1988). Fertility was reduced due to decreased pollen viability. Some of the modifications in the morphology of plants obtained by transformation with *rol* genes are highly desirable for ornamental plant breeders. Similar to *A. rhizogenes*-transformed plants, the dwarfing or the increased number of flowers in some species obtained with *rolA*, *B* and *C* are desirable traits for new varieties. The increased rooting capacity, as well as the early flowering—which was not obtained with *A. rhizogenes*, are very important for both improving survival and reducing the time of culture before being sold. Although we could not compare the effect of transformation with *A. rhizogenes* or *rol* genes in different species, the overall effects, termed hairy-root symptoms, are obtained by both types of transformation. Specific differences, such as the modification of the flower shape, obtained only in transformations with *A. rhizogenes*, or the time of flowering, which can be delayed with the T-DNA of *A. rhizogenes* but with *rol* genes is almost always advanced, may be due to the presence/absence of *aux* genes (in the case of transformation with an agropine-type strain), other genes of the T-DNA, or even species-specific effects of *rol* genes. Introducing *rol* genes separately into plants may help to discriminate their effects in hairy-root syndrome.

5.2. Transgenic plants with rolA

Transgenic tobacco plants with *rolA* presented highly wrinkled leaves with a low length-to-width ratio, were stunted due to the short internodes, and had condensed inflorescences with larger flowers (Schmülling et al., 1988; Sinkar et al., 1988); however, they could also display delayed flowering with small and abnormal flowers, and severely reduced male fertility (Sun et al., 1991a; Martin-Tanguy et al., 1993; Michael and Spena, 1995). *rolA*-transgenic tomato plants, which had long internodes and a small root system, also displayed small and wrinkled leaves and small flowers, showing protruded pistils and reduced pollen viability (van Altvorst et al., 1992). However, a large increase in the number of flowers was observed in *rolA*-tomatoes.

Studies with mutations in this locus showed *rolA* to be determinant of the abnormal wrinkled phenotype of plants transgenic with the T_L-DNA of *A. rhizogenes* (Sinkar et al., 1988), probably due to differential growth of tissues of the leaf blade (Michael and Spena, 1995). Although in some cases the number of flowers, which are usually smaller, increases, they display a delay in flowering and decreased fertility, both of which are horticulturally undesirable. Moreover, *rolA* does not lead to an improved rooting, in the studied species. This may be why until now no ornamental flowering plant has been transformed with the *rolA* gene.

5.3. Transgenic plants with rolB

Transgenic tobacco plants with *rolB* showed alterations in leaf morphology, abundant adventitious rooting, with branched and plagiotropic roots (Cardarelli et al., 1987a), and bigger flowers, with protruded pistils, and early leaf necrosis when *rolB* was driven by the CaMV-35S promoter (Schmülling et al., 1988). Fertility was not reported to be reduced (Schmülling et al., 1988). In addition to the changes in leaf morphology (wider leaves as in tobacco) and increased rooting, *rolB* under its native promoter led to a reduction in

internode length and apical dominance when expressed in tomato. Flower production and size were also reduced, as was pollen viability (van Altvorst et al., 1992). However, when *rolB* was introduced into rose (Table 2), lateral shoot formation was severely decreased, indicating the induction of apical dominance, although this phenotype is of a single transformant and cannot be closely linked to the introduced gene (van der Salm et al., 1996, 1997). The above trait, although not desirable for roses, might be useful for other species, since their budding could be prevented. When *rolB* was under the control of an inducible promoter, tobacco plants were phenotypically indistinguishable from the wild type. Its induction caused stunted plants, which did not develop floral meristems and showed necrotic and wrinkled leaves. However, once induction was stopped, healthy shoots developed again, even from affected meristems (Röder et al., 1994).

Overall, *rolB* effects also depend on the transformed species. In the described cases, leaves were wider, apical dominance was either reduced or increased, flower induction and development might be reduced, with larger or smaller flowers, and pollen viability was found to decrease in some cases. However, the occasional stunted phenotype, together with increased rooting, which has typically been associated with this gene among the *rol* genes, might be desirable in some ornamental species and this should certainly be tested. Moreover, since the effects of *rolB* under a constitutive or even native promoter cannot be controlled, inducible promoters could be useful in modulating the manifestation of a particular phenotype, for example, during a specific period of plant development.

5.4. Transgenic plants with rolC

The first studies of *rolC* were also performed on tobacco. The phenotypic alterations of *rolC*-transgenic tobacco plants consisted of reduced apical dominance leading to increased branching, dwarfed plants with short internodes, altered leaf morphology, often with reduced area due to their narrower shape, early flowering, reduced flower size, reduced seed production and pollen viability, and when *rolC* was constitutively expressed, there were even male sterility and pale green leaves in some cases (Schmülling et al., 1988; Oono et al., 1990; Nilsson et al., 1993b; Scorza et al., 1994). However, the root length did not differ between transgenic and control plants (Scorza et al., 1994). These alterations have also been reported for other *rolC*-transformed herbaceous species, such as potato (Fladung, 1990; Schmülling et al., 1993) and belladonna, where increased flowering and normal fertility, in addition to advanced flowering, was noteworthy (Kurioka et al., 1992), and woody species like hybrid aspen (Nilsson et al., 1996b) and pear tree (Bell et al., 1999), in which flowering has yet to be studied. This gene increased the rooting rate of trifoliate orange and Japanese persimmon cuttings, which, moreover, have potential as dwarfing rootstocks (Kaneyoshi and Kobayashi, 1999; Koshita et al., 2002). The *rolC* gene has also been used to transform several ornamental flowering species (Table 2).

Carnation is one of the world's top four cut-flower crops. *Dianthus caryophyllus* cv. White Sim transformed with *rolC*, under the constitutive CaMV-35S promoter, displayed a slightly dwarfed phenotype without altered leaf morphology (Ovadis et al., 1999; Zuker et al., 2001) (Fig. 2). The enhanced lateral shoot development of *rolC*-transgenic carnation plants led to a yield of stem cuttings that was up to 1.5-fold higher than in controls (Zuker

Fig. 2. Morphological alterations and root formation in *rolC*-transgenic carnation plants. Left, control (*uidA*-transgenic) carnations; middle and right, *rolC*-transgenic carnations.

et al., 2001). Interestingly, the *rolC* gene led to better rooting ability in these cuttings. Moreover, *rolC*-carnations produced threefold more flowering stems and only one line (out of 18 transgenic lines) presented smaller flowers. Male fertility could not be tested, since the cultivar was male-sterile. However, in carnation this would not be a problem as carnation plants are multiplied vegetatively. Thus, *rolC* clearly improves horticultural traits in carnation plants.

Chrysanthemum, another of the world's top cut flowers, is available in a great variety of colors and forms and is now becoming popular as a pot plant. For this reason, plant architecture and size need to be manipulated. Introduction of the *rolC* gene, under the CaMV-35S promoter, in the variety White Snowdon of *Chrysanthemum morifolium* produced one transgenic line with dramatic changes in plant and flower architecture (Mitiouchkina and Dolgov, 2000). This *rolC* line displayed dwarfing characteristics, shorter internodes and suppressed apical dominance with an increase in lateral branching, all contributing to an overall bushy appearance. Leaf blades were narrower and paler than control leaves. Interestingly, *rolC*-chrysanthemums presented a great number of flowers, although they were smaller than controls. Moreover, petals were wider and their form was changed. The changes obtained with the insertion of the *rolC* gene corresponded to the ideal pot plant described by Mol et al. (1995), with a dense layer of flowers covering the green parts of the plant.

When *rolC* was introduced constitutively under the CaMV-35S promoter in osteospermum, *O. ecklonis*, it caused a plant height similar to that of the control as well as an erect plant habit, as occurred with the introduction of *rolA*, *B* and *C* together; however, pale green leaves were obtained (Giovannini et al., 1999b; Allavena et al., 2000). 35S-*rolC*-plants displayed earlier flowering and more flowers, up to three times the control, sometimes with longer vase life; thus, these were the same flowering traits as in *rolABC*-transformants but more marked. Flower size was reduced, as with *rolABC*, and fertility was also reduced or even inhibited. Interestingly, the levels of the RolC protein in the studied lines correlated with the strength of their *rolC* phenotype.

Painted tongue or velvet trumpet flower, *Salpiglossis sinuata*, is an annual plant with trumpet-shaped flowers, similar to petunia. Transgenic plants with *rolC* under its native promoter were shorter, had more branches, and therefore a compact growth habit, and presented narrower leaves and smaller flowers with respect to control plants. Moreover, the flowers were male-sterile (Lee et al., 1996).

Petunia is one of the most popular ornamental flowers and is used mainly as a pot plant. *Petunia axillaris*×(*P. axillaris*×*P. hybrida*) cv. Mitchell was transformed with *rolC* driven by the CaMV-35S promoter (Winefield et al., 1999). Several phenotypic alterations, which correlated with the levels of *rolC* transcript, were obtained in transgenic plants. They were shorter, due to decreased internode length, and displayed a decrease in apical dominance leading to increased branching, showing a bushy growth habit. The *rolC*-petunia plants presented either a reduction in leaf area, in plants with the most severe phenotypes, or an increase in leaf size, in this case together with an increase in the number of leaves. Transgenic plants also had smaller flowers, and most of them flowered several days before controls. However, no differences in the number of flowers were recorded. Both pollen viability and female fertility were severely reduced. Therefore, the influence of *rolC* on petunias, causing compact and bushy plants with advanced flowering, might be used to obtain dwarf varieties.

Regal pelargonium, *Pelargonium*×*domesticum*, is very popular as a garden plant. The cultivar Dubonnet was transformed with the *rolC* gene driven by CaMV-35S (Boase et al., 2004). Among the four transformants that presented phenotypical alterations, one displayed a super-dwarf phenotype, whereas the other three exhibited a dwarf phenotype; all of them showed a reduction in height and leaf size. Both the petal area and the flower diameter were also reduced in the *rolC*-regal pelargoniums. Moreover, these transgenic plants displayed a two- to threefold higher number of flowers per plant, as well as early flowering.

Rose, *R. hybrida* cv. Madame G. Delbard ®, was transformed with *rolC* under its own promoter (Souq et al., 1996). The transgenic rose plants were dwarfed, sometimes with wrinkled leaves and with multiple stems arising from the base of the main stem. In some lines, the flowers were similar to those of the control plants but exhibited abnormal sexual organs; thorns were small and numerous and leaves were chlorotic. In others, the flowers were smaller than the controls, with non-fertilizable pistils, and exhibited changes in color. The root system of *rolC*-roses was reduced, they were highly sensitive to insects and diseases and, after pruning, the new stems failed to flower. Moreover, many plants displayed a drying-out phenomenon with consequent high death rate.

In most of these flowering plants, the *rolC* gene was driven by the constitutive promoter CaMV-35S to obtain more pronounced changes in phenotype, as described in other species (Schmülling et al., 1988; Kurioka et al., 1992; Guivarc'h et al., 1996). Moreover, a correlation between the *rolC* phenotype and the level of gene expression has been reported in 35S-*rolC*-transgenic plants of belladonna (Kurioka et al., 1992), aspen (Nilsson et al., 1996b), osteospermum (Giovannini et al., 1999b) and petunia (Winefield et al., 1999). This correlation enables a range of new cultivars to be developed through the selection of individual transgenic lines and specific promoters.

The most generalizable traits of *rolC*-ornamental plants are dwarfness, due to the shorter internodes, and reduced apical dominance with an increase in the lateral shoots, leading to a more compact phenotype. Moreover, *rolC*-transgenic plants usually displayed smaller leaves, which were often narrower than the controls. Pale green leaves were observed a few times in ornamentals. Among ornamentals, it is noteworthy that carnation plants displayed increased rooting ability (Zuker et al., 2001). This trait, highly desirable in species that are propagated with cuttings and usually assigned to *rolB*, has also recently been related to *rolC* in two fruit trees (Kaneyoshi and Kobayashi, 1999; Koshita et al., 2002), but not in the model plant tobacco. In many cases, the number of flowers was higher, a trait that has not been described in *rolC*-tobacco (Schmülling et al., 1988; Scorza et al., 1994). Flower size was almost always reduced, but with the same shape, while time to flowering was sometimes reduced but never delayed. Fertility, as with all *rol* genes, was reduced or totally inhibited. The increase in the number of flowers was only sometimes observed in *rolABC*-ornamental plants, but was frequent with *rolC*, although in some cases it could be an indirect effect of the improved lateral branching, as suggested by Giovannini et al. (1999a). Moreover, the early flowering, also observed in some *rolABC*-plants, was displayed by *rolC*-plants, in contrast to plants transformed with *rolA* or *rolB*. Thus, these improved flowering traits seem to be related to the *rolC* gene. The smaller flowers, obtained sporadically with any transformation but almost always with the *rolC*, may also reflect an effect of this gene. The action of the *rolC* gene in altering the plant growth habit, causing dwarfness and compact but vigorous growth, improving the rooting capacity and promoting flowering makes it a highly useful tool for obtaining horticulturally and commercially important traits. Moreover, undesirable pleiotropic effects, except for reduced fertility, are absent, provided that a certain degree of dwarfness is also one of the objectives. This is why *rolC* is the most widely used, among the *rol* genes, in transforming ornamental flower species.

5.5. Transgenic plants with rolD

rolD is the least studied *rol* gene. When introduced into carrot it produced an extremely dwarfed phenotype (Limami et al., 1998). Interestingly, tobacco plants transformed with *rolD* displayed strongly stimulated and advanced flowering, in accordance with the results in tissue culture (Mauro et al., 1996). Thus, early flowering was followed by the production of a large number of lateral inflorescences. The only alteration in the morphology of *rolD* plants was the smaller and thinner leaves compared with non-transformed plants, since the root system of these *rolD*-tobacco plants showed no differences with respect to controls. Similarly, *rolD*-tomato plants flowered earlier and

displayed more inflorescences than control plants, with consequently higher fruit yield (Bettini et al., 2003). While *rolD* may prove interesting for genetic improvement in ornamentals, its effects in various species need to be better characterized.

6. Secondary metabolism of *rol*-transgenic plants

Transformation of tissues or plants with *A. rhizogenes*, as well as transformation with *rol* genes, may produce, in addition to hairy roots, alterations in the plants' secondary metabolism. Some of these products, which could be of interest to the pharmaceutical industry, are synthesized mainly in the roots (Sevón and Oksman-Caldentey, 2002). Scopolamine, the most valuable tropane alkaloid used in medicine, is produced in the roots of several species, such as *Atropa belladonna* and *Hyoscyamus muticus*. The latter was transformed with *A. rhizogenes* containing the gene encoding the enzyme that synthesizes the desired metabolite, the result being the production of hairy roots with enhanced levels of scopolamine (Jouhikainen et al., 1999). Another example is the valepotriates, active compounds obtained from the roots of *Valeriana wallichii*. Its transformation with *A. rhizogenes* generated hairy roots which produced a two- to threefold increase in valepotriate levels (Banerjee et al., 1998). Roots of tobacco lines transformed with *rolA*, *B* and *C* together or with *rolC* alone showed more growing capacity and produced more nicotine, alkaloid synthesized in the roots, compared with controls (Palazón et al., 1998). Ginsenoside production in hairy roots of ginseng, *Panax ginseng*, transformed with *A. rhizogenes* was twofold higher than in the control culture, and *rolC*-transgenic roots produced three times more ginsenosides (Bulgakov et al., 1998).

The indirect effect of Ri plasmid T-DNA on the increase in essential oils, and thus on the fragrance of certain plants, may be an additional benefit of *rol* genes when introduced into ornamental plants such as lemon geranium (Pellegrineschi et al., 1994). The use of *rol* genes, together with other biosynthetic genes of odorous components, opens up new perspectives on improving scent in ornamental flowers.

7. Hormonal balance of *rol*-transgenic plants

The *rol* genes may affect either the metabolism of plant hormones or the sensitivity of plant cells to hormones (Binns and Costantino, 1998; Meyer et al., 2000). As a group, *rol* genes have been suggested to cause an increase in auxin sensitivity, as in *rolABC*-transformed tobacco, tomato and rose leaves (Spena et al., 1987; Spanò et al., 1988; van Altvorst et al., 1992; van der Salm et al., 1997). Each of them has also been analyzed individually in order to elucidate how they produce their effect on plant growth and development.

The RolA protein, suggested to act as a transcription factor (Meyer et al., 2000), has been suggested to be implicated in the metabolism of gibberellins, as a reduction in their content has been found in *rolA*-transgenic tobacco plants (Dehio et al., 1993). This could explain the dwarfing of these plants, since a similar phenotype was obtained by applying inhibitors of gibberellin synthesis (Dehio et al., 1993), although the wild phenotype was

not completely restored with the application of gibberellins (Schmülling et al., 1993). The *rolA* gene was also reported to be responsible for changes in polyamine metabolism by inhibiting their conjugation (Sun et al., 1991a; Martin-Tanguy et al., 1996). Moreover, measurement of their transmembrane potential difference in response to auxins showed *rolA*-tobacco protoplasts to be more sensitive to auxins (Maurel et al., 1991).

The capability of *rolB*-transgenic plants to give rise to adventitious roots has led to suggestions that the auxin activity in cells expressing this gene is affected (Spena et al., 1987; Schmülling et al., 1988). RolB protein was suggested to be a β-glucosidase able to increase the levels of free, and thus active, IAA (the natural endogenous auxin) by releasing it from its inactive glucose conjugates in vitro (Estruch et al., 1991c). However, in plant tissues IAA-glucosides were shown not to be substrates for RolB (Nilsson et al., 1993a). These results strongly suggest that RolB is not involved in the regulation of hormone metabolism. The auxin effects observed in *rolB*-transformed plants could perhaps be due to an altered perception of the hormone stimuli. In this regard, *rolB*-transgenic tobacco protoplasts, as well as those of *rolA* and *rolC*, were shown, by analyzing their transmembrane potential in response to auxins, to have increased auxin sensitivity, the effect being strongest in *rolB*-cells (Maurel et al., 1991, 1994). This might be due to the several-fold increment in the auxin-binding capacity of *rolB*-transformed cells as compared to non-transformed ones (Filippini et al., 1994). Moreover, the tyrosine phosphatase activity of RolB, located in the plasma membrane, may account for the perturbation of the hormone's signal perception/transduction pathway, thus altering the auxin sensitivity of *rolB*-cells (Filippini et al., 1996).

Some of the morphological phenotypes of *rolC*-transformed plants, such as reduced apical dominance and enhanced lateral shoot development, are suggestive of cytokinin activity (Schmülling et al., 1988; Zuker et al., 2001). RolC has been reported to be a cytosolic β-glucosidase that releases free forms of cytokinins from their inactive glucosidic conjugates in vitro (Estruch et al., 1991a,b). However, when free cytokinins and their glucosides were quantified in 35S-*rolC*-transgenic plants, it was shown that RolC did not hydrolyze cytokinin glucosides (Faiss et al., 1996; Nilsson et al., 1996b). Moreover, contradictory data have been reported on the levels of cytokinins in *rolC*-plants: they increase, decrease or remain stable, depending on the species and tissues being analyzed. The internal pool of cytokinins in *rolC*-transformed tobacco plants was found to be affected either negatively (Nilsson et al., 1993b), or not at all (Schmülling et al., 1993; Faiss et al., 1996). In contrast, an up to fourfold increase in cytokinin levels was quantified in potato plants expressing *rolC* (Schmülling et al., 1993), and zeatin riboside was found to be increased in *rolC*-hybrid aspen, despite the slight decrease in overall cytokinin levels (Nilsson et al., 1996b; Fladung et al., 1997). In ornamentals, *rolC*-transgenic chrysanthemums presented, during flower formation, an 80-fold higher content of the cytokinins zeatin and zeatin riboside in apices compared to controls (Mitiouchkina and Dolgov, 2000). In *rolC*-carnation tissues we reported an enhanced cytokinin-like effect, whereby both petal and leaf explants of the studied lines were able to generate a higher number of shoots per explant as compared to control plants (Casanova et al., 2003). Further measurements of cytokinin content in carnation petals showed that the most abundant cytokinin in *rolC*-petals was isopentenyladenine (iP) (Casanova et al., 2004). iP levels were 7-, 4.5- and 3.5-fold higher than those of control petals in transgenic lines

*rolC*1, *rolC*2 and *rolC*4, respectively. These values correlated with the adventitious shoot regeneration in petal explants. However, *rolC*-carnation leaves presented the same levels of iP as their non-transformed counterparts.

In contrast, the root-stimulating action of *rolC* in both tissues and cuttings cannot be explained by a cytokinin effect, but rather by an auxin effect of that gene (Schmülling et al., 1988; Zuker et al., 2001). *rolC*-carnation explants, in addition to displaying enhanced shoot regeneration, also presented higher root regeneration than controls, indicating an auxin-like effect of *rolC* on carnation tissues (Casanova et al., 2003). The measurement of IAA levels in *rolC*-tobacco, potato and carnation plants showed no differences with respect to control plants (Nilsson et al., 1993b; Schmülling et al., 1993, Casanova et al., 2004) and there was even a decrease in *rolC*-hybrid aspen and chrysanthemum (Nilsson et al., 1996b; Mitiouchkina and Dolgov, 2000). Thus, this auxin-like effect might be caused by an increase in auxin sensitivity. Accordingly, *rolC*-transgenic tobacco protoplasts were shown, by measuring their transmembrane potential hyperpolarization in response to auxins, to be more sensitive to auxins than their wild-type counterparts (Maurel et al., 1991).

Levels of gibberellins, specifically active GA_1, were found to decrease in *rolC*-tobacco, potato and hybrid aspen tissues (Nilsson et al., 1993b, 1996b; Schmülling et al., 1993). However, the phenotype, in particular early flowering, could not be completely reversed by the application of gibberellins (Schmülling et al., 1993). RolC has also been suggested to act by creating a sink for sucrose, necessary to induce root primordia (Nilsson and Olsson, 1997). Accordingly, sucrose was found to be a strong inducer of the *rolC* promoter (Yokoyama et al., 1994; Nilsson et al., 1996a).

rolD was suggested to exert its effect on increased flowering through changes in the concentration of plant hormones in transformed plants (Mauro et al., 1996). Recent biochemical assays have shown that *rolD* encodes an ornithine cyclodeaminase able to catalyze the conversion of ornithine to proline (Trovato et al., 2001). The high proline concentration in tomato flowers (Schwacke et al., 1999) has led some authors to argue for a proline-mediated role of *rolD* in flowering (Trovato et al., 2001).

In conclusion, changes in cytokinin, auxin or gibberellin levels might constitute secondary effects of *rol* genes on hormone metabolism (Schmülling et al., 1993; Faiss et al., 1996; Nilsson et al., 1996b). However, despite much research over the years, and some evidence assigning the effects of *rol* genes to plant hormone effects, the biochemical functions of these genes remain poorly understood, reflecting the complexity of the system. Nevertheless, they are suitable tools for modifying plant development.

8. Applicability of *rol* genes in floriculture

The transformation of ornamental flowering plants with a combination of *rol* genes produces floriculturally desirable traits, such as dwarf and bushy phenotypes. These traits are of interest in creating new varieties, such as pot-plant species from bigger ones, while improved rooting ability is a useful characteristic for any commercial plant, especially woody ornamentals that are recalcitrant to rooting. The *rol* genes can be used not only to modify the morphology of a plant, but also to influence the process of flowering. The

promotion of flowering, by both increasing the number of flowers and advancing time of flowering, is a major desirable trait in floriculture and can be achieved by introducing *rolC*, as well as the less studied *rolD*. The latter traits translate into less time to selling, increased consumer demand due to the presence of more flowers, and, in the case of cut flowers, greater profits because of increased quantities. Moreover, changes in flower shape are constantly being sought by breeders to produce novel varieties. It should be remembered that in some cases undesirable traits, such as reduced fertility, may also be obtained. However, this does not affect plants which are propagated vegetatively, and the multiplication of seed-propagated plants with, for example, male but not female sterility can be achieved by pollinating transgenic plants with the pollen of non-transformed ones. As a whole, *rol* genes are a powerful tool for achieving specific desirable characteristics. The *rolC* gene is the most highly recommended for use, since many desirable traits have been obtained in the studied ornamental species, and the least-studied *rol* gene, *rolD*, presents promising applications. Although *rolA* and *rolB* require further study in this regard, they are not recommended for introduction into ornamental species, since with them, many abnormalities are obtained.

9. Future trends

The demand for flowers with new plant architecture, attractive colors, stronger fragrance and better post-harvest keeping quality drives breeders to continuously create new varieties, for the economically important floriculture market. In the year 2002, worldwide exports of floricultural products reached $9.0 billion (http://pathfastpublishing.com). Biotechnological techniques, such as genetic engineering, are a useful addition to classical breeding for the introduction of novel traits into plants and the production of new varieties. Gene availability no longer hinders the introduction of new traits and successful transformation is being reported for an ever-increasing number of species. The first transgenic flower was reported in 1987 by Meyer et al., and around 30 ornamental species have now been transformed successfully (Deroles et al., 2002). However, whereas several transgenic crops have already been introduced commercially, among ornamental flowers only carnations with a range of violet colors, including cv. Moondust and cv. Moonshadow, produced by Florigene (Australia), are currently on the market.

In the case of floricultural crops, molecular regulation of flowering and floral traits, as well as alterations in plant shape, via the application of genes such as *rol* genes, all contribute to the improvement of the ornamental plants. Currently, the number of *rol*-transgenic plants continues to increase, and in many cases, beneficial traits are obtained. Thus, *rol* genes, particularly *rolC* and the promising *rolD*, should be introduced into more ornamental species, since they may prove highly useful in yielding improved floricultural traits. However, further research is needed to determine the effects of *rol* proteins and their interaction with other proteins, organs and environmental factors, in order to understand all the different phenotypical and biochemical effects they may produce in transgenic plants. Consequently, increased knowledge about the functions of these genes would greatly benefit their application in both floriculture and horticulture. In the near future, molecular plant breeding will complement classical breeding, with its obvious shortcomings, for the

improvement of commercial species, and many *rol*-transformed species may prove useful in breeding programs to generate ornamentals with new and improved traits.

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References

Allavena A, Giovannini A, Berio T, Spena A, Zottini M, Accotto GP, et al. Genetic engineering of *Osteospermum* spp: a case story The 19th International Symposium on Improvement of Ornamental Plants. Acta Hortic 2000;508:129–33.

Altamura MM. *Agrobacterium rhizogenes rolB* and *rolD* genes: regulation and involvement in plant development. Plant Cell, Tissue Organ Cult 2004;77:89–101.

Altamura MM, Capitani F, Gazza L, Capone I, Costantino P. The plant oncogene *rolB* stimulates the formation of flower and root meristemoids in tobacco thin cell layers. New Phytol 1994;126:283–93.

Altamura MM, D'Angeli S, Capitani F. The protein of *rolB* gene enhances shoot formation in tobacco leaf explants and thin cell layers from plants in different physiological stages. J Exp Bot 1998;49:1139–46.

Angenent GC, Colombo L. Molecular control of ovule development. Trends Plant Sci 1996;1:228–32.

Banerjee S, Rahman L, Uniyal GC, Ahuja PS. Enhanced production of valepotriates by *Agrobacterium rhizogenes* induced hairy root cultures of *Valeriana wallichii* DC. Plant Sci 1998;131:203–8.

Banfield MJ, Brady RL. The structure of *Antirrhinum* centroradialis protein (CEN) suggests a role as a kinase regulator. J Mol Biol 2000;297:1159–70.

Bell RL, Scorza R, Srinivasan C, Webb K. Transformation of "Beurre Bosc" pear with the *rolC* gene. J Am Soc Hortic Sci 1999;124:570–4.

Ben-Meir H, Zuker A, Weiss D, Vainstein A. Molecular control of floral pigmentation: Anthocyanins. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 253–72.

Bettini P, Michelotti S, Bindi D, Giannini R, Capuana M, Buiatti M. Pleiotropic effect of the insertion of the *Agrobacterium rhizogenes rolD* gene in tomato (*Lycopersicon esculentum* Mill). Theor Appl Genet 2003;107:831–6.

Binns AN, Costantino P. The Agrobacterium oncogenes. In: Spaink H, Kondorosi A, Hooykaas PJJ, editors. The Rhizobiaceae. Dordrecht: Kluwer Press; 1998. p. 251–66.

Boase MR, Winefield CS, Lill TA, Bendall MJ. Transgenic regal pelargoniums that express the *rolC* gene from *Agrobacterium rhizogenes* exhibit a dwarf floral and vegetative phenotype. In Vitro Cell Dev Biol 2004;40:46–50.

Bovy AG, Angenent GC, Dons HJM, van Altvorst AC. Heterologous expression of the *Arabidopsis etr1-1* allele inhibits the senescence of carnation flowers. Mol Breed 1999;5:301–8.

Budar F, Deboeck F, van Montagu M, Hernalsteens JP. Introduction and expression of the octopine T-DNA oncogenes in tobacco plants and their progeny. Plant Sci 1986;46:195–206.

Bulgakov VP, Khodakovskaya MV, Labetskaya NV, Chernoded GK, Zhuravlev YN. The impact of plant *rolC* oncogene on ginsenoside production by gingseng hairy root cultures. Phytochemistry 1998;49:1929–34.

Capone I, Cardarelli M, Trovato M, Costantino P. Upstream non-coding region which confers polar expression to Ri plasmid root inducing gene *rolB*. Mol Gen Genet 1989;216:239–44.

Cardarelli M, Mariotti D, Pomponi M, Spanò L, Capone I, Costantino P. *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. Mol Gen Genet 1987a;209:475–80.

Cardarelli M, Spanò L, Mariotti D, Mauro L, van Sluys MA, Costantino P. The role of auxin in hairy root induction. Mol Gen Genet 1987b;208:457–63.

Casanova E, Zuker A, Trillas MI, Moysset L, Vainstein A. The *rolC* gene in carnation exhibits cytokinin- and auxin-like activities. Sci Hortic 2003;97:321–31.

Casanova E, Valdés AE, Zuker A, Fernández B, Vainstein A, Trillas MI, et al. *rolC*-transgenic carnation plants: adventitious organogenesis and levels of endogenous auxin and cytokinins. Plant Sci 2004;167:551–60.

Cassells AC. Tissue culture for ornamental breeding. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 139–53.

Choi PS, Kim YD, Choi KM, Chung HJ, Choi DW, Liu JR. Plant regeneration from hairy-root cultures transformed by infection with *Agrobacterium rhizogenes* in *Catharanthus roseus*. Plant Cell Rep 2004;22:828–31.

Christey MC. Transgenic crop plants using *Agrobacterium rhizogenes* mediated transformation. In: Doran PM, editor. Hairy roots: culture and applications. Amsterdam: Harwood Academic Publishers; 1997. p. 99–111.

Christey MC. Use of Ri-mediated transformation for production of transgenic plants. In Vitro Cell Dev Biol 2001;37:687–700.

Coen ES, Meyerowitz EM. The war of the whorls: genetic interactions controlling flower development. Nature 1991;353:31–7.

Cui M, Takayanagi K, Kamada H, Nishimura S, Handa T. Transformation of *Antirrhinum majus* L by a *rol*-type multi-auto-transformation (MAT) vector system. Plant Sci 2000;159:273–80.

Cui M, Takayanagi K, Kamada H, Nishimura S, Handa T. Efficient shoot regeneration from hairy roots of *Antirrhinum majus* L transformed by the *rol* type MAT vector system. Plant Cell Rep 2001;20:55–9.

Cunningham FX, Gantt E. Molecular control of floral pigmentation: Carotenoids. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 273–93.

Daimon H, Mii M. Plant regeneration and tiophene production in hairy root cultures of *Rudbeckia hirta* L used as an antagonist plant to nematodes. Jpn J Crop Sci 1995;64:650–5.

Dehio C, Grossmann K, Schell J, Schmülling T. Phenotype and hormonal status of transgenic tobacco plants overexpressing the *rolA* gene of *Agrobacterium rhizogenes* T-DNA. Plant Mol Biol 1993;23:1199–210.

Deroles SC, Boase MR, Lee CE, Peters TA. Gene transfer to plants. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 155–96.

Dudareva N. Molecular control of floral fragance. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 295–309.

Dudareva N, Murfitt LM, Mann CJ, Gorenstein N, Kolosova N, Kish CM, et al. Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers. Plant Cell 2000;12:949–61.

Dudareva N, Martin D, Kish CM, Kolosova N, Gorenstein N, Fäldt J, et al. (E)-β-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell 2003;15:1227–41.

Esposito S, Colucci MG, Frusciante L, Filippone E, Lorito M, Bressan RA. Antifungal transgenes expression in *Petunia hybrida*. The 19th International Symposium on Improvement of Ornamental Plants. Acta Hortic 2000;508:157–61.

Estruch JJ, Chriqui D, Grossmann K, Schell J, Spena A. The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates. EMBO J 1991a;10:2889–95.

Estruch JJ, Parets-Soler A, Schmülling T, Spena A. Cytosolic localization in transgenic plants of the *rolC* peptide from *Agrobacterium rhizogenes*. Plant Mol Biol 1991b;17:547–50.

Estruch JJ, Schell J, Spena A. The protein encoded by *rolB* plant oncogene hydrolyses indole glucosides. EMBO J 1991c;10:3125–8.

Faiss M, Strnad M, Redig P, Dolezal K, Hanus J, van Onckelen H, et al. Chemically induced expression of the *rolC*-encoded β-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: *rolC* does not hydrolyze endogenous cytokinin glucosides in planta. Plant J 1996;10:33–46.

Filippini F, Lo Schiavo F, Terzi M, Costantino P, Trovato M. The plant oncogene *rolB* alters binding of auxin to plant cell membranes. Plant Cell Physiol 1994;35:767–71.

Filippini F, Rossi V, Marin O, Trovato M, Costantino P, Downey PM, et al. A plant oncogene as a phosphatase. Nature 1996;379:499–500.

Fladung M. Transformation of diploid and tetraploid potato clones with the *rolC* gene of *Agrobacterium rhizogenes* and characterization of transgenic plants. Plant Breed 1990;104:295–304.

Fladung M, Grossmann K, Ahuja MR. Alterations in hormonal and developmental characteristics in transgenic *Populus* conditioned by the *rolC* gene from *Agrobacterium rhizogenes*. J Plant Physiol 1997;150:420–7.

Fukui Y, Tanaka N, Kusumi T, Iwashita T, Nomoto K. A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3′,5′-hydroxylase gene. Phytochemistry 2003;63:15–23.

Furner IJ, Huffman GA, Amasino RM, Garfinkel DJ, Gordon MP, Ester EW. An *Agrobacterium* transformation in the evolution of the genus *Nicotiana*. Nature 1986;319:422–7.

Gaudin V, Vrain T, Jouanin L. Bacterial genes modifying hormonal balances in plants. Plant Physiol Biochem 1994;32:11–29.

Geng S, Ma M, Ye H-C, Liu B-Y, Li G-F, Chong K. Effects of *ipt* gene expression on the physiological and chemical characteristics of *Artemisa annua* L. Plant Sci 2001;160:691–8.

Giovannini A, Pecchioni N, Allavena A. Genetic transformation of lisianthus (*Eustoma grandiflorum* Griseb) by *Agrobacterium rhizogenes*. J Genet Breed 1996;50:33–40.

Giovannini A, Pecchioni N, Rabaglio M, Allavena A. Characterization of ornamental *Datura* plants transformed by *Agrobacterium rhizogenes*. In Vitro Cell Dev Biol 1997;33:101–6.

Giovannini A, Mascarello C, Allavena A. Effects of *rol* genes on flowering in *Osteospermum ecklonis*. Flower Newsl 1999a;28:49–53.

Giovannini A, Zottini M, Morreale G, Spena A, Allavena A. Ornamental traits modification by *rol* genes in *Osteospermum ecklonis* transformed with *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol 1999b;35:70–5.

Godo T, Tsujii O, Ishikawa K, Mii M. Fertile transgenic plants of *Nierembergia scoparia* Sendtner obtained by a mikimopine type strain of *Agrobacterium rhizogenes*. Sci Hortic 1997;68:101–11.

Gubrium EK, Clevenger DJ, Clark DG, Barrett JE, Nell TA. Reproduction and horticultural perfomance of transgenic ethylene-insensitive petunias. J Am Soc Hortic Sci 2000;125:277–81.

Guivarc'h A, Spena A, Noin M, Besnard C, Chriqui D. The pleiotropic effects induced by the *rolC* gene in transgenic plants are caused by expression restricted to protophloem and companion cells. Transgenic Res 1996;5:3–11.

Handa T. Genetic transformation of *Antirrhinum majus* L and inheritance altered phenotype induced by Ri T-DNA. Plant Sci 1992a;81:199–206.

Handa T. Regeneration and characterization of praire gentian (*Eustoma grandiflorum*) plants transformed by *Agrobacterium rhizogenes*. Plant Tissue Cult Lett 1992b;9:10–4.

Handa T, Deroles S. Transgenic *Eustoma grandiflorum* (Lisianthus). In: Bajaj YPS, editor. Transgenic Crops III Biotechnology in agriculture and forestry, vol. 48. Berlin: Springer-Verlag; 2001. p. 107–22.

Handa T, Sugimura T, Kato E, Kamada H, Takayanagi K. Genetic transformation of *Eustoma grandiflorum* with *rol* genes. Genetic Improvement of Horticultural Crops by Biotechnology. Acta Hortic 1995;392:209–18.

Hatta M, Beyl CA, Garton S, Diner AM. Induction of roots on jujube softwood cuttings using *Agrobacterium rhizogenes*. J Hortic Sci 1996;71:881–6.

Horn W. Breeding methods and breeding research. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 47–83.

Hoshino Y, Mii M. Bialaphos stimulatess shoot regeneration from hairy roots of snapdragon (*Antirrhinum majus* L) transformed by *Agrobacterium rhizogenes*. Plant Cell Rep 1998;17:256–61.

Hosokawa K, Matsuki R, Oikawa Y, Yamamura S. Genetic transformation of gentian using wild-type *Agrobacterium rhizogenes*. Plant Cell, Tissue Organ Cult 1997;51:137–40.

Jandrew J, Clark DG. Selectively induced nutrient deficiency in transgenic P_{SAG12}-*IPT*, P_{SAG13}-*IPT* and P_{SAG12}-*Kn1* petunias. HortScience 2001;36:518–9.

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey K-M. Enhacement of scopolamine production in *Hyoscyamus muticus* L hairy root cultures by genetic engineering. Planta 1999;208:545–51.

Kakimoto T. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. Plant Cell Physiol 2001;42:677–85.

Kamo K, Hammond J, Roh M. Transformation of *Gladiolus* for disease resistance. J Kor Soc Hort Sci 1997;38: 188–193.

Kaneyoshi J, Kobayashi S. Characteristics of transgenic trifoliate orange (*Poncirus trifoliata* Raf) possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid. J Jpn Soc Hortic Sci 1999;68:734–8.

Kiyokawa S, Kikuchi Y, Kamada H, Harada H. Genetic transformation of *Begonia tuberhybrida* by Ri *rol* genes. Plant Cell Rep 1996;15:606–9.

Klee HJ, Horsch RB, Hinchee MA, Hein MB, Hoffmann NL. The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Genes Dev 1987;1:86–96.

Knudsen JT, Tollsten L, Bergström G. Floral scents—a check list of volatile compounds isolated by head-space techniques. Phytochemistry 1993;33:253–80.

Koike Y, Hoshino Y, Mii M, Nakano M. Horticultural characterization of *Angelonia salicariifolia* plants transformed with wild-type strains of *Agrobacterium rhizogenes*. Plant Cell Rep 2003;21:981–7.

Koornneef M, Stam P. Changing the paradigms in plant breeding. Plant Physiol 2001;125:156–9.

Koshita Y, Nakamura Y, Kobayashi S, Morinaga K. Introduction of the *rolC* gene into the genome of the Japanese persimmon causes dwarfism. J Jpn Soc Hortic Sci 2002;71:529–31.

Kosugi Y, Waki K, Iwazaki Y, Tsuruno N, Mochizuki A, Yoshioka T, et al. Senescence and gene expression of transgenic non-ethylene-producing carnation flowers. J Jpn Soc Hortic Sci 2002;71:638–42.

Kurioka Y, Suzuki Y, Kamada H, Harada H. Promotion of flowering and morphological alterations in *Atropa belladonna* transformed with a CaMV 35S-*rolC* chimeric gene of the Ri plasmid. Plant Cell Rep 1992;12:1–6.

Lavy M, Zuker A, Lewinsohn E, Larkov O, Ravid U, Vainstein A, et al. Linalool and linalool oxide production in transgenic carnation flowers expressing the *Clarkia breweri* linalool synthase gene. Mol Breed 2002;9:103–11.

Lee C, Wang L, Ke S, Qin M, Cheng Z-M. Expression of the *rolC* gene in transgenic plants of *Salpiglossis sinuata* L. HortScience 1996;31:571.

Limami MA, Sun L-Y, Douat C, Helgeson J, Tepfer D. Natural genetic transformation by *Agrobacterium rhizogenes*. Plant Physiol 1998;118:543–50.

Lorito M, Del Sorbo G, Scala F. Molecular approaches for increasing plant resistance to biotic and abiotic stresses. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 197–218.

Lücker J, Bouwmeester HJ, Schwab W, Blaas J, van der Plas LHW, Verhoeven HA. Expression of *Clarkia* S-linalool synthase in transgenic petunia plants results in the accumulation of S-linalyl-β-D-glucopyranoside. Plant J 2001;27:315–24.

Ma H. To be, or not to be, a flower—control of floral meristem identity. Trends Genet 1998;14:26–32.

Ma H, dePamphilis C. The ABCs of floral evolution. Cell 2000;101:5–8.

Machácková I, Sergeeva L, Ondrej M, Zaltsman O, Konstantinova T, Eder J, et al. Growth pattern, tuber formation and hormonal balance in in vitro potato plants carrying *ipt* gene. Plant Growth Regul 1997;21:27–36.

Marchant R, Davey MR, Lucas JA, Lamb CJ, Dixon RA, Power JB. Expression of a chitinase transgene in rose (*Rosa hybrida* L) reduces development of blackspot disease (*Diplocarpon rosae* Wolf). Mol Breed 1998;4:187–94.

Martin GB, Bogdanove AJ, Sessa G. Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 2003;54:23–61.

Martin-Tanguy J, Corbineau F, Burtin D, Ben-Hayyim G, Tepfer D. Genetic transformation with a derivative of *rolC* from *Agrobacterium rhizogenes* and treatment with α-aminoisobutyric acid produce similar phenotypes and reduce ethylene production and the accumulation of water-insoluble polyamine-hydroxycinnamic acid conjugates in tobacco flowers. Plant Sci 1993;93:63–76.

Martin-Tanguy J, Sun L-Y, Burtin D, Vernoy R, Rossin N, Tepfer D. Attenuation of the phenotype caused by the root-inducing, left-hand, transferred DNA and its *rolA* gene. Plant Physiol 1996;111:259–67.

Maurel C, Barbier-Brygoo H, Spena A, Tempé J, Guern J. Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. Plant Physiol 1991;97:212–6.

Maurel C, Leblanc N, Barbier-Brygoo H, Perrot-Rechenmann C, Bouvier-Durand M, Guern J. Alterations of auxin perception in *rolB*-transformed tobacco protoplasts. Plant Physiol 1994;105:1209–15.

Mauro ML, Trovato M, De Paolis A, Gallelli A, Costantino P, Altamura MM. The plant oncogene *rolD* stimulates flowering in transgenic tobacco plants. Dev Biol 1996;180:693–700.

Mercuri A, Bruna S, De Benedetti L, Burchi G, Schiva T. Modification of plant architechture in *Limonium* spp induced by *rol* genes. Plant Cell, Tissue Organ Cult 2001;65:247–53.

Mercuri A, Anfosso L, Burchi G, Bruna S, De Benedetti L, Schiva T. *Rol* genes and new genotypes of *Limonium gmelinii* through *Agrobacterium*-mediated transformation. The 26th International Horticultural Congress: Elegant Science in Floriculture. Acta Hortic 2003a;624:455–62.

Mercuri A, De Benedetti L, Bruna S, Bregliano R, Bianchini C, Foglia G, et al. *Agrobacterium*-mediated transformation with *rol* genes of *Lilium longiflorum* Thunb. The 21th International Symposium on Classical versus Molecular Breeding of Ornamentals. Acta Hortic 2003b;612:129–36.

Meyer P, Heidmann I, Forkmann G, Saedler H. A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature 1987;330:677–8.

Meyer AD, Tempé J, Costantino P. Hairy root: A molecular overview. Functional analysis of *Agrobacterium rhizogenes* T-DNA genes. In: Stacey G, Keen NT, editors. Plant-microbe interactions. St. Paul: APS Press; 2000. p. 93–139.

Michael T, Spena A. Plant oncogenes *rolA*, *B*, and *C* from *Agrobacterium rhizogenes*. In: Gartland KMA, Davey MR, editors. *Agrobacterium* protocols. Methods Mol Biol, vol. 44. Totowa: Humana Press; 1995. p. 207–22.

Mitiouchkina TY, Dolgov SV. Modification of chrysanthemum flower and plant architecture by *rolC* gene from *Agrobacterium rhizogenes* introduction. The 19th International Symposium on Improvement of Ornamental Plants. Acta Hortic 2000;508:163–9.

Mol JNM, Holton TA, Koes RE. Floriculture: genetic engineering of commercial traits. Trends Biotechnol 1995;13:350–5.

Momcilovic I, Grubisic D, Kojic M, Neskovic M. *Agrobacterium rhizogenes*-mediated transformation and plant regeneration of four *Gentiana* species. Plant Cell, Tissue Organ Cult 1997;50:1–6.

Morandini P, Salamini F. Plant biotechnology and breeding: allied for years to come. Trends Plant Sci 2003;8:70–5.

Mouradov A, Cremer F, Coupland G. Control of flowering time: interacting pathways as a basis for diversity. Plant Cell 2002;S111–S130 [Supplement].

Müller R, Stummann BM, Serek M. Characterization of an ethylene receptor family with differential expression in rose (*Rosa hybrida* L) flowers. Plant Cell Rep 2000;19:1232–9.

Nielsen K, Deroles S, Markham KR, Bradley MJ, Podivinsky E, Manson D. Antisense flavonol synthase alters copigmentation and flower color in lisianthus. Mol Breed 2002;9:217–29.

Nilsson O, Olsson O. Getting to the root: The role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol Plant 1997;100:463–73.

Nilsson O, Crozier A, Schmülling T, Sandberg G, Olsson O. Indole-3-acetic acid homeostasis in transgenic tobacco plants expressing the *Agrobacterium rhizogenes rolB* gene. Plant J 1993a;3:681–9.

Nilsson O, Moritz T, Imbault N, Sandberg G, Olsson O. Hormonal characterization of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* TL-DNA. Plant Physiol 1993b;102:363–71.

Nilsson O, Little CHA, Sandberg G, Olsson O. Expression of two heterologous promoters, *Agrobacterium rhizogenes rolC* and cauliflower mosaic virus 35S, in the stem of transgenic hybrid aspen plants during the annual cycle of growth and dormancy. Plant Mol Biol 1996a;31:887–95.

Nilsson O, Moritz T, Sundberg B, Sandberg G, Olsson O. Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fasciation. Plant Physiol 1996b;112:493–502.

Oono Y, Kanaya K, Uchimiya H. Early flowering in transgenic tobacco plants possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid. Jpn J Genet 1990;65:7–16.

Otani M, Shimada T, Kamada H, Teruya H, Mii M. Fertile transgenic plants of *Ipomoea trichocarpa* Ell induced by different strains of *Agrobacterium rhizogenes*. Plant Sci 1996;116:169–75.

Ovadis M, Zuker A, Tzfira T, Ahroni A, Shklarman E, Scovel G, et al. Generation of transgenic carnation plants with novel characteristics by combining microprojectile bombardment with *Agrobacterium tumefaciens* transformation. In: Altman A, Izhar S, Ziv M, editors. Plant biotechnology and in vitro biology in the 21st century. Dordrecht: Kluwer Academic Publishers; 1999. p. 189–92.

Palazón J, Cusidó RM, Roig C, Piñol MT. Expression of the *rolC* gene and nicotine production in transgenic roots and their regenerated plants. Plant Cell Rep 1998;17:384–90.

Pawlicki-Jullian N, Sedira M, Welander M. The use of *Agrobacterium rhizogenes* transformed roots to obtain transgenic shoots of the apple rootstock Jork 9. Plant Cell, Tissue Organ Cult 2002;70:163–71.

Pellegrineschi A, Damon J-P, Valtorta N, Paillard N, Tepfer D. Improvement of ornamental characters and fragrance production in lemon-scented geranium through genetic transformation by *Agrobacterium rhizogenes*. Bio/Technology 1994;12:64–8.

Pellegrineschi A, Davolio-Mariani O. *Agrobacterium rhizogenes*-mediated transformation of scented geranium. Plant Cell, Tissue Organ Cult 1996;47:79–86.

Puddephat IJ, Robinson HT, Fenning TM, Barbara DJ, Morton A, Pink DAC. Recovery of phenotypically normal transgenic plants of *Brassica oleracea* upon *Agrobacterium rhizogenes*-mediated co-transformation and selection of transformed hairy roots by GUS assay. Mol Breed 2001;7:229–42.

Röder F, Schmülling T, Gatz C. Efficiency of the tetracycline-dependent gene expression system: complete suppression and efficient induction of the *rolB* phenotype in transgenic plants. Mol Gen Genet 1994;243:32–8.

Savin KW, Baunidette SC, Graham MW, Michael MZ, Nugent GD, Lu C-Y, et al. Antisense ACC oxidase RNA delays carnation petal senescence. HortScience 1995;30:970–2.

Schell J, First NL, Vasil IK. Prospects and limitations of agricultural biotechnologies: An update. In: Altman A, editor. Agricultural biotechnology. New York: Marcel Dekker; 1998. p. 743–8.

Schmülling T, Schell J, Spena A. Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J 1988;7:2621–9.

Schmülling T, Beinsberger S, De Greef J, Schell J, van Onckelen H, Spena A. Construction of a heat-inducible chimaeric gene to increase the cytokinin content in transgenic plant tissue. FEBS Lett 1989;249:401–6.

Schmülling T, Fladung M, Grossmann K, Schell J. Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. Plant J 1993;3:371–82.

Schuler TH, Poppy GM, Kerry BR, Denholm I. Insect-resistant transgenic plants. Trends Biotechnol 1998;16:168–75.

Schwacke R, Grallath S, Breitkreuz KE, Stransky E, Stransky H, Frommer WB, et al. LePro T1, a transporter for proline, glycine betaine, and γ-amino butyric acid in tomato pollen. Plant Cell 1999;11:377–91.

Scorza R, Zimmerman TW, Cordts JM, Footen KJ, Ravelonandro M. Horticultural characteristics of transgenic tobacco expressing the *rolC* gene from *Agrobacterium rhizogenes*. J Am Soc Hortic Sci 1994;119:1091–8.

Senior I, Holford P, Cooley RN, Newbury HJ. Transformation of *Antirrhinum majus* using *Agrobacterium rhizogenes*. J Exp Bot 1995;46:1233–9.

Sergeeva LI, de Bruijn SM, Koot-Gronsveld EAM, Navratil O, Vreugdenhil D. Tuber morphology and starch accumulation are independent phenomena: Evidence from *ipt*-transgenic potato lines. Physiol Plant 2000;108:435–43.

Sevón N, Oksman-Caldentey K-M. *Agrobacterium rhizogenes*-mediated transformation: Root cultures as source of alkaloids. Planta Med 2002;68:859–68.

Shen WH, Davioud E, David C, Barbier-Brygoo H, Tempé J, Guern J. High sensitivity to auxin is a common feature of hairy root. Plant Physiol 1990;94:554–60.

Shibuya K, Nagata M, Tanikawa N, Yoshioka T, Hashiba T, Satoh S. Comparison of mRNA levels of three ethylene receptors in senescing flowers of carnation (*Dianthus caryophyllus* L). J Exp Bot 2002;53:399–406.

Sinkar VP, Pythoud F, White FF, Nester EW, Gordon MP. *rolA* locus of the Ri plasmid directs developmental abnormalities in transgenic tobacco plants. Genes Dev 1988;2:688–97.

Sitbon F, Sundberg B, Olsson O, Sandberg G. Free and conjugated indoleacetic acid (IAA) contents in transgenic tobacco plants expressing the *iaaM* and *iaaH* IAA biosynthesis genes from *Agrobacterium tumefaciens*. Plant Physiol 1991;95:480–5.

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G. Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. Plant Physiol 1992a;99:1062–9.

Sitbon F, Little CHA, Olsson O, Sandberg G. Correlation between the expression of T-DNA IAA biosynthetic genes from developmentally regulated promoters and the distribution of IAA in different organs of transgenic tobacco. Physiol Plant 1992b;85:679–88.

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D. Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J Biol Chem 1986;261:108–21.

Smart CM, Scofield SR, Bevan MW, Dyer TA. Delayed leaf senescence in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium*. Plant Cell 1991;3:647–56.

Smigocki A, Owens LD. Cytokinin-to-auxin ratios and morphology of shoots and tissues transformed by a chimeric isopentenyl transferase gene. Plant Physiol 1989;91:808–11.

Soltis DE, Soltis PS, Albert VA, Oppenheimer DG, dePamphilis CW, Ma H, et al. Missing links: the genetic architecture of flower and floral diversification. Trends Plant Sci 2002;7:22–31.

Souq F, Coutos-Thevenot P, Yean H, Delbard G, Maziere Y, Barbe JP, et al. Genetic transformation of roses, 2 examples: one on morphogenesis, the other on anthocyanin biosynthetic pathway. Second International Symposium on Roses. Acta Hortic 1996;424:381–8.

Spanò L, Mariotti D, Cardarelli M, Branca C, Costantino P. Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. Plant Physiol 1988;87:479–83.

Spena A, Schmülling T, Koncz C, Schell J. Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants. EMBO J 1987;6:3891–9.

Stearns JC, Glick BR. Transgenic plants with altered ethylene biosynthesis or perception. Biotechnol Adv 2003;21:193–210.

Stummer BE, Smith SE, Langridge P. Genetic transformation of *Verticordia grandis* (Myrtaceae) using wild-type *Agrobacterium rhizogenes* and binary *Agrobacterium* vectors. Plant Sci 1995;111:51–62.

Suginuma C, Akihama T. Transformation of gentian with *Agrobacterium rhizogenes.* Genetic Improvement of Horticultural Crops by Biotechnology. Acta Hortic 1995;392:153–60.

Sun L-Y, Monneuse M-O, Martin-Tanguy J, Tepfer D. Changes in flowering and the accumulation of polyamines and hydroxycinnamic acid-polyamine conjugates in tobacco plants transformed by the *rolA* locus from the Ri TL-DNA of *Agrobacterium rhizogenes*. Plant Sci 1991a;80:145–56.

Sun L-Y, Touraud G, Charbonnier C, Tepfer D. Modification of phenotype in Belgian endive (*Cichorium intybus*) through genetic transformation by *Agrobacterium rhizogenes*: conversion from biennal to annual flowering. Transgenic Res 1991b;1:14–22.

Sung ZR, Chen L, Moon Y-H, Lertpiriyapong K. Mechanisms of floral repression in *Arabidopsis*. Curr Opin Plant Biol 2003;6:29–35.

Takei K, Sakakibara H, Sugiyama T. Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J Biol Chem 2001;276:26405–10.

Tepfer D. Transformation of several species of higher plants by *Agrobacterium rhizogenes*: Sexual transmission of the transformed genotype and phenotype. Cell 1984;37:959–67.

Thompson JE, Wang T-W. Molecular genetics of flower senescence. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 311–27.

Trovato M, Maras B, Linhares F, Costantino P. The plant oncogene *rolD* encodes a functional ornithine cyclodeaminase. Proc Natl Acad Sci U S A 2001;98:13449–53.

Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002.

van Altvorst AC, Bino RJ, van Dijk AJ, Lamers AMJ, Lindhout WH, van der Mark F, et al. Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. Plant Sci 1992;83:77–85.

van der Krol AR, Chua N-H. Flower development in petunia. Plant Cell 1993;5:1195–203.

van der Salm TPM, Hänish ten Cate CH, Dons HJM. Prospects for applications of *rol* genes for crop improvement. Plant Mol Biol Report 1996;14:207–28.

van der Salm TPM, van der Toorn CJG, Bouwer R, Hänisch ten Cate CH, Dons HJM. Production of *rol* gene transformed plants of *Rosa hybrida* L and characterization of their rooting ability. Mol Breed 1997;3:39–47.

van der Salm TPM, Bouwer R, van Dijk AJ, Keizer LCP, Hänisch ten Cate CH, van der Plas LHW, et al. Stimulation of scion bud release by *rol* gene transformed rootstocks of *Rosa hybrida* L. J Exp Bot 1998;49:847–52.

Vishnevetsky M, Meyerowitz EM. Molecular control of flower development. In: Vainstein A, editor. Breeding for ornamentals: classical and molecular approaches. Dordrecht: Kluwer Academic Publishers; 2002. p. 239–52.

Wang J, Letham DS, Cornish E, Stevenson KR. Studies of cytokinin action and metabolism using tobacco plants expressing either the *ipt* or the *GUS* gene controlled by a chalcone synthase promoter I Developmental features of the transgenic plants. Aust J Plant Physiol 1997;24:661–72.

Webster MA, Gilmartin PM. A comparison of early floral ontogeny in wild-type and floral homeotic mutant phenotypes of *Primula*. Planta 2003;216:903–17.

White FF, Taylor BH, Huffman GA, Gordon MP, Nester EW. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J Bacteriol 1985;164:33–44.

Winefield C, Lewis D, Arathoon S, Deroles S. Alterations of *Petunia* plant form through the introduction of the *rolC* gene from *Agrobacterium rhizogenes*. Mol Breed 1999;5:543–51.

Yokoyama R, Hirose T, Fujii N, Aspuria ET, Kato A, Uchimiya H. The *rolC* promoter of *Agrobacterium rhizogenes* Ri plasmid is activated by sucrose in transgenic tobacco plants. Mol Gen Genet 1994;244:15–22.

Yu D, Kotilainen M, Pöllänen E, Mehto M, Elomaa P, Helariutta Y, et al. Organ identity genes and modified patterns of flower development in *Gerbera hybrida* (Asteraceae). Plant J 1999;17:51–62.

Zik M, Irish VF. Flower development: Initiation, differentiation, and diversification. Annu Rev Cell Dev Biol 2003;19:119–40.

Zuker A, Tzfira T, Vainstein A. Genetic engineering for cut-flower improvement. Biotechnol Adv 1998;16:33–79.

Zuker A, Tzfira T, Scovel G, Ovadis M, Shklarman E, Itzhaki H, et al. *rolC*-transgenic carnation with improved agronomic traits: Quantitative and qualitative analyses of greenhouse-grown plants. J Am Soc Hortic Sci 2001;126:13–8.

Zuker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, Itzhaki H, et al. Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Mol Breed 2002;9:33–41.

RESUM DELS RESULTATS I DISCUSSIÓ

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1. Relacions genètiques entre els cultivars de clavell estudiats

Els cultivars de l'espècie *D. caryophyllus* L. (White Sim, Red Sim, Pallas, Bianca Neve i Early Sam) han quedat classificats genèticament en un sol grup, sense cap divisió entre el classificat fenotípicament com a *spray* (Early Sam) i els classificats com a estàndards. Així, les anàlisis RAPD indiquen que els clavells estàndard i els *spray* no són dues línies genètiques. Dins del grup dels *D. caryophyllus*, els clavells Sim s'han reflectit com els més propers genèticament, ja que pertanyen tots a una línia genètica amb variacions principalment en els gens que afecten la pigmentació de la flor (Holley i Baker 1963). El cultivar Pulcino, tot i ser un híbrid interespecífic, ha quedat classificat com a molt proper genèticament al Pallas, dins del grup dels *D. caryophyllus*, ja que probablement en algun moment del procés d'obtenció s'hagi creuat amb el Pallas per heredar-ne o estabilitzar un color tan difícil d'obtenir en clavell com és el groc. Les classificacions basades en el fenotip, doncs, no corresponen sempre amb classificacions genètiques, tal com també s'ha demostrat amb anàlisis RFLP (*restriction fragment length polymorphism*) amb altres cultivars del tipus mediterrani i *spray* (Vainstein et al. 1991). Els híbrids interespecífics Inorsa R-24 i Mei Ling han quedat separats genèticament del grup *D. caryophyllus* i entre ells estan tant separats com ho estan del grup *D. caryophyllus*, a causa de l'origen d'ambdós cultivars. Anàlisis RFLP d'altres híbrids interespecífics també reflecteix que aquests, generalment classificats en la categoria fenotípica de plantes nanes o plantes de test, són els que mostren la menor similaritat genètica tant entre si com amb els cultivars de les categories de *D. caryophyllus* (Vainstein et al. 1991).

2. Efecte del genotip i l'explant en l'organogènesi adventícia del clavell

En l'organogènesi *in vitro* del clavell s'ha trobat un efecte del genotip, l'explant, el medi de cultiu i les condicions de cultiu. L'organogènesi adventícia s'ha avaluat mesurant el percentatge d'explants que han donat organogènesi, i el nombre d'òrgans (tiges o rels) per cada explant regeneratiu. Aquestes dues variables quasi sempre van lligades, ja que augmenten i disminueixen en paral·lel. Així, la capacitat organogènica o de regeneració d'òrgans s'ha representat en una sola variable global, que seria el nombre d'òrgans regenerats per cada explant cultivat, fent un promig entre els regeneratius i els no regeneratius.

El genotip és determinant el l'organogènesi adventícia obtinguda. El fet que els tres híbrids interespecífics, el cultivar Mei Ling, l'Inorsa R-24 i el Pulcino, siguin els que menys capacitat regenerativa tenen no ens permet correlacionar aquestes dues característiques, ja que necessitaríem estudiar molts més cutivars per poder-ho afirmar. En concordança, l'estudi d'altres híbrids interespecífics no ha donat regeneració a partir de pètals i només un grau

de regeneració molt reduït en fulla (van Altvorst et al. 1992b). En les nostres condicions experimentals, el cultivar White Sim ha estat el més regeneratiu tant en explants de pètals com de fulles, seguit de l'Early Sam, amb una menor capacitat de regenerar tiges i rels en ambdós explants. Altres estudis amb explants de pètals de cultivars del grup Sim també han mostrat que tenen el major potencial organogènic, però no les fulles (van Altvorst et al. 1992b). L'elevada capacitat regenerativa dels explants de White Sim pot fins i tot incrementar-se quan plantes d'aquest cultivar es transformen amb el gen *rolC* (veieu apartat 5.1, p.163).

En els cultivars White Sim i Early Sam, les fulles tendeixen a ser lleugerament més regeneratives que els pètals, ja que han regenerat tiges i rels en un medi sense reguladors del creixement. En el medi amb només NAA (0.5 μM), els pètals no han regenerat rels, mentre que les fulles, a part de regenerar algunes tiges, han format moltes rels. Trobem, doncs, un efecte de l'explant en la capacitat de regenerar tiges i rels, tal com ja s'havia descrit per altres cultivars de clavell (Frey i Janick 1991, van Altvorst et al. 1992b). Les fulles, però, han regenerat més tiges hiperhídriques, sobretot en presència de TDZ (0.5 μM). Aquest regulador del creixement tendeix a generar plàntules hiperhídriques en clavell (Genkov et al. 1997) i en altres espècies (Huetteman i Preece 1993, Kadota i Niimi 2003).

L'època de l'any també influeix en el potencial organogènic dels explants de pètals de clavell. En pètals del cultivar White Sim s'ha observat una regeneració de 2.8 tiges per pètal a l'hivern, de 4.6 tiges per pètal a la tardor i de 8.5 tiges per pètal a la primavera. La màxima regeneració a partir de pètals ha coincidit amb l'època de màxima floració tant en White Sim (estiu) com en Early Sam (primavera) (dades no publicades). Les variacions estacionals podrien ser degudes a variacions internes de les hormones o bé de la sensibilitat a aquestes, tot i que no podem descartar un efecte d'altres condicions experimentals del cultiu.

En els cultivars i explants estudiats, la regeneració es dóna a través de la formació directa d'òrgans a la base dels explants, ja que és la que conté més cèl·lules amb capacitat de divisió, d'acord amb els resultats de van Altvorst et al. (1992b, 1994). No obstant, en alguns pètals de White Sim la superfície organogènica s'ha expandit cap a parts més centrals del pètal (veieu Introducció, Figura 2), resultat que només s'havia observat en els mateixos pètals cultivats en medi líquid, en presència de BA (Fisher et al. 1993). El TDZ probablement hagi estat la causa d'aquest increment en la zona organogènica, però també podria ser causa de la interacció entre l'elevada activitat del TDZ com a CK i l'alta capacitat regenerativa del cultivar White Sim.

En explants de pètals de White Sim cultivats 3 dies en el medi amb 0.5 μM d'NAA i 0.5 μM de TDZ s'ha donat la desdiferenciació i l'inici de la divisió d'algunes cèl·lules de la zona adaxial epidèrmica i subepidèrmica, i esporàdicament en cèl·lules de prop dels vasos conductors. Altres autors descriuen que els primordis adventicis provenen bàsicament de la regió vascular (Frey i Janick 1991). Els primordis caulinars han començat a emergir de l'explant al 7è dia de cultiu en el medi esmentat. En medis amb menor concentració de TDZ (0.005 i 0.05 μM) aquest procés s'ha donat més lentament, i el 7è dia encara no s'han observat primordis. En aquests pètals de White Sim, la proporció de zones meristemàtiques el 7è dia de cultiu es correlaciona amb el nombre de tiges obtingudes al 30è dia de cultiu, així un estudi histològic al 7è dia permet predir la capacitat de regeneració de tiges d'aquests explants. Això ha fet que aquest dia es considerés clau en el procés organogènic en els pètals de clavell White Sim.

3. Efecte de l'ambient físic del cultiu en l'organogènesi adventícia del clavell i en el grau d'hiperhidricitat de les tiges regenerades

La regeneració de tiges a partir d'explants de pètals dels cultivars White Sim i Early Sam ha estat menor en els pètals cultivats en tubs ventilats que en els cultivats en tubs hermètics. A més, aquestes tiges han presentat un pes fresc, un pes sec i un contingut d'aigua també inferior a les tiges obtingudes en els tubs hermètics, en els que pràcticament no s'ha obtingut cap tija hiperhídrica degut a que el medi estava solidificat amb 8 g l^{-1} d'agar. Aquestes diferències es poden atribuir a la menor RH mesurada en els tubs ventilats. La disminució de la RH del cultiu en medis sòlids, ja sigui amb dessecants o per ventilació, ha reduït el nombre de plàntules de clavell hiperhídriques, i n'ha disminuït el seu creixement en els cultivars Ceris royallete i Sam's Pride (Ziv et al. 1983, Hakkaart i Versluijs 1983) i la taxa de propagació en els cultivars Nelken i Tanga (Majada et al. 1997). En plàntules de clavell, la ventilació augmenta la funcionalitat dels estomes i la concentració de ceres epicuticulars, millorant notablement la seva capacitat de supervivència *ex vitro* (Majada et al. 1998, 2001). Les plàntules de *Rosa* també han mostrat una reducció tant en el seu creixement com en la taxa de propagació en ventilar els pots de cultiu (Sallanon i Maziere 1992), mentre que en plàntules de *Brassica oleracea* o *Lycopersicon esculentum* no només n'ha disminuït la hiperhidricitat, sinó que n'ha augmentat el creixement (Zobayed et al. 1999, Mills i Tal 2004). En plàntules de clavell cultivades en medi líquid, en el que s'obté molta hiperhidricitat, la ventilació ha permès reduir la hiperhidricitat, tot mantenint la taxa de creixement i de propagació (Ziv et al. 1983, Majada et al. 1997).

La capacitat regenerativa dels pètals de White Sim ha disminuït 4 vegades amb l'increment de la concentració d'agar de 2 a 12 g l^{-1} en el medi, possiblement a causa de la reducció de l'aigua disponible, ja que el potencial matricial del medi de cultiu disminueix amb l'increment de la concentració d'agar (Smith i Spomer 1995). L'organogènesi adventícia en explants d'altres espècies també s'ha reduït amb l'increment de la concentració d'agent gelificant en el medi (Brown et al. 1979, Bornman i Vogelman 1984, Castro-Concha et al. 1990, Owens i Wozniak 1991), així com també la taxa de propagació de plàntules de clavell (Ziv et al. 1983, Yadav et al. 2003) i d'altres espècies (Debergh et al. 1981, Singha 1982, Mackay i Kitto 1988, Turner i Singha 1990) excepte en *Pyrus communis*, en el que la taxa de propagació incrementa (Singha 1982, Turner i Singha 1990). La capacitat organogènica dels pètals de White Sim ha estat superior en els medis sòlids (2, 4, 6 i 8 g l^{-1} d'agar) respecte al medi líquid, probablement degut a l'anòxia causada per la distribució irregular del medi líquid en la base del pot de cultiu. En pètals de clavell cultivats en 2 ml de medi líquid l'efecte en la regeneració ha estat el contrari (Fisher et al. 1993).

La hiperhidricitat de les tiges de clavell regenerades ha disminuït amb l'increment d'agar en el medi, de 0 a 12 g l^{-1}, amb un canvi significatiu entre els 4 g l^{-1} d'agar, on totes les tiges han estat hiperhídriques, i els 8 g l^{-1}, on la majoria han estat sanes. La reducció del nombre de plàntules hiperhídriques amb l'increment de l'agent gelificant també s'ha descrit en altres espècies, però en la majoria d'estudis es considera un caràcter qualitatiu que és assignat de manera visual (Debergh et al. 1981, Ziv et al. 1983, Bornman i Vogelman 1984, Mackay i Kitto 1988, Pasqualetto et al. 1988, Castro-Concha et al. 1990, Turner i Singha 1990, Yadav et al. 2003). La hiperhidricitat de les tiges de clavell regenerades ha estat quantificada pel seu percentatge d'aigua, que ha disminuït amb la reducció

d'aquesta anomalia. Plàntules hiperhídriques de clavell i altres espècies també solen tenir un major contingut d'aigua que les sanes (Kevers et al. 1984, Turner i Singha 1990, Genkov et al. 1997, Olmos et al. 1997). Paral·lelament, una reducció del pes fresc i el pes sec de les tiges, del nombre de capes de cèl·lules parenquimàtiques de les fulles i del tamany d'aquestes cèl·lules també ha indicat una disminució en el grau d'hiperhidricitat de les tiges adventícies de clavell. D'acord amb això, el tamany de les cèl·lules de fulles hiperhídriques dels cultivars Domingo i Nelken de clavell també ha estat més gran que el de les cèl·lules de fulles normals (Olmos i Hellín 1998, Majada et al. 2000). Aquesta hipertròfia de les cèl·lules provoca un creixement anormal dels teixits (Werker i Leshem 1987), probablement per un defecte en la lignificació de les parets cel·lulars, que permetria un increment en l'absorció d'aigua del medi (Gaspar et al. 1987, Olmos et al. 1997, Piqueras et al. 2002).

El fet que les fulles de clavells desenvolupades en el medi amb 12 g l^{-1} d'agar presentin un gran acúmul de grànuls de midó podria ser una resposta a condicions estressants, causades per la falta d'aigua, que causarien un endarreriment en la mobilització de fotoassimilats. D'acord amb això, plàntules de clavell del cultivar Nelken cultivades en recipients ventilats també han mostrat un acúmul de midó (Majada et al. 2000).

4. Implicació de les auxines i citocinines en l'organogènesi adventícia del clavell

El tipus de regulador del creixement del medi, el balanç i la seva concentració afecten de manera dràstica l'organogènesi adventícia del clavell. En explants de fulles de White Sim i Early Sam, el TDZ (0.5 μM) ha estat molt més efectiu que la BA, en la mateixa concentració, tant en la inducció de tiges com en la inhibició de la formació de rels. Aquesta gran efectivitat del TDZ respecte la BA s'havia demostrat en la inducció de l'organogènesi de tiges en pètals del cultivar de clavell Scania (Nakano et al. 1994), i també en altres espècies (Huetteman i Preece 1993, Mundhara i Rashid 2002, Vikrant i Rashid 2002, Sharma et al. 2004). El TDZ és especialment útil en el cultiu d'espècies llenyoses, força recalcitrants al cultiu *in vitro* (Huetteman i Preece 1993, Hosseini-Nasr i Rashid 2003, Singh et al. 2003, Ledbetter i Preece 2004, Haensch 2004). La major efectivitat d'aquest derivat de la fenilurea pot ser deguda a que la capacitat de l'explant de metabolitzar-lo és molt lenta (Mok i Mok 1985), mentre que la BA es conjuga ràpidament en els teixits (Feito et al. 1995, Centeno et al. 1998, 2003). En pètals de White Sim, el TDZ sol (0.5 i 5.0 μM) ha estat efectiu induint l'organogènesi de tiges, d'acord amb altres cultivars de clavell (Frey i Janick 1991) i amb altres espècies (Fiola et al. 1990, Malik i Saxena 1992, Huetteman i Preece 1993, Ricci et al. 2001, Singh et al. 2003). En canvi, en els cultivars Early Sam, Mei Ling, Inorsa R-24 i Pulcino, a part del TDZ s'ha requerit NAA (0.5 o 5.0 μM) per poder regenerar tiges.

En els cultivars i explants estudiats s'ha trobat que les auxines i les CKs del medi de cultiu interaccionen entre si. El tipus d'organogènesi obtinguda en els explants depèn del balanç entre CKs (BA o TDZ) i auxines (NAA), ja que quan han incrementat les auxines, tot decantant el balanç CK/auxina cap a aquestes hormones, ha augmentat la formació de rels, mentre que amb l'increment de les CKs, ha augmentat la caulogènesi adventícia. Aquests resultats estan d'acord amb el model clàssic de regulació hormonal de la morfogènesi *in vitro* proposat per

Skoog i Miller (1957). Però per un mateix balanç CK/auxina, diferents concentracions de reguladors també afecten l'organogènesi adventícia. Amb un balanç d'1, per exemple, la regeneració de tiges i de rels ha estat més gran amb les concentracions més altes de reguladors (veieu Capítol 2, Taula 1). Eklöf et al. (2000) també han suggerit que algunes respostes es poden atribuir no només al balanç hormonal CK/auxina, sinó també a la concentració absoluta de cadascuna d'aquestes hormones.

En la regeneració de tiges en pètals dels cultivars estudiats, que ha augmentat amb l'increment de les concentracions (0, 0.5 i 5.0 μM) de TDZ i d'NAA, aquests reguladors del creixement han mostrat un efecte sinèrgic, ja que la caulogènesi s'ha multiplicat en afegir auxina en el medi (veieu Capítol 2, Taula 1). Aquesta sinèrgia no ha estat observada en explants de clavell d'altres cultivars (Frey i Janick 1991, Watad et al. 1996).

Donat que amb l'increment de la concentració de TDZ en el medi han augmentat tant la proporció de zones meristemàtiques en els explants de pètals de White Sim el 7è dia de cultiu, com el nombre de tiges regenerades d'aquests pètals al cap d'un mes, és molt probable que el TDZ exerceixi un efecte de CK per si mateix en la inducció de l'organogènesi adventícia de tiges de clavell. En aquest sentit, s'ha demostrat que el TDZ s'uneix al receptor de CKs CRE1, amb la mateixa afinitat que la iP i la Z (Yamada et al. 2001). El fet que la BA s'hi uneixi amb menys afinitat també seria una explicació per la que el TDZ presenta una activitat de CK més elevada que la BA. A part de l'efecte directe, els resultats de la quantificació i immunolocalització de CKs endògenes el 7è dia de cultiu (dia representatiu del resultat de l'organogènesi) ens han mostrat que el TDZ té un efecte en aquestes hormones i, per tant, també exerceix un efecte indirecte en els processos que indueix.

4.1. Efecte del TDZ i l'NAA en la concentració d'IAA i de citocinines durant la inducció de l'organogènesi adventícia de tiges

La concentració de CKs totals, suma de les tres bases lliures i els seus ribòsids, pràcticament no ha variat entre els explants de pètals de White Sim cultivats 7 dies amb diferents concentracions de TDZ, en presència d'NAA (0.5 μM), però sí que s'han donat canvis en CKs específiques. En els explants de pètals cultivats amb baixes concentracions de TDZ (0.005 μM), amb poques cèl·lules meristemàtiques, s'hi ha observat un increment de ZR, que podria ser l'estímul per l'inici de l'activitat mitòtica. D'acord amb això, en suspensions de cèl·lules BY-2 de *N. tabacum* amb divisió sincronitzada s'ha trobat un acúmul de Z i ZR a la fase S i a la mitosi (Redig et al. 1996, Dobrev et al. 2002), tot i que només la Z pot reiniciar la divisió en aquestes cèl·lules amb la mitosi inhibida (Laureys et al. 1998). Explants de *N. tabacum* cultivats en un medi de desdiferenciació, per induir-hi divisió cel·lular, també han mostrat un gran increment de Z el 7è dia de cultiu (Boucheron et al. 2002).

D'altra banda, amb concentracions més elevades de TDZ (0.5 μM), en explants de pètals amb una gran proliferació cel·lular i un inici de diferenciació de les tiges adventícies, s'hi han duplicat els nivells d'iP. Aquest efecte del TDZ pot ser degut a la inhibició competitiva (Kamínek i Armstrong 1990, Bilyeu et al. 2001) o no competitiva (Hare i van Staden 1994) de l'activitat CK oxidasa, causada per les fenilurees (Mok i Mok 2001). D'acord amb els nostres resultats, durant l'embriogènesi estimulada pel TDZ en hipocòtils de *Pelargonium* x *hortorum* i en plançons

d'*Arachis hypogaea*, la iP, la Z i la DHZ han augmentat durant la primera setmana de cultiu (Hutchinson et al. 1996, Victor et al. 1999). En canvi, la BA no ha provocat canvis en les CKs endògenes ni en plàntules de *Chenopodium rubrum* ni d'*Actinidia deliciosa* cultivades *in vitro* (Feito et al. 1995, Blazková et al. 2001). Pel que fa a la importància de la iP en la morfogènesi, altres autors han suggerit que un increment en la relació CKs tipus iP / CKs tipus Z i DHZ és indicativa d'una major capacitat organogènica o embriogènica d'explants de *Corylus avellana* (Centeno et al. 1997, Andrés et al. 2002). Un valor elevat d'aquest índex també s'ha trobat durant els estadis juvenils del desenvolupament en *Pinus radiata* i *Prunus persica* (Valdés et al. 2002, Moncaleán et al. 2002). La transformació de plantes de clavell amb el gen *rolC* també incrementa el contingut d'iP en els pètals, així com la seva capacitat organogènica (veieu apartat 5.2, p.165).

La disminució del DHZR observada en els explants de pètals el 7è dia de cultiu és independent de la concentració de TDZ en el medi i, per tant, es pot atribuir a la presència d'NAA (0.5 μM). D'acord amb això, l'increment en la concentració d'auxines, bé aplicades exògenament o com a resultat de l'expressió dels gens *iaaM* i *iaaH* en teixits transgènics, provoca una reducció significativa dels nivells endògens de CKs, ja sigui inhibint la seva biosíntesi o promovent la seva inactivació (Zhang et al. 1995, Kamínek et al. 1997, Zazímalová et al. 1999, Eklöf et al. 2000, Swarup et al. 2002, Nordström et al. 2004). La disminució del DHZR també podria ser deguda a que en el procés d'organogènesi es requerissin més les CKs tipus iP i tipus Z, la qual cosa provocaria que hi hagués menys conversió cap a les CKs tipus DHZ.

L'increment de l'IAA en els explants de pètals cultivats 7 dies *in vitro*, respecte als pètals frescos, també pot atribuir-se a l'NAA (0.5 μM) del medi, ja que no ha variat en les diferents concentracions de TDZ assajades. Amb una setmana de cultiu amb TDZ tampoc s'ha alterat l'IAA durant l'embriogènesi somàtica en cotilèdons d'*A. hypogaea* (Murthy et al. 1995). En canvi, l'IAA sí que ha augmentat al 7è dia de cultiu durant el procés embriogènic en hipocòtils de *P.* x *hortorum* (Hutchinson et al. 1996). Durant la inducció de l'embriogènesi somàtica en *Helianthus annuus* i durant la desdiferenciació d'explants de *N. tabacum* s'han trobat increments transitoris d'IAA previs al 7è dia de cultiu (Thomas et al. 2002, Boucheron et al. 2002). Tot i que els nivells d'IAA s'han mantingut constants en els explants de pètals amb diversos graus de proliferació cel·lular, no es pot descartar que l'IAA estigués concentrat en les cèl·lules en divisió, ja que les auxines activen la progressió del cicle cel·lular, juntament amb les CKs (D'Agostino i Kieber 1999, Frank i Schmülling 1999, Stals i Inzé 2001, Boniotti i Griffith 2002). En fulles d'*N. tabacum* l'IAA s'acumula principalment en zones mersitemàtiques, i està menys concentrat en zones de creixement (Ljung et al. 2001). I en embrions de *H. annuus* l'IAA s'ha localitzat a l'extrem dels àpexs de les rels (Thomas et al. 2002).

4.2. Immunolocalització de citocinines endògenes durant l'organogènesi adventícia de tiges induïda pel TDZ i l'NAA

Els tres tipus de ribòsids de CKs, l'iPR, el ZR i el DHZR, tenen una distribució molt semblant en explants de pètals de White Sim el 7è dia del cultiu *in vitro*. S'han trobat molt concentrats en els primordis caulinars adventicis, especialment en la zona apical. L'immunomarcatge ha estat inferior a la part adaxial del pètals, que és la zona amb

cèl·lules que tenen la capacitat de proliferar, tal com s'ha observat en els estudis histològics (veieu Capítol 2, Figura 1). Anàlogament, en àpexs de tiges de diferents espècies s'hi ha localitzat una alta concentració de CKs: iPR i ZR en *L. esculentum* (Sossountzov et al. 1988), Z en *Prunus persica* (Bitonti et al. 2002), Z i iP en *Sinapis alba* (Jacqmard et al. 2002) i les tres bases lliures, iP, Z i DHZ, en *N. tabacum* (Dewitte et al. 1999). Durant la inducció de la floració en *S. alba* ha incrementat la iP per tota la zona apical, tot indicant la seva intervenció en la diferenciació de flors (Jacqmard et al. 2002). També s'han localitzat iP i Z en cèl·lules en divisió, o amb l'habilitat de proliferar, d'embrions somàtics de *Dactylis glomerata* en desenvolupament (Ivanova et al. 1994) i els tres ribòsids de les CKs en l'àpex de la rel, el meristema caulinar i les cèl·lules dels cotilèdons d'embrions de *Tilia cordata* (Kärkönen i Simola 1999). Pel que fa al DHZR en els pètals de clavell, malgrat la disminució detectada en la quantificació, la seva localització en el primordi caulinar suggereix que té un paper rellevant en el procés d'organogènesi adventícia, junt amb els altres ribòsids de CK, dels quals tampoc s'ha quantificat cap increment en els pètals estudiats.

El fet que haguem localitzat les CKs en l'àpex del primordi caulinar i en zones amb capacitat de proliferació ens suggereix que estan intervenint en la divisió cel·lular. La seva ubicació en la resta del primordi, com per exemple en cèl·lules xilemàtiques en diferenciació tal com també ha descrit Azmi et al. (2001), mostraria la seva possible implicació en la diferenciació d'alguns teixits de la futura tija adventícia de clavell. D'acord amb això, plantes de *N. tabacum* i *Arabidopsis* amb baixos nivells de CKs endògenes han mostrat un endarreriment en el creixement i desenvolupament de la part aèria degut a la reducció de la proliferació cel·lular en el meristema apical, tot indicant que les CKs regulen la divisió cel·lular, però que també podrien estar implicades en processos de diferenciació (Werner et al. 2001, 2003). Recentment, s'ha trobat un punt de connexió entre la divisió cel·lular i la diferenciació de tiges. Estudis amb plantes de *N. tabacum* transformades amb el gen *cdc25* de llevat han mostrat que la fosfatasa Cdc25, que és activada per CKs, no és només promotora el cicle cel·lular, sinó que també promou la diferenciació de tiges adventícies *in vitro* (Suchomelová et al. 2004).

5. Efecte del gen *rolC* en l'organogènesi adventícia del clavell i en els nivells d'auxines i citocinines endògenes de les plantes transformades

5.1. Implicació del gen *rolC* en l'organogènesi adventícia

El gen *rolC* d'*Agrobacterium rhizogenes* incorporat en plantes de clavells del cultivar White Sim ha incrementat la capacitat dels explants de pètals i fulles de produir tiges adventícies, manifestant un efecte de CK en les quatre línies transgèniques estudiades (*rolC*1, *rolC*2, *rolC*3 i *rolC*4). El mateix gen ha estat capaç d'augmentar el nombre de rels adventícies produïdes pels explants, manifestant també un efecte d'auxina. Aquests efectes han estat variables depenent de la línia transgènica. L'activitat de CK del gen *rolC* està d'acord amb el fenotip de les parts aèries de les plantes de clavell transformades amb *rolC*, en les que es redueix la dominància apical, amb el consegüent increment de la ramificació lateral (Ovadis et al. 1999, Zuker et al. 2001). Aquest fenotip també s'observa en la majoria de

plantes d'altres espècies transformades amb *rolC*, com de *N. tabacum* (Schmülling et al. 1988, Oono et al. 1990, Nilsson et al. 1993, Scorza et al. 1994), *Solanum tuberosum* (Fladung 1990, Schmülling et al. 1993), *Atropa belladonna* (Kurioka et al. 1992), *Populus tremula x Populus tremuloides* (Nilsson et al. 1996), *P. communis* (Bell et al. 1999) i, entre les ornamentals, en *Salpiglossis sinuata* (Lee et al. 1996), *Rosa* (Souq et al. 1996), *Petunia* (Winefield et al. 1999), *Chrysanthemum morifolium* (Mitiouchkina i Dolgov 2000) i *Pelargonium* x *domesticum* (Boase et al. 2004). L'efecte d'auxina del gen *rolC* en la rizogènesi adventícia està d'acord amb el fenotip de les plantes de clavell transformades amb *rolC*, les quals arrelen amb molta més facilitat que les control (Ovadis et al. 1999, Zuker et al. 2001). Aquesta característica també s'ha descrit en plantes de *Poncirus trifoliata* (Kaneyoshi i Kobayashi 1999) i *Diospyros kaki* (Koshita et al. 2002) transformades amb *rolC*. D'altra banda, rels de *N. tabacum* transformades amb aquest gen tenen una major capacitat de creixement *in vitro* (Schmülling et al. 1988), efecte assignat al *rolC* en la malaltia de les rels velloses (White et al. 1985).

En el medi sense reguladors del creixement, però, el gen *rolC* no ha incrementat ni la rizogènesi ni la caulogènesi en cap dels explants de clavell estudiats. De la mateixa manera, en fulles de *Kalanchoe diagremontiana* cultivades sense hormones el *rolC* tampoc hi ha fet cap efecte (Spena et al. 1987), mentre que en fulles de *N. tabacum* hi ha promogut la rizogènesi (Spena et al. 1987). Així, hi ha una interacció entre el *rolC* i el genotip de cada espècie. En el cas del clavell, doncs, el *rolC* requereix reguladors del creixement en el medi per estimular la rizogènesi i la caulogènesi en els explants. Pel que fa a la caulogènesi, les diferències més notables entre línies s'han obtingut en els medis més regeneratius (0.5 μM NAA i 0.5 μM), on ha incrementat fins a 3 vegades. I pel que fa a la rizogènesi, que ha augmentat fins a 19 vegades en les línies *rolC*, són els medis menys òptims els que han mostrat majors diferències (veieu Capítol 5, Taules 1 i 2). Per tant, el gen *rolC* també interacciona amb els reguladors del creixement del medi en la inducció de l'organogènesi adventícia.

Les quatre línies *rolC* de clavell han mostrat diferències en l'organogènesi adventícia, la qual, a la vegada, ha presentat una gran relació amb el fenotip de la línia corresponent. La línia *rolC*1 és la que té més capacitat de regeneració d'òrgans i un fenotip més extrem, mentre que les *rolC*2, *rolC*3 i *rolC*4 tenen un comportament intermig entre la línia control i la *rolC*1. Les diferències fenotípiques entre línies transgèniques també s'ha trobat en plantes d'altres espècies transformades amb *rolC* (Giovannini et al. 1999, Mitiouchkina i Dolgov 2000, Boase et al. 2004). Les diferències trobades entre les línies *rolC* de clavell podrien ser degudes a un nombre diferent de còpies del gen, ja que la línia *rolC*1 en té dues, mentre que la *rolC*2, i també la *rolC*3, en tenen una (Zuker et al. 2001). En algunes espècies, però, s'ha descrit que els nivells d'expressió del gen *rolC* es correlacionen amb els fenotips obtinguts en les plantes (Kurioka et al. 1992, Nilsson et al. 1996, Giovannini et al. 1999, Winefield et al. 1999). En els clavells, tant la línia *rolC*2 com la *rolC*4 expressen nivells semblants de la proteïna, coincidint amb que tenen una capacitat regenerativa molt similar. En canvi, en la línia *rolC*1, de la que s'esperava més expressió, no s'hi ha detectat la proteïna RolC. Així, hi podria haver hagut un silenciament del gen *rolC* en aquesta línia. El silenciament de transgens és un fenomen força freqüent a llarg termini, que es produeix principalment amb la introducció de transgens que ja estan en el genoma i, sobretot, en els que tenen un elevat nivell de transcripció (Fagard i Vaucheret 2000, Vaucheret et al. 2001, Susi et al. 2004). El fet que la línia transgènica *rolC*1 tingui dues còpies del

transgen i que el promotor d'aquest gen sigui constitutiu (35S) està d'acord amb la possibilitat del silenciament. Tot i així, caldrien més estudis per confirmar aquesta hipòtesi i, en cas afirmatiu, per conèixer si el silenciament és transcripcional o post-transcripcional (Herve Vaucheret, comunicació personal). Donat que la immunodetecció de la proteïna RolC es feu 2 anys després dels últims experiments d'organogènesi adventícia, 3.5 anys després de la determinació hormonal i 7 anys després de l'obtenció de les línies transgèniques, aquest silenciament no invalida els resultats de l'organogènesi ni del contingut hormonal que hem obtingut de la línia *rolC*1.

5.2. Alteracions del contingut d'IAA i citocinines endògens induïdes pel *rolC*

El mode d'acció de la proteïna RolC en les plantes transgèniques encara no s'ha aclarit. Tanmateix, els fenotips *rolC* indiquen que els efectes bioquímics de la proteïna podrien ser en les hormones endògenes, especialment en les auxines i les CK, ja sigui alterant-ne el metabolisme o bé modificant la sensibilitat dels teixits a aquestes hormones. Tot i que *in vitro* la proteïna RolC mostra una activitat η-glucosidasa que pot catabolitzar glucòsids de CKs i alliberar bases lliures de CKs (Estruch et al. 1991a), estudis posteriors han demostrat que aquest procés no es dóna *in vivo* (Nilsson et al. 1993, Nilsson et al. 1996, Faiss et al. 1996), probablement degut a la compartimentalització d'aquesta proteïna en el citoplasma (Estruch et al. 1991b) i a la ubicació dels glucòsids de les CKs en el vacúol (Coenen i Lomax 1997).

En les bases de fulles i pètals frescos de les línies de clavell *rolC*1, *rolC*2 i *rolC*4, ni els nivells de CKs totals ni la relació CKs/IAA s'han correlacionat amb l'increment de l'organogènesi adventícia. En aquest sentit trobem dades contradictòries pel que fa als nivells totals de CKs en plantes transformades amb *rolC*. En plantes *rolC* de *N. tabacum* els nivells globals de CKs s'han mantingut (Schmülling et al. 1993, Faiss et al. 1996) o han disminuït (Nilsson et al. 1993), disminució que també s'ha observat en plantes transformades de *P. tremula* x *P. tremuloides* (Nilsson et al. 1996, Fladung et al. 1997). Només en plantes de *S. tuberosum* transformades amb *rolC* han incrementat les CKs, fins a 4 vegades (Schmülling et al. 1993).

El gen *rolC* en els clavells provoca canvis en determinades CKs, unes o altres depenent de l'òrgan estudiat (fulles o pètals). Els increments trobats en CKs específiques poden ser la causa d'un efecte indirecte del *rolC* en el metabolisme hormonal, ja que amb l'expressió constitutiva del transgen un efecte directe de la proteïna RolC produiria els mateixos efectes en òrgans diferents.

Les bases de les fulles de les línies *rolC* han presentat nivells més baixos de Z i un major contigut de ZR, de fins a 3 vegades, però aquests canvis no s'han correlacionat amb l'increment en la regeneració de rels i tiges adventícies. Tanmateix, els valors del ZR poden ser importants per l'increment de l'organogènesi de tiges, ja que les CKs tipus Z s'han associat amb divisió cel·lular en diversos sistemes (veieu apartat 4.1, p.161). Tant en fulles de *S. tuberosum* com en àpexs de tiges de *P. tremula* x *P. tremuloides* amb *rolC* també s'ha trobat un increment de ZR (Schmülling et al. 1993, Nilsson et al. 1996). Contràriament, fulles joves de *N. tabacum* amb *rolC* tenen un contingut més baix de ZR (Nilsson et al. 1993). Durant el període de floració, plantes *rolC* de *C. morifolium* presenten 80 vegades més CKs tipus Z en els àpexs (Mitiouchkina i Dolgov 2000).

En les bases dels pètals la diferència més important entre les línies trangèniques i el control ha estat l'elevat contingut d'iP, tot sent 7, 4.5 i 3 vegades més alt en les línies *rolC*1, *rolC*2 i *rolC*4, respectivament. A més, els nivells d'aquesta CK s'han correlacionat positivament amb la regeneració de tiges en aquests explants. Donat que la concentració d'iP ha augmentat en pètals de White Sim no transformats el 7è dia del cultiu (veieu apartat 4.1, p.161), aquests resultats indiquen que la iP està implicada en l'organogènesi de tiges en aquests explants. Així, una relació elevada CKs tipus iP / CKs tipus Z i DHZ en els pètals de clavell indica una elevada competència organogènica, tal com s'ha trobat en explants de *C. avellana* (Andrés et al. 2002). No s'han quantificat increments d'iP en cap altra planta o òrgan transformats amb *rolC*, incloent les fulles *rolC* de clavell. En canvi, els nivells d'iPR han estat més elevats en tots els òrgans de plantes de *S. tuberosum* amb *rolC* (Schmülling et al. 1993).

L'increment d'iP dels pètals *rolC* no es reflecteix en la localització *in situ* d'aquesta CK. Ni en pètals frescos control ni en pètals *rolC*1, no s'ha observat marcatge d'iP a nivell histològic. A nivell subcel·lular, en ambdós tipus de pètals s'ha trobat la iP concentrada bàsicament en el nucli, mentre que en el citoplasma s'hi ha trobat un marcatge molt dèbil. De manera semblant als nostres resultats, CKs tipus iP i tipus Z s'han localitzat en el nucli i en el citoplasma de cèl·lules d'embrions de *D. glomerata* (Ivanova et al. 1994) i de *T. cordata* (Kärkönen i Simola 1999), i de cèl·lules dels àpexs de la tija i la rel de *L. esculentum* (Sossountzov et al. 1988). En canvi, en àpexs de tija de *N. tabacum* la iP només s'ha ubicat en el citoplasma i la Z en el nucli (Dewitte et al. 1999). Donat que en els pètals frescos pràcticament no hi ha activitat meristemàtica, la localització nuclear de la iP indicaria un efecte d'aquesta CK en la regulació de l'expressió de gens, tal com ha descrit Schmülling et al. (1997) per les CKs, gens que intervindrien en processos no relacionats amb l'activitat mitòtica.

Els nivells d'IAA no han estan afectats ni en fulles ni en pètals de plantes de clavell transgèniques amb *rolC*, en comparació amb les control. En concordança, plantes *rolC* de *N. tabacum* i *S. tuberosum* també presenten els mateixos nivells d'aquesta auxina (Nilsson et al. 1993, Schmülling et al. 1993), mentre que en plantes transgèniques de *P. tremula* x *P. tremuloides* i *C. morifolium* els nivells són fins i tot inferiors (Nilsson et al. 1996, Mitiouchkina i Dolgov 2000). En explants de plantes transformades amb els gens *rolABC*, com *N. tabacum*, *K. diagremontiana*, *L. esculentum* i *Rosa*, s'ha incrementat la rizogènesi adventícia, i s'ha proposat que els gens *rol* combinats provoquen un increment en la sensibilitat a les auxines (Spena et al. 1987, Spanò et al. 1988, van Altvorst et al. 1992a, van der Salm et al. 1997). Estudis de sensibilitat en protoplasts de *L. esculentum* i *Catharanthus trichophyllus* transformats amb aquests gens *rol* confirmen aquesta hipòtesi (Shen et al. 1990). Anàlogament, suggerim que l'efecte d'auxina del *rolC* en la rizogènesi adventícia en les plantes de clavell podria ser degut a un increment de la sensibilitat a les auxines. Està àmpliament acceptat que la sensibilitat a les auxines dels gens *rol* actuant junts és deguda al gen *rolB* (Nilsson i Olsson 1997, Meyer et al. 2000). D'acord amb la nostra proposta, les mesures del potencial de membrana de protoplasts *rolC* de *N. tabacum* mostren que són més sensibles a les auxines que els control, encara que en els *rolB* aquest efecte és molt més pronunciat (Maurel et al. 1991, 1994).

6. Aplicació dels gens *rol* a la floricultura

En els últims anys, l'avenç de l'enginyeria genètica ha permès obtenir plantes transgèniques amb alteracions en trets específics. Pel que fa a les plantes ornamentals amb flor transformades amb *A. rhizogenes* o amb els seus gens *rol* (*rolA*, *rolB*, *rolC* i *rolD*), s'ha trobat que han adquirit trets atractius en floricultura, que afecten la morfologia de la planta i influeixen en el procés de floració, els quals poden interessar tant a l'hora d'obtenir noves varietats com des del punt de vista horticultural.

Les plantes ornamentals transformades amb l'*A. rhizogenes* presenten el fenotip de les rels velloses. En general, és un fenotip nanitzat, amb la dominància apical reduïda i el consegüent increment de la ramificació lateral, les fulles petites i arrugades, amb més capacitat d'arrelament, la floració alterada (quasi sempre endarrerida o fins i tot inhibida), i en alguns casos amb un increment del nombre de flors i una modificació de la seva forma. La fertilitat queda reduïda, principalment la masculina, o a vegades inhibida. Aquest és un tret general per totes les plantes transformades amb els gens *rol* i el desavantatge més gran de l'ús d'aquests gens. Però això no afectaria ni les plantes que es multipliquen vegetativament, ni les plantes transgèniques amb només esterilitat masculina, ja que podrien ser propagades tot sent pol·linitzades amb plantes no transformades i part de la descendència mantindria les mateixes característiques que la planta transgènica.

El fenotip de les rels velloses causat per *A. rhizogenes* en plantes ornamentals és molt similar a l'obtingut amb els seus gens *rolA*, *B* i *C* actuant alhora, excepte que aquest últim cas s'avança la floració en algunes espècies, i no es modifica la forma de les flors. Així, la modificació de la morfologia floral i la floració tardana en plantes transformades amb *A. rhizogenes* podrien ser degudes a la presència d'altres gens del T-DNA, com ara els gens *aux*, en cas d'haver utilitzat soques d' *A. rhizogenes* del tipus agropina.

Pel que fa als gens *rol* per separat, el *rolA* no es recomana per ser introduït en espècies ornamentals, ja que s'obtenen plantes amb moltes anormalitats, com ara fulles arrugades, un sistema radicular reduït i/o floració tardana. I el gen *rolB*, encara que incrementi la capacitat d'arrelament, bé hauria d'estar controlat per determinats promotors o bé s'hauria d'introduir conjuntament amb altres gens *rol*, com el *rolC*, per tal de compensar els efectes no desitjats.

Entre els gens *rol*, el *rolC* és el que provoca més avantatges pel que fa a la millora de trets ornamentals i horticulturals. El nanisme i l'increment de les tiges laterals provoquen un fenotip compacte. Sorprenentment, aquestes plantes poden tenir la capacitat d'arrelament millorada, tret clàssicament associat amb el *rolB*, i pràcticament no mostren trets desavantatjosos, excepte la disminució de la fertilitat. Les plantes *rolC* produeixen més flors, les quals són més petites, i tenen una floració avançada, característiques molt importants en plantes ornamentals, ja que impliquen un millor rendiment del cultiu. El gen *rolD*, que és el menys estudiat entre els gens *rol*, també promou la floració, tot avançant-la i incrementant el nombre de flors. Així, presenta aplicacions beneficioses per plantes ornamentals i fins i tot per l'horticultura, ja que també incrementa la producció de fruits.

Força trets obtinguts amb els gens *rol* poden generalitzar-se, mentre que altres estan associats a cada espècie i a cada línia transgènica, tal com també s'ha trobat en clavell. Tot i que cal determinar la funció o funcions

de les proteïnes Rol per tal d'entendre els efectes bioquímics i fenotípics que produeixen en les plantes transgèniques, els gens *rol* són útils per la millora de plantes ornamentals amb flor i també poden ser-ho en altres branques de l'agricultura.

CONCLUSIONS

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1) Els cultivars de *Dianthus caryophyllus* L. classificats fenotípicament com a estàndards (White Sim, Red Sim, Pallas i Bianca Neve) i com a *spray* (Early Sam), i també l'híbrid interespecífic Pulcino, constitueixen genèticament un sol grup. Els híbrids interespecífics Mei Ling i Inorsa R-24 estan genèticament separats del grup *D. caryophyllus* i entre si.

2) L'organogènesi de tiges i de rels adventícies en el clavell depèn del genotip i l'explant. El cultivar White Sim és el més regeneratiu, seguit de l'Early Sam i dels híbrids interespecífics Mei Ling, Inorsa R-24 i Pulcino, que són els menys regeneratius. Les fulles són lleugerament més regeneratives que els pètals. L'organogènesi és directa en els cultivars i explants estudiats i en les condicions assajades.

3) La capacitat de regeneració de tiges i de rels adventícies en explants de pètals i fulles de clavell està afectada pel tipus, balanç i concentració d'auxines i citocinines (CKs) del medi de cultiu.
   - ∉ El N-fenil-N'-1,2,3-tiadiazol-5-ilurea (TDZ) és més efectiu que la N^6-benziladenina (BA) a la mateixa concentració (0.5 μM) en induir l'organogènesi de tiges adventícies i en inhibir la formació de rels en explants de fulles.
   - ∉ El TDZ sol (0.5 i 5.0 μM) és efectiu en la inducció de tiges adventícies en explants de pètals del cultivar White Sim, mentre que pels altres cultivars es requereix l'auxina àcid 1-naftalenacètic (NAA).
   - ∉ El balanç CK/auxina del medi regula la caulogènesi i la rizogènesi en ambdós tipus d'explants. Per un mateix balanç de TDZ i NAA, concentracions més elevades d'aquests reguladors del creixement incrementen la regeneració tant de tiges com de rels adventícies en explants de pètals.
   - ∉ El TDZ i l'NAA (0, 0.5 i 5.0 μM) tenen efectes sinèrgics en promoure l'organogènesi de tiges en explants de pètals.

4) La capacitat de regeneració de tiges adventícies en explants de pètals de clavell depèn de la humitat relativa i del grau de solidificació del medi de cultiu.
   - ∉ En disminuir la humitat relativa (entre 4.5 i 6.5 %) dels pots de cultiu, per ventilació, es redueix la capacitat regenerativa dels explants de pètals de White Sim i Early Sam, i el contingut d'aigua de les tiges regenerades.
   - ∉ Amb l'increment de la concentració d'agar (de 2 a 12 g l^{-1}) en el medi es redueix la capacitat de regeneració de tiges dels explants de pètals de White Sim, però també disminueix la hiperhidricitat d'aquestes tiges.
   - ∉ La hiperhidricitat de les tiges pot ser quantificada amb el seu percentatge d'aigua, el seu pes fresc i sec, el nombre de capes de cèl·lules parenquimàtiques de les fulles i el tamany d'aquestes cèl·lules.

5) El TDZ (0, 0.005, 0.05 i 0.5 μM), en presència d'NAA (0.5 μM), estimula l'organogènesi adventícia de tiges en explants de pètals de clavell White Sim de forma directa i també de forma indirecta alterant les CKs endògenes.
   - ∉ El TDZ indueix l'organogènesi de tiges de manera dependent de la concentració, tot indicant que aquest regulador exerceix un efecte directe en aquest procés.
   - ∉ La proporció de zones meristemàtiques en explants de pètals de White Sim el 7è dia de cultiu també depèn de la concentració de TDZ, i permet predir la capacitat de regeneració de tiges d'aquests explants.
   - ∉ El TDZ indueix l'organogènesi de tiges de forma indirecta, tot incrementant els nivells de la N^6-isopenteniladenina (iP) i del ribòsid de zeatina (ZR) endògens, al 7è dia de cultiu. L'increment de ZR estimulat pel TDZ (0.005 μM) estaria relacionat amb l'inici de l'activitat meristemàtica. L'augment d'iP induït pel TDZ (0.5 μM) estaria implicat amb una elevada proliferació cel·lular i un inici de diferenciació dels primordis caulinars.
   - ∉ La disminució del ribòsid de la dihidrozeatina (DHZR) i l'increment de l'àcid indol-3-acètic (IAA) dels mateixos explants al 7è dia de cultiu s'atribueixen a l'NAA del medi, donat que no varien amb les diferents concentracions de TDZ.

6) En explants de pètals de clavell White Sim, els ribòsids de CKs (N^6-isopenteniladenosina (iPR), ZR i DHZR) s'han localitzat en els primordis caulinars adventicis en formació, especialment concentrats en l'àpex, i en regions amb capacitat proliferativa, tot indicant la participació d'aquestes CKs en la divisió cel·lular, i possiblement en la diferenciació, en el procés d'organogènesi adventícia de tiges.

7) El gen *rolC* d'*Agrobacterium rhizogenes* introduït en plantes de clavell White Sim incrementa la capacitat dels explants de pètals i fulles de produir tiges adventícies, tot manifestant un efecte de CK, i també augmenta el nombre de rels adventícies produïdes pels explants, tot exhibint una activitat d'auxina. La caulogènesi i la rizogènesi es manifesten en major o menor grau depenent de la línia transgènica, i presenten una gran relació amb el fenotip de cadascuna de les línies.

8) El gen *rolC* introduït en plantes de clavell White Sim altera el contingut de determinades CKs endògenes de manera dependent de l'òrgan, tot indicant un efecte indirecte d'aquest transgen amb expressió constitutiva.
   - ∉ Les fulles transgèniques presenten un major contingut de ZR, de fins a 3 vegades més, depenent de la línia transgènica.
   - ∉ Els pètals transgènics presenten nivells d'iP fins a 7 vegades més elevats, que es correlacionen amb la resposta caulogènica dels pètals de cada línia transgènica. La iP es troba concentrada en el nucli de les cèl·lules.

9) La incorporació del gen *rolC* en plantes de clavell White Sim no afecta els nivells d'IAA ni en pètals ni en fulles. Així, l'increment de la rizogènesi adventícia en explants transgènics s'explicaria per un increment de la sensibilitat a l'NAA exogen.

10) Els gens *rol*, sols o combinats, provoquen alteracions en la morfologia i la fisiologia de les plantes transformades, algunes de les quals són avantatjoses i d'altres, com la reducció o la inhibició de la fertilitat obtinguda en quasi totes les transformacions, no ho són.
    - ∉ El fenotip obtingut amb la introducció de la combinació *rolABC*, presenta algun avantatge respecte la transformació amb l'*A. rhizogenes*, com ara l'avançament de la floració en plantes d'algunes espècies, però també manté alguns desavantatges del fenotip de les rels velloses, com ara les fulles arrugades o la reducció de la fertilitat masculina.
    - ∉ El gen *rolC* és el més recomanable per ser introduït en plantes ornamentals amb flor, ja que es poden obtenir molts trets beneficiosos, com ara el nanisme i un increment en la ramificació lateral, produint un creixement compacte, un nombre major de flors, encara que més petites, i una floració avançada (uns dels trets més avantatjosos en floricultura), i fins i tot més bon arrelament.
    - ∉ El gen *rolD*, el gen *rol* menys estudiat, pot tenir aplicacions en plantes ornamentals, ja que també incrementa i avança la floració.
    - ∉ El *rolA* i el *rolB* no són recomanables, ja que provoquen moltes anomalies en les plantes tranformades.

ANNEXOS

Annex1. Mètode de quantificació d'hormones vegetals

Per tal d'analitzar el contingut endogen d'àcid indol-3-acètic (IAA) i de les citocinines (CKs) N^6-isopenteniladenina (iP), zeatina (Z), dihidrozeatina (DHZ) i els seus ribòsids (iPR, ZR i DHZR), les bases dels pètals i les fulles dels clavells, congelats amb nitrogen líquid i liofilitzats, se sotmeteren a un procés d'extracció, purificació, separació i quantificació d'aquestes hormones, segons el mètode de Fernández et al. (1995). Aquestes anàlisis hormonals es realitzaren amb la Dra. Belén Fernández, del Laboratori de Fisiologia Vegetal de la Universitat d'Oviedo. Tot el procés es realitzà en condicions de llum tènue per evitar la fotoxidació i la consegüent inactivació de l'IAA.

Extracció

1. Les hormones s'extragueren en fase líquida, tot utilitzant un solvent orgànic. Per això, es trituraren 100 mg de material vegetal liofilitzat per mostra, utilitzant un morter, i es barrejaren amb 25 ml de metanol al 80% (v/v), al que se li afegí 10 mg l^{-1} de *t*-butilhidroxitoluè (BHT) com a antioxidant. Prèviament a la seva utilització, el BHT es dissolgué en etanol en una dilució de 10 mg BHT ml^{-1}. Aquest antioxidant s'utilitza sobretot per evitar l'oxidació de l'IAA. El procés d'extracció es realizà en agitació invertida a 4 ºC i a la foscor durant 14 h.
2. Es realitzà una primera filtració al buit de l'extracte a través de placa porosa i filtres de fibra de vidre (Whatman GF/A 3, λ = 3.7 cm), i es guardà el filtrat.
3. S'efectuà una reextracció del material vegetal amb 15 ml del solvent utilitzat en la primera extracció, en agitació invertida a 4 ºC i a la foscor durant 7 h. Es tornà a filtrar i s'uniren els dos extractes metanòlics.
4. Posteriorment a l'extracció s'afegiren els marcadors radioactius 3-[5(n)-^{3}H] IAA (999 GBq $mmol^{-1}$) i [8-^{14}C] N^6-benziladenina (BA) (2.0 GBq $mmol^{-1}$) (Amersham Ibérica S.A.) a cadascuna de les mostres, per tal que la mesura inicial fos de 25000 dpm per cadacun dels isòtops. Així, la radioactivitat emesa per cada mostra al final del procés, mesurada amb un comptador de centelleig (Wallac 1409), permeté quantificar les pèrdues produïdes durant l'anàlisi.
5. Els extractes es concentraren en un rotavapor, en el que s'evaporà el metanol (en condicions de buit i a una temperatura de 30 ºC).
6. Les solucions aquoses resultants se centrifugaren (Centrikon T-124) per a la seva clarificació, a 14500 *g* i 5 ºC durant 26 min. Es guardaren els sobrenedants i els sediments es redissolgueren en 1.5 ml d'aigua destil·lada i se centrifugaren 10 min més a les mateixes condicions.
7. Els sobrenadants procedents de les dues centrifugacions foren reduïts a un volum de 5 ml amb un concentrador d'alt buit (Savant SC-200).

Purificació

La purificació de les hormones es realitzà en dues fases: una prepurificació, o extracció en fase sòlida, en columnes de fase reversa (amb una fase estacionària apolar) i una cromatografia d'immunoafinitat.

1. Els 5 ml d'extracte aquós de cada mostra s'ajustaren a pH 3 amb àcid acètic diluït, per transformar les hormones en apolars, abans de prepurificar-los en columnes de fase reversa Sep-Pak C18 (Waters Associates).
2. Abans de ser utilitzades, les columnes va ser condicionades amb 10 ml de metanol al 80% (v/v) i equilibrades amb 10 ml d'aigua. Després de fer passar les mostres, les fitohormones retingudes en les columnes foren eluïdes amb 10 ml de metanol al 80% (v/v), dissolvent orgànic apolar.
3. Els extractes metanòlics s'assecaren totalment en un concentrador d'alt buit (Savant SC-200).
4. Per la seva purificació, les mostres es redissolgueren posteriorment en 2 ml de tampó fosfat salí (PBS) 10 mM, a pH 7.4, i es feren passar per una columna d'immunoafinitat a les CKs, constituïda per anticossos policlonals anti-CKs del tipus iP, Z i DHZ, obtinguts tal com es descriu a Fernández et al. (1995). Com que les CKs quedaren retingudes a la columna pels anticossos, aquest pas ens permeté separar les CKs de l'IAA. L'IAA, que no queda retingut a la columna, fou recuperat amb el pas de 30 ml de PBS. Després es rentà la columna amb aigua destil·lada, que fou descartada. La velocitat de pas del PBS i de l'aigua per la columna fou regulada mitjançant una bomba peristàltica de forma constant a 1.5 ml min^{-1}.
5. Les CKs, retingudes en la columna, s'eluiren amb el pas de 30 ml de metanol d'elevada puresa (99.9%).
6. Les fraccions de PBS, en les que es trobava l'IAA, s'ajustaren a pH 3 amb HCl diluït al 50 % (v/v) i a continuació es fraccionaren 4 vegades en ampolles de decantació amb èter dietílic fins a un volum final d'èter de 50 ml.
7. Les dues fraccions (IAA i CKs) de cada mostra s'assecaren completament amb un corrent de nitrogen i un concentrador d'alt buit (Savant SC-200), respectivament.

Separació de les CKs

Les CKs se separaren mitjançant cromatografia líquida d'alta resolució (HPLC) de fase reversa, seguint una modificació del mètode descrit per Horgan i Scott (1987) (Fernández et al. 1995), amb un cromatògraf líquid Waters 600 connectat a un detector UV Waters 996 a una longitud d'ona de 270 nm.

La separació de les sis CKs fou imprescindible per poder-les quantificar, ja que que els anticossos dels que es disposava eren policlonals. Les reaccions creuades d'aquests anticossos per les CKs es troben a la Taula 1.

1. Les mostres de les CKs es dissolgueren amb 500 µl d'acetonitril i acetat de trietilamoni (TEAA) 40 mM a pH 7 (5% v/v), condicions inicials de la cromatografia.

Taula 1. Reaccions creuades (%) dels tres antisèrums policlonals per les CKs (Fernández et al. 1995).

CK	Anti-ZR	Anti-DHZR	Anti-iPR
ZR	100	22.9	-
Z	39.3	15	-
DHZR	7.3	100	-
DHZ	27.5	50.5	-
iPR	-	-	100
iP	-	-	77.6

2. Les mostres dissoltes es filtraren amb filtres Millex-HV_{13} (Millipore) de 0.45 μm i a cada mostra s'afegiren 2 μg de kinetina. Aquesta CK sintètica s'afegí com estàndard intern.
3. El volum de mostra injectat en el cromatògraf fou sempre de 0.2 ml i el flux dels solvents fou d'1.5 ml min^{-1}. S'utilitzaren columnes de fase reversa Kromasil 100 C18 (150 mm de longitud x 4.6 mm de diàmetre) amb un tamany de partícula interna de 5 μm. La utilització de columnes de fase reversa, amb la fase estacionària apolar, permet la separació de substàncies per la seva polaritat. Els solvents utilitzats com a fase mòbil foren acetonitril i TEAA 40 mM a pH 7 amb un gradient des d'un 5% (v/v) a un 20% (v/v) d'acetonitril en 40 min. D'aquesta manera, primer de tot s'eluiren les CKs més polars i, en anar incrementant la proporció del solvent apolar, anaren eluint-se les CKs més apolars.

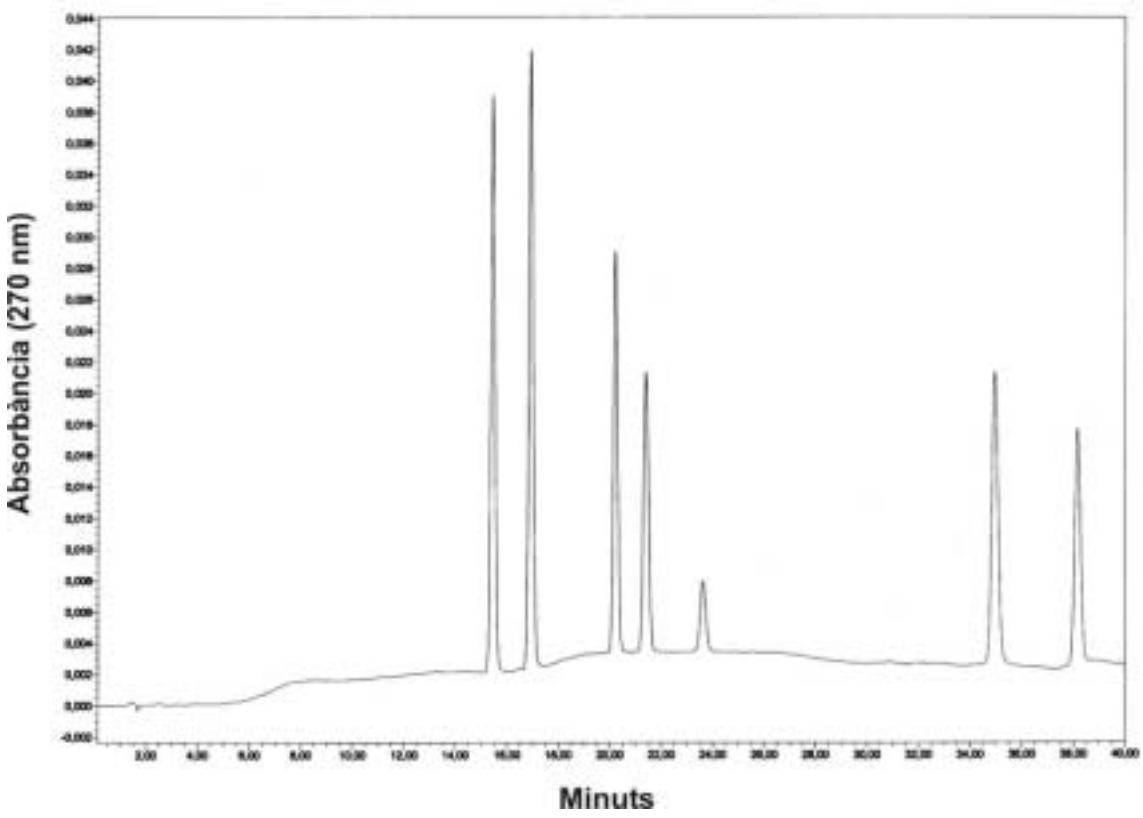

Fig. 1. Cromatograma del temps de retenció (min) de les CKs corresponents al patró, separades per HPLC de fase reversa segons la tècnica de Fernández et al. (1995), obtingut amb l'absorbància a 270 nm. D'esquerra a dreta: Z, DHZ, ZR, DHZR, kinetina, iP i iPR.

4. Prèviament a la injecció de les mostres en el cromatògraf s'injectà el patró, amb les sis CKs a quantificar concentrades i també amb la kinetina dissoltes en els solvents corresponents a les condicions inicials de la cromatografia (1 μg ml^{-1} de cada CK). L'absorbància a l'UV de les fraccions eluïdes dels patrons ens mostrà el temps de retenció de cada CK a la columna (Fig. 1) i, per tant, el minut en que s'havia de col·lectar cadascuna de les CKs en passar les mostres.
5. S'injectaren les mostres en el cromatògraf. Les CKs endògenes, en trobar-se a baixes concentracions, estan per sota del límit de detecció de l'HPLC i no se n'observen els pics d'absorbància. Així, la kinetina afegida a cada mostra serveix per corroborar el temps de retenció de les CKs endògenes, ja que en estar-hi molt concentrada se'n pot observar el pic d'absorbància. Les fraccions eluïdes de les CKs es recolliren en un col.lector en alíquotes d'1.5 ml i s'assecaren a alta velocitat i buit (Savant SC-200).

Quantificació

Per la quantificació de l'IAA i de les CKs s'utilitzà un assaig de l'enzim immunoadsorbit (ELISA) de competició, sobre microplaques (F96 MaxiSorp™, Nunc). En aquest assaig s'estableix una reacció de competitivitat per la unió a l'anticòs entre una quantitat constant de traçador (hormona lligada a la fosfatasa alcalina) i la quantitat desconeguda d'hormona que conté la mostra. En afegir paranitrofenil fosfat, substrat de la fosfatasa alcalina, es produeix paranitrofenol, substància colorejada l'absorbància de la qual es determina a una longitud d'ona de 405 nm. D'aquesta forma la quantitat de color és inversament proporcional a la quantitat d'antigen, en el nostre cas d'hormona, present a la mostra.

Quantificació de les CKs

Les sis CKs ja separades de cada mostra foren analitzades en sis plaques ELISA, una per cada CK. L'assaig ELISA es realizà segons el protocol descrit per Centeno et al. (1996), tot utilitzant els mateixos anticossos policlonals (Taula 1) que en la columna d'immunoafinitat: anti-ZR per valorar la Z i el ZR, anti-DHZR per valorar la DHZ i el DHZR, i anti-iPR per valorar la iP i la iPR.

1. Es posà cadascun dels anticossos, dissolts en tampó bicarbonat 50 mM, pH 7.4, als pouets de dues plaques ELISA (200 μl per pouet) i s'incubaren a 4ºC tota la nit. Seguidament les plaques es rentaren tres vegades amb tampó tris(hidroximetil)aminometà salí (TBS) 25 mM amb $MgCl_2 \cdot 6H_2O$ 1.0 mM, pH 7.5.
2. Després s'incubaren amb albúmina de sèrum boví (BSA) al 0.1% (p/v) amb el tampó TBS (250 μl per pouet), durant 30 min, i es rentaren tres vegades amb el tampó TBS.

3. Les mostres de cada CKs foren dissoltes en TBS 25 mM, pH 7.5: la Z, la DHZ i els seus ribòsids es dissolgueren en 800 µl de tampó, mentre que la iP i la iPR es dissolgueren en 1000 µl. Cada mostra fou aplicada per triplicat (100 µl per pouet) en la placa amb l'anticòs corresponent i s'incubà a temperatura ambient durant 30 min. A la vegada, a cada placa s'aplicaren les CKs (estàndards) corresponents a l'anticòs per realitzar la corba patró (veieu el punt 7).
4. Els traçadors, ribòsids de les CKs units a la fosfatasa alcalina, foren dissolts en el tampó TBS amb gelatina al 0.1% (p/v). Passats els 30 min d'incubació de les mostres i els estàndards, s'afegiren les solucions traçadores (100 µl per pouet) a les plaques amb les CKs correponents i s'incubaren a 4ºC durant 2.5 h. A continuació, les plaques es rentaren tres vegades amb el tampó TBS.
5. El substrat de la fosfatasa alcalina, el paranitrofenil fosfat, es dissolgué en una solució de dietanolamina 0.9 M amb $MgCl_2 \cdot 6H_2O$ 0.3 mM, pH 9.8, (1.4 mg ml^{-1}) i s'afegí a totes les plaques (200 µl per pouet), que es deixaren incubar a 37ºC entre 45 i 60 min, fins que agafaren color.
6. Es va mesurar l'absorbància de cada microplaca a 405 nm en un lector de microplaques (Perkin-Elmer Lambda Reader).
7. Per determinar la quantitat de CKs cal fer, per cada microplaca, una corba patró amb la CK corresponent a l'anticòs d'aquella placa. Aquesta es construeix amb quantitats creixents de ZR, DHZR i iPR, des de 0.02 a 100 pmol/0.1 ml de TBS. Per tal de conèixer la màxima unió del traçador als anticossos (B_0) en la corba patró s'inclouen diversos pouets que contenen únicament tampó TBS. Per determinar les unions no específiques dels anticossos (NSB) s'utiliza un excés de CK (100 pmol/0.1 ml de TBS). El percentatge d'unió del traçador (%B) en cada pouet d'estàndard o mostra es calcula de la següent manera:

   $$\%B = (DO_{405} \text{ mostra o patró} - DO_{405} \text{ NSB} / DO_{405} B_0 - DO_{405} \text{ NSB}) \times 100$$

   La corba patró es genera utilizant el % d'unió (%B) en cada cas i el logaritme de la concentració total del regulador (pmol/ml), i ajustant una corba sigmoide. Aquesta corba pot ajustar-se a una recta mitjançant la transformació Log-Logit dels valors %B.

   $$\text{Logit } \%B = \text{Ln } [\%B / (100 - \%B)]$$

8. Els nivells d'hormones presents a les mostres es determinaren per interpolació del percentatge d'unió en les mostres a partir de la recta patró.
9. Les dades obtingudes per cada CK es corregiren d'acord amb el percentatge d'entrecreuament de cada anticòs amb l'antigen corresponent (Taula 1, Fernández et al. 1995), amb el factor de dilució de la mostra i amb el percentatge de recuperació obtingut per la BA marcada radioactivament.

Quantificació de l'IAA

En el cas de l'IAA es disposava d'anticossos monoclonals, que reaccionen amb aquesta molècula metilada. Per tant, prèviament al test immunològic no fou necessari el pas de separació.

1. La fracció que contenia l'IAA es metilà amb diazometà per tal d'augmentar la seva estabilitat química i reduir les pèrdues d'hormona (Neill i Horgan 1987).
2. Després de la metilació l'IAA es quantificà mitjançant un ELISA, utilizant un anticòs monoclonal anti-IAA metilat amb un rang de sensibilitat de 0.5-100 pmol/0.1 ml (Agdia™). Aquests assaig ELISA és de tipus indirecte idèntic al descrit anteriorment per les CKs i s'efectuà seguint el protocol adjunt al producte. La quantitat de color obtinguda en la reacció és inversament proporcional a la quantitat d'hormona present a la mostra, deduïda de la corba patró (0.05 pmol-500 pmol/0.1 ml de TBS).
3. Els valors obtinguts per l'IAA en els immunoassatjos es corregiren d'acord amb el factor de dilució de la mostra i amb el percentatge de recuperació mesurat per l'IAA marcat radioactivament.

Annex 2. Mètode d'immunolocalització d'hormones vegetals

El processament del material vegetal, bases de pètals, així com l'immunomarcatge de les citocinines (CKs) en les seccions transversals semifines i ultrafines, es realitzà seguint una modificació del mètode de Sossountzov et al. (1988), i amb la col·laboració de la Unitat de Microscòpia Electrònica del Serveis Cientificotècnics de la Universitat de Barcelona. Es van usar anticossos policlonals anti-CKs, obtinguts contra els ribòsids de les CKs (OlChemIm Ltd., Txèquia). Cadascun dels tres anticossos, anti-iPR, anti-ZR i anti-DHZR, té reaccions creuades amb la base lliure corresponent (N^6-isopenteniladenina (iP), zeatina (Z) i dihidrozeatina (DHZ)) (Taula 1). El mètode de fixació és el que ens permetrà que quedin retingudes bé les bases lliures bé els ribòsids de les CKs (Sossountzov et al. 1988, Sotta et al. 1992).

Taula 1. Reaccions creuades (%) de diferents CKs amb els anticossos policlonals anti-CKs (OlChemIm Ltd., Txèquia).

CK	Anti-ZR	Anti-DHZR	Anti-iPR
ZR	100	-	-
Z	37.0	-	-
DHZR	2.4	100	-
DHZ	1.5	45.0	-
iPR	-	-	100
iP	-	-	52.9

Processament dels teixits

1. Per la fixació dels ribòsids de les CKs es realizà el procés A, mentre que per la fixació de les bases lliures de les CKs es dugué a terme el procés B (Sossountzov et al. 1988, Sotta et al. 1992, Dewitte et al. 1999).

   A. Les mostres foren sotmeses al mètode del periodat-borohidrid (Sossountzov et al. 1988, Dewitte et al. 1999), ja que permet als ribòsids de les CKs unir-se a les proteïnes cel·lulars. Les mostres foren submergides en metaperiodat sòdic 20 mM, dissolt en tampó carbonat-bicarbonat 50 mM, pH 9.6, a temperatura ambient durant 2 h. Això fa que es doni una reacció d'oxidació dels ribòsids, i que les funcions dialdehid produïdes per l'obertura de l'anell de ribosa reaccionin amb els grups amino de les proteïnes cel·lulars. Les bases de Schiff produïdes s'estabilitzaren amb borohidrid sòdic 2 mM en tampò Tris, pH 7.6, durant 1 h, després de la qual les mostres es rentaren amb tampó fosfat 0.1 M, pH 7.4. Ambdues solucions foren preparades just abans d'utilitzar-se. Posteriorment les mostres foren fixades en paraformaldehid al 4% (v/v) i glutaraldehid al 0.1% (v/v) en tampó fosfat 0.1 M, pH 7.4, tot fent el buit a 4ºC durant 4-6 dies.

B. Per la detecció de les bases lliures de les CKs, les mostres foren fixades directament en paraformaldehid al 4% (v/v) i glutaraldehid al 0.1% (v/v) en tampó fosfat 0.1 M, pH 7.4, tot fent el buit a 4ºC durant 4-6 dies. Tant en aquest cas com en l'anterior, s'utilitzà aquest fixador en comptes d'un amb una major proporció de glutaraldehid, ja que aquest últim, malgrat tenir excel.lents propietats relatives a la preservació morfològica, no assegura una bona conservació de la reactivitat immunològica. En canvi, es pot dir que el paraformaldehid conserva acceptablement una bona part dels antígens tissulars ja que no modifica l'estructura secundària de les proteïnes i d'aquesta manera és menys susceptible d'impedir l'accés dels anticossos als epítops.

2. Les mostres es rentaren amb tampó fosfat 0.1 M (4 x 10 min) i amb aigua destil·lada (2 x 5 min).
3. Seguidament, les mostres se sotmeteren a una deshidratació mitjançant la immersió en una sèrie de solucions amb concentracions creixents d'acetona amb aigua: 50 % (v/v) (1 x 10 min), 70 % (2 x 10 min), 90 % (3 x 10 min), 96 % (3 x 10 min) i 100 % (3 x 15 min).
4. Les mostres foren incloses en resina Spurr (1969), amb solucions amb concentracions creixents de resina amb acetona: 25 % (v/v) (mínim 4 h), 50 % (mínim 4 h), 75 % (mínim 4 h) i 100 % (3 x mínim 12 h), en agitació i a temperatura ambient, excepte l'últim pas que es feu a 4ºC.
5. Finalment, les mostres es dipositaren en un motlle de silicona en el que s'hi afegí la resina. La polimerització es produí a 60ºC durant 2 dies.
6. A partir dels blocs obtinguts s'obtingueren seccions semifines (1 μm) per dur a terme l'estudi a nivell tissular. Un cop seleccionades les àrees d'interès es realitzaren les seccions ultrafines (50-60 nm), pels estudis cel·lulars. Les seccions es van obtenir mitjançant un ultramicròtom (Ultracut E, Reichert-Jung), amb ganivetes de vidre per tallar les seccions semifines i ganivetes de diamant (Diatome) per tallar les ultrafines.

Procediments immunocitoquímics

A nivell tissular

La immunolocalització per l'observació al microscopi òptic es dugué a terme en seccions semifines col·locades en portaobjectes prèviament gelatinitzats.

1. Les seccions es tractaren durant 10 min amb peròxid d'hidrogen al 10% (v/v), per tal de permeabilitzar la resina als anticossos, i es rentaren amb aigua destil·lada (3 x 5 min).
2. A continuació s'incubaren en la solució de bloqueig: tampó fosfat salí (PBS) 0.1 M, pH 7.4, amb albúmina de sèrum boví (BSA) a l'1% (p/v) i Tween-20 al 0.05% (v/v), a temperatura ambient durant 15 min. El tractament de bloqueig de les seccions amb una proteïna permet neutralitzar grups aldehids i saturar possibles unions no específiques dels anticossos.

3. Posteriorment, les seccions s'incubaren en 40 μl de l'anticòs primari corresponent, en una cambra humida a 4ºC durant tota una nit. Els anticossos anti-iPR, anti-ZR i anti-DHZR s'utilitzaren a una concentració de 0.996 μg μl^{-1}, 1.436 μg μl^{-1} i 0.884 μg μl^{-1}, respectivament, en la solució de bloqueig.
4. Seguidament, les seccions es rentaren amb de solució de bloqueig (3 x 5 min), per tal d'eliminar els anticossos que no s'havien unit a la mostra, i s'incubaren en Proteïna A conjugada amb or col·loïdal (5 nm) diluïda 1:85 en PBS 0.1 M, pH 7.4, amb BSA al 5% i Tween-20 al 0.05%, a temperatura ambient durant 1h.
5. Les seccions foren rentades amb PBS 0.1 M, pH 7.4, (3 x 5 min) i amb aigua destil·lada (4 x 5 min) i, un cop seques, se sotmeteren a una reacció d'amplificació amb plata (British BioCell International kit), durant 14 min, seguint el protocol adjunt al producte. La precipitació de la plata al voltant de les partícules d'or es visualitza com un senyal brillant quan s'observa en un microscopi òptic amb llum reflectida. La plata té l'avantatge que la marca que s'obté perdura al llarg del temps.

A nivell cel·lular

L'immunomarcatge per l'observació al microscopi electrònic es dugué a terme en seccions ultrafines col·locades en reixetes d'or amb una membrana de formwar.

1. Les seccions es tractaren 5 min amb peròxid d'hidrogen al 10%, per tal de permeabilitzar la resina als anticossos, i es rentaren amb aigua destil·lada (3 x 5 min).
2. Les seccions foren incubades en la solució de bloqueig: PBS 0.1 M, pH 7.4, amb BSA a l'1% (p/v) i Tween-20 al 0.05% (v/v), a temperatura ambient durant 15 min.
3. Les mostres s'incubaren en 20 μl de l'anticòs primari anti-iPR en una dilució 0.498 μg μl^{-1} en la solució de bloqueig, en una cambra humida a 4ºC durant tota una nit.
4. Posteriorment es rentaren amb la solució de bloqueig (3 x 5 min) i s'incubaren en Proteïna A conjugada amb or col·loïdal (10 nm), diluïda 1:70 en PBS 0.1 M, pH 7.4, amb BSA al 5% i Tween-20 al 0.05%, a temperatura ambient durant 1h.
5. Les mostres es rentaren amb PBS 0.1 M, pH 7.4, (3 x 5 min) i amb aigua destil·lada (4 x 5 min), i la marca d'or s'amplificà amb plata (British BioCell International kit) durant 1 min.
6. Les mostres es contrastaren amb acetat d'uranil durant 15 min i citrat de plom (Reynolds 1963) durant 3 min.
7. Les reixetes s'observaren en un microscopi electrònic de transmissió JEOL 1010 a 80 kV. Donat que la plata és electrodensa, les partícules de plata es visualitzen com a taques negres en la mostra. Malgrat que l'or col·loïdal també és electrodens, l'amplificació amb plata ens permet una visualització del marcatge a una escala més gran.

En ambdós casos els controls per l'especificitat de l'immunomarcatge foren el reemplaçament de la incubació amb l'anticòs primari per la incubació amb la solució de bloqueig sense aquest anticòs primari.

BIBLIOGRAFIA

(Introducció, Resum dels resultats i discussió i Annexos)

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Aloni R (1995) The induction of vascular tissue by auxin and cytokinin. In: Davies PJ (ed) Plant Hormones: Physiology, Biochemistry and Molecular Biology (pp. 531-546). Kluwer Academic Publishers, Dordrecht, The Netherlands

Andrés H, Fernández B, Rodríguez R, Rodríguez A (2002) Phytohormone contents in *Corylus avellana* and their relationship to age and other developmental processes. Plant Cell Tiss. Org. Cult. 70:173-180

Arigita L, Sánchez-Tamés R, González A (2003) 1-Methylcyclopropane and ethylene as regulators of *in vitro* organogenesis in kiwi explants. Plant Growth Regul. 40:59-64

Astot C, Dolezal K, Nordström A, Wang Q, Kunkel T, Moritz T, Chua N-H, Sandberg G (2000) An alternative cytokinin biosynthesis pathway. Proc. Natl. Acad. Sci. USA 97:14778-14783

Attfield EM, Evans PK (1991a) Developmental pattern of root and shoot organogenesis in cultured leaf explants of *Nicotiana tabacum* cv. Xanthi nc. J. Exp. Bot. 42:51-57

Attfield EM, Evans PK (1991b) Stages in the initiation of root and shoot organogenesis in cultured leaf explants of *Nicotiana tabacum* cv. Xanthi nc. J. Exp. Bot. 42:59-63

Azmi A, Dewitte W, van Onckelen HA, Chriqui D (2001) In situ localization of endogenous cytokinins during shooty tumor development on *Eucalyptus globulus* Labill. Planta 213:29-36

Bandurski RS, Cohen JD, Slovin JP, Reinecke DM (1995) Auxin biosynthesis and metabolism. In: Davies PJ (ed) Plant Hormones: Physiology, Biochemistry and Molecular Biology (pp. 39-65). Kluwer Academic Publishers, Dordrecht, The Netherlands

Bartel B, LeClere S, Magidin M, Zolman BJK (2001) Inputs to the active indole-3-acetic acid pool: *de novo* synthesis, conjugate hydrolysis, and indole-3-butyric acid ß-oxidation. J. Plant Growth Regul. 20:198-216

Bell RL, Scorza R, Srinivasan C, Webb K (1999) Transformation of "Beurre Bosc" pear with the *rolC* gene. J. Amer. Soc. Hort. Sci. 124:570-574

Bilyeu KD, Cole JL, Laskey JG, Riekhof WR, Esparza TJ, Kramer MD, Morris RO (2001) Molecular and biochemical characterization of a cytokinin oxidase from maize. Plant Physiol. 125:378-386

Binns AN, Costantino P (1998) The *Agrobacterium* oncogenes. In: Spaink H, Kondorosi A, Hooykaas PJJ (eds) The Rhizobiaceae (pp. 251-266). Kluwer Press, Dordrecht, The Netherlands

Bitonti MB, Cozza R, Chiappetta A, Giannino D, Castiglione MR, Dewitte W, Mariotti D, van Onckelen H, Innocenti AM (2002) Distinct nuclear organization, DNA methylation pattern and cytokinin distribution mark juvenile, juvenile-like and adult vegetative apical meristems in peach (*Prunus persica* (L.) Barsch). J. Exp. Bot. 53:1047-1054

Blazková A, Macháčková I, Eder J, Krekule J (2001) Benzyladenine-induced inhibition of flowering in *Chenopodium rubrum in vitro* is not related to the levels of isoprenoid cytokinins. Plant Growth Regul. 34:159-166

Boase MR, Winefield CS, Lill TA, Bendall MJ (2004) Transgenic regal pelargoniums that express the *rol*C gene from *Agrobacterium rhizogenes* exhibit a dwarf floral and vegetative phenotype. In Vitro Cell. Dev. Biol. 40:46-50

Boniotti MB, Griffith ME (2002) "Cross-talk" between cell division cycle and development in plants. Plant Cell 14:11-16

Bornman CH, Vogelmann TC (1984) Effect of rigidity of gel medium on benziladenine-induced adventitious bud formation and vitrification in vitro in *Picea abies*. Physiol. Plant. 61:505-512

Boucheron E, Guivarc'h A, Azmi A, Dewitte W, van Onckelen HA, Chriqui D (2002) Competency of *Nicotiana tabacum* L. stem tissues to dedifferentiate is associated with differential levels of cell cycle gene expression and endogenous cytokinins. Planta 215:267-278

Brown DCW, Leung DWM, Thorpe TA (1979) Osmotic requirement for shoot formation in tobacco callus. Physiol. Plant. 46:36-41

Burritt DJ, Leung DWM (1996) Organogenesis in cultured petiole explants of *Begonia* x *erythrophylla*: the timing and specificity of the inductive stimuli. J. Exp. Bot. 47:557-567

Capelle SC, Mok DWS, Kirchner SC, Mok MC (1983) Effects of thidiazuron on cytokinin autonomy and the metabolism of N^{6}-(÷^{2}-isopentenyl)[8-^{14}C]adenosine in callus tissues of *Phaseolus lunatus* L. Plant Physiol. 73:796-802

Cassells AC (2002) Tissue culture for ornamental breeding. In: Vainstein A (ed) Breeding For Ornamentals: Classical and Molecular Approaches (pp. 139-153). Kluwer Academic Publishers, Dordrecht, The Netherlands

Castro-Concha L, Loyola-Vargas VM, Chan JL, Robert ML (1990) Glutamate dehydrogenase activity in normal and vitrified plants of *Agave tequilana* Weber propagated in vitro. Plant Cell Tiss. Org. Cult. 22:147-151

Catterou M, Dubois F, Smets R, Vaniet S, Kichey T, van Onckelen H, Sangwan-Norreel BS, Sangwan RS (2002) *hoc*: an *Arabidopsis* mutant overproducing cytokinins and expressing high *in vitro* organogenic capacity. Plant J. 30:273-287

Celenza JL, Grisafi PL, Fink GR (1995) A pathway for lateral root formation in *Arabidopsis thaliana*. Genes Dev. 9:2131-2142

Centeno ML, Rodríguez A, Feito I, Fernández B (1996) Relationship between endogenous auxin and cytokinin levels and the morphogenic responses in *Actinidia deliciosa* tissue cultures. Plant Cell Rep. 16:58-62

Centeno ML, Rodríguez A, Berros B (1997) Endogenous hormonal content and somatic embryogenic capacity of *Corylus avellana* L. cotyledons. Plant Cell Rep. 17:139-144

Centeno ML, Rodríguez A, Albuerne MA, Feito I, Fernández B (1998) Uptake, distribution and metabolism of 6-benzyladenine and cytokinin content during callus initiation from *Actinidia deliciosa* tissues. J. Plant Physiol. 152:480-486

Centeno ML, Rodríguez A, Feito I, Sánchez-Tamés R, Fernández B (2003) Uptake and metabolism of N^6-benzyladenine and 1-naphthalenaeacetic acid and dynamics of indole-3-acetic acid and cytokinins in two callus lines of *Actidinia deliciosa* differing in growth and shoot organogenesis. Physiol. Plant. 118:579-588

Chen C-M (1997) Cytokinin biosynthesis and interconversion. Physiol. Plant. 101:665-673

Chriqui D, Guivarc'h A, Dewitte W, Prinsen E, van Onckelen H (1996) *Rol* genes and root initiation and development. Plant and Soil 187:47-55

Christey MC (1997) Transgenic crop plants using *Agrobacterium rhizogenes*-mediated transformation. In: Doran PM (ed) Hairy roots: Culture and Applications (pp. 99-111). Harwood Academic Publishers, Amsterdam, The Netherlands

Christey MC (2001) Use of Ri-mediated transformation for production of transgenic plants. In Vitro Cell. Dev. Biol. 37:687-700

Christianson ML, Warnick DA (1983) Competence and determination in the process of *in vitro* shoot organogenesis. Devel. Biol. 95:288-293

Cleland RE (1999) Introduction: Nature, occurrence and functioning of plant hormones. In: Hooykaas PJJ, Hall MA, Libbenga KR (eds) Biochemistry and Molecular Biology of Plant Hormones (pp. 3-22). Elsevier Science B.V., Amsterdam, The Netherlands

Coenen C, Lomax TL (1997) Auxin-cytokinin interactions in higher plants: old problems and new tools. Trends Plant Sci. 2:351-356

Collin HA, Edwards S (1998) Plant Cell Culture. BIOS Scientific Publishers Ltd, Springer-Verlag, New York

Crozier A, Kamiya Y, Bishop G, Yokota T (2000) Biosynthesis of hormones and elicitor molecules. In: Buchanan B, Gruissem W, Jones R (eds) Biochemistry and Molecular Biology of Plants American Society of Plant Physiologists, Rockville, Maryland, USA

D'Agostino IB, Kieber JJ (1999) Molecular mechanisms of cytokinin action. Curr. Opin. Plant Biol. 2:359-364

Davies PJ (1995) The plant hormones: Their nature, occurrence, and functions. In: Davies PJ (ed) Plant Hormones: Physiology, Biochemistry and Molecular Biology (pp. 1-12). Kluwer Academic Publishers, Dordrecht, The Netherlands

Davis MJ, Baker R, Hanan JJ (1977) Clonal multiplication of carnation by micropropagation. J. Amer. Soc. Hort. Sci. 102:48-53

Debergh P, Harbaoui Y, Lemeur R (1981) Mass propagation of globe artichoke (*Cynara scolymus*): Evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53:181-187

Debergh P, Aitken-Christie J, Cohen D, Grout B, von Arnold S, Zimmerman R, Ziv M (1992) Reconsideration of the term 'vitrification' as used in micropropagation. Plant Cell Tiss. Org. Cult. 30:135-140

Demmink JF, Custers JBM, Bergervoet JHW (1987) Gynogenesis to bypass crossing barriers between diploid and tetraploid *Dianthus* species. Acta Hort. 216:343-344

Deroles SC, Boase MR, Lee CE, Peters TA (2002) Gene transfer to plants. In: Vainstein A (ed) Breeding For Ornamentals: Classical and Molecular Approaches (pp. 155-196). Kluwer Academic Publishers, Dordrecht, The Netherlands

Dewitte W, Chiappetta A, Azmi A, Witters E, Strnad M, Rembur J, Noin M, Chriqui D, van Onckelen HA (1999) Dynamics of cytokinins in apical shoot meristems of a day-neutral tobacco during floral transition and flower formation. Plant Physiol. 119:111-121

Dewitte W, Murray JAH (2003) The plant cell cycle. Annu. Rev. Plant Biol. 54:235-264

Dhaliwal HS, Ramesar-Fortner NS, Yeung EC, Thorpe TA (2003) Competence, determination, and meristemoid plasticity in tobacco organogenesis in vitro. Can. J. Bot. 81:611-621

Dobrev P, Motyka V, Gaudinová A, Malbeck J, Trávnícková A, Kamínek M, Vanková R (2002) Transient accumulation of *cis*- and *trans*-zeatin type cytokinins and its relation to cytokinin oxidase activity during cell cycle of synchronized tobacco BY-2 cells. Plant Physiol. Biochem. 40:333-337

Earle ED, Langhans RW (1975) Carnation propagation from shoots tips cultured in liquid medium. HortSci. 10:608-610

Eklöf S, Astot C, Sitbon F, Moritz T, Olsson O, Sandberg G (2000) Transgenic tobacco plants co-expressing *Agrobacterium iaa* and *ipt* genes have wild-type hormone levels but display both auxin- and cytokinin-overproducing phenotypes. Plant J. 23:279-284

Estruch JJ, Chriqui D, Grossmann K, Schell J, Spena A (1991a) The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates. EMBO J. 10:2889-2895

Estruch JJ, Parets-Soler A, Schmülling T, Spena A (1991b) Cytosolic localization in transgenic plants of the *rolC* peptide from *Agrobacterium rhizogenes*. Plant Mol. Biol. 17:547-550

Fagard M, Vaucheret H (2000) (Trans)gene silencing in plants: How many mechanisms? Annu. Rev. Plant Physiol. Plant Mol. Biol. 51:167-194

Faiss M, Strnad M, Redig P, Dolezal K, Hanus J, van Onckelen H, Schmülling T (1996) Chemically induced expression of the *rolC*-encoded η-glucosidase in transgenic tobacco plants and analysis of cytokinin metabolism: rolC does not hydrolyze endogenous cytokinin glucosides in planta. Plant J. 10:33-46

Fal MA, Majada JP, González A, Sánchez-Tamés R (1999) Differences between *Dianthus caryophyllus* L. cultivar in *in vitro* growth and morphogenesis are related to their ethylene production. Plant Growth Regul. 27:131-136

Feito I, Rodríguez A, Centeno ML, Sánchez-Tamés R, Fernández B (1995) Effect of applied benzyladenine on endogenous cytokinin content during the early stages of bud development of kiwifruit. Physiol. Plant. 95:241-246

Fernández B, Centeno ML, Feito I, Sánchez-Tamés R, Rodríguez A (1995) Simultaneous analysis of cytokinins, auxins and abscisic acid by combined immunoaffinity chromatography, high performance liquid chromatography and immunoassay. Phytochem. Anal. 6:49-54

Fiola JA, Hassan MA, Swartz HJ, Bors RH, McNicols R (1990) Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoots organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell Tiss. Org. Cult. 20:223-228

Fisher M, Ziv M, Vainstein A (1993) An efficient method for adventitious shoot regeneration from cultured carnation petals. Sci. Hort. 53:231-237

Fladung M (1990) Transformation of diploid and tetraploid potato clones with the *rolC* gene of *Agrobacterium rhizogenes* and characterization of transgenic plants. Plant Breed. 104:295-304

Fladung M, Grossmann K, Ahuja MR (1997) Alterations in hormonal and developmental characteristics in transgenic *Populus* conditioned by the *rolC* gene from *Agrobacterium rhizogenes*. J. Plant Physiol. 150:420-427

Francis D, Sorrell DA (2001) The interface between the cell cycle and plant growth regulators: a mini review. Plant Growth Regul. 33:1-12

Franck T, Kevers C, Gaspar T, Dommes J, Deby C, Greimers R, Serteyn D, Deby-Dupond G (2004) Hyperhydricity of *Prunus avium* shoots cultured on gelrite: a controlled stress response. Plant Physiol. Biochem. 42:519-527

Frank M, Schmülling T (1999) Cytokinin cycles cells. Trends Plant Sci. 4:243-244

Frey L, Janick J (1991) Organogenesis in carnation. J. Amer. Soc. Hort. Sci. 116:1108-1112

Frey L, Saranga Y, Janick J (1992) Somatic embryogenesis in carnation. HortSci. 27:63-65

Fujiwara K, Kozai T (1995) Physical microenvironment and its effects. In: Aitken-Christie J, Kozai T, Smith ML (eds) Automation and Environmental Control in Plant Tissue Culture (pp. 319-369). Kluwer Academic Publishers, Dordrecht, The Netherlands

Fukui Y, Tanaka N, Kusumi T, Iwashita T, Nomoto K (2003) A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3',5'-hydroxylase gene. Phytochemistry 63:15-23

Gaspar T, Kevers C, Debergh P, Maene L, Paques M, Boxus P (1987) Vitrification: morphological, physiological, and ecological aspects. In: Bonga JM, Durzan DJ (eds) Cell and Tissue Culture in Forestry. Vol.1. General Principles and Biotechnology (pp. 152-166). Martinus Nijhoff Publishers, Dordrecht, The Netherlands

Gaudin V, Vrain T, Jouanin L (1994) Bacterial genes modifying hormonal balances in plants. Plant Physiol. Biochem. 32:11-29

Gautheret RJ (1939) Sur la possibilité de réaliser la culture indéfinie des tissue de tubercules de carotte. C. R. Hebd. Seances Acad. Sci. 208:118-121

Genkov T, Tsoneva P, Ivanova I (1997) Effect of cytokinins on photosynthetic pigments and chlorophyllase activity in in vitro cultures of axillary buds of *Dianthus caryophyllus* L. J. Plant Growth Regul. 16:169-172

George EF (1993) Plant Propagation by Tissue Culture. Exegetics Ltd., England

Gimelli F, Ginatta G, Venturo R, Positano S, Buiatti M (1984) Plantlet regeneration from petals and floral induction *in vitro* in the Mediterranean carnation (*Dianthus caryophyllus* L.). Riv. Ortoflorofrutt. It. 68:107-121

Giovannini A, Zottini M, Morreale G, Spena A, Allavena A (1999) Ornamental traits modification by *rol* genes in *Osteospermum ecklonis* transformed with *Agrobacterium tumefaciens*. In Vitro Cell. Dev. Biol. 35:70-75

Hackett WP, Anderson JM (1967) Aseptic multiplication and maintenance of differentiated carnation shoot tissue derived from shoot apices. Proc. Amer. Soc. Hort. Sci. 90:365-369

Haensch K-T (2004) Thidiazuron-induced morphogenetic response in petiole cultures of *Pelagonium* x *hortorum* and *Pelargonium* x *domesticum* and its histological analysis. Plant Cell Rep. 23:211-217

Hakkaart FA, Versluijs JMA (1983) Some factors affecting glassiness in carnation meristem tip cultures. Neth. J. Plant Pathol. 89:47-53

Hare PD, van Staden J (1994) Inhibitory effect of thidiazuron on the activity of cytokinin oxidase isolated from soybean callus. Plant Cell Physiol. 35:1121-1125

Hemerly AS, Ferreira P, de Almeida Engler J, van Montagu M, Engler G, Inzé D (1993) *cdc2a* expression in Arabidopsis is linked with competence for cell division. Plant Cell 5:1711-1723

Holley WD, Baker R (1963) Carnation Production. Including the History, Breeding, Culture and Marketing of Carnations. M.C.Brown, Dubuque, Iowa, USA

Horgan R, Scott IM (1987) Cytokinins. In: Rivier L, Crozier A (eds) Principles and Practice of Plant Hormone Analysis (pp. 303-365). Academic Press, London, UK

Horn W (2002) Breeding methods and breeding research. In: Vainstein A (ed) Breeding For Ornamentals: Classical and Molecular Approaches (pp. 47-83). Kluwer Academic Publishers, Dordrecht, The Netherlands

Hosseini-Nasr M, Rashid A (2003) Thidiazuron-induced high-frequency shoot regeneration from root region of *Robinia pseudoacacia* L. seedlings. Biol. Plant. 47:593-596

Howell SH, Lall S, Che P (2003) Cytokinins and shoot development. Trends Plant Sci. 8:453-459

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33:105-119

Hutchinson MJ, Saxena PK (1996) Role of purine metabolism in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium x hortorum*) hypocotyl cultures. Physiol. Plant. 98:517-522

Hutchinson MJ, Krishnaraj S, Saxena PK (1996) Morphological and physiological changes during thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium x hortorum* Bailey) hypocotyl cultures. Int. J. Plant Sci. 157:440-446

Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. Nature 409:1060-1063

Ivanova I, Todorov IT, Atanassova L, Dewitte W, van Onckelen HA (1994) Co-localization of cytokinins with proteins related to cell proliferation in developing somatic embryos of *Dactylis glomerata* L. J. Exp. Bot. 45:1009-1017

Jacqmard A, Houssa C, Bernier G (1994) Regulation of cell cycle by cytokinins. In: Mok DWS, Mok MC (eds) Cytokinins: Chemistry, Activity, and Function (pp. 197-215). CRC Press, Boca Raton, FL, USA

Jacqmard A, Detry N, Dewitte W, van Onckelen HA, Bernier G (2002) *In situ* localisation of cytokinins in the shoot apical meristem of *Sinapis alba* at floral transition. Planta 214:970-973

Jelaska S, Sutina R (1977) Maintained culture of multiple plantlets from carnation shoot tips. Acta Hort. 78:333-340

Jelenska J, Deckert J, Kondorosi E, Legocki AB (2000) Mitotic B-type cyclins are differentially regulated by phytohormones and during yellow lupine nodule development. Plant Sci. 150:29-39

Kadota M, Niimi Y (2003) Effects of cytokinin types and their concentrations on shoot proliferation and hyperhydricity in *in vitro* pear cultivar shoots. Plant Cell Tiss. Org. Cult. 72:261-265

Kakehi M (1979) Studies on the tissue culture of carnation. V. Induction of redifferentiated plants from the petal tissue. Bull. Hiroshima Agric. Coll. 6:159-166

Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. Plant Cell Physiol. 42: 677-685

Kakimoto T (2003) Perception and signal transduction of cytokinins. Annu. Rev. Plant Biol. 54:605-627

Kallak H, Hilpus I, Virumäe K (1996) Influence of genotype and growth regulators on morphogenetic processes in carnation shoot apex cultures. Sci. Hort. 65:181-189

Kamínek M, Armstrong DJ (1990) Genotypic variation in cytokinin oxidase from *Phaseolus* callus cultures. Plant Physiol. 93:1530-1538

Kamínek M, Motyka V, Vanková R (1997) Regulation of cytokinin content in plant cells. Physiol. Plant. 101:689-700

Kaneyoshi J, Kobayashi S (1999) Characteristics of transgenic trifoliate orange (*Poncirus trifoliata* Raf.) possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid. J. Jpn. Soc. Hort. Sci. 68:734-738

Kärkönen A, Simola LK (1999) Localization of cytokinins in somatic and zygotic embryos of *Tilia cordata* using immunocytochemistry. Physiol. Plant. 105:356-366

Kevers C, Coumans M, Coumans-Gillès M-F, Gaspar T (1984) Physiological and biochemical events leading to vitrification of plants cultured in vitro. Physiol. Plant. 61:69-74

Kevers C, Frank T, Strasser RJ, Dommes J, Gaspar T (2004) Hyperhydricity of micropropagated shoots: a typically stress-induced change of physiological state. Plant Cell Tiss. Org. Cult. 77:181-191

Koshita Y, Nakamura Y, Kobayashi S, Morinaga K (2002) Introduction of the *rolC* gene into the genome of the Japanese persimmon causes dwarfism. J. Jpn. Soc. Hort. Sci. 71:529-531

Kosugi Y, Waki K, Iwazaki Y, Tsuruno N, Mochizuki A, Yoshioka T, Hashiba T, Satoh S (2002) Senescence and gene expression of transgenic non-ethylene-producing carnation flowers. J. Jpn. Soc. Hort. Sci. 71:638-642

Krikorian AD (1995) Hormones in tissue culture and micropropagation. In: Davies PJ (ed) Plant Hormones: Physiology, Biochemistry and Molecular Biology (pp. 774-796). Kluwer Academic Publishers, Dordrecht, The Netherlands

Kurioka Y, Suzuki Y, Kamada H, Harada H (1992) Promotion of flowering and morphological alterations in *Atropa belladonna* transformed with a CaMV 35S-*rolC* chimeric gene of the Ri plasmid. Plant Cell Rep. 12:1-6

Landrieu I, da Costa M, De Veylder L, Dewitte F, Vandepoele K, Hassan S, Wieruszeski JM, Faure JD, van Montagu M, Inzé D, Lippens G (2004) A small CDC25 dual-specificity tyrosine-phosphatase isoform in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 101:13380-13385

Laureys F, Dewitte W, Witters E, van Montagu M, Inzé D, van Onckelen HA (1998) Zeatin is indispensable for the G_2-M transition in tobacco BY-2 cells. FEBS Lett. 426:29-32

Laureys F, Smets R, Lenjou M, van Bockstaele D, Inzé D, van Onckelen HA (1999) A low content in zeatin type cytokinins is not restrictive for the occurrence of G_1/S transition in tobacco BY-2 cells. FEBS Lett. 460:123-128

Lavy M, Zuker A, Lewinsohn E, Larkov O, Ravid U, Vainstein A, Weiss D (2002) Linalool and linalool oxide production in transgenic carnation flowers expressing the *Clarkia breweri* linalool synthase gene. Mol. Breed. 9:103-111

Ledbetter DI, Preece JE (2004) Thidiazuron stimulates adventitious shoot production from *Hydrangea quercifolia* Bartr. leaf explants. Sci. Hort. 101:121-126

Lee C, Wang L, Ke S, Qin M, Cheng Z-M (1996) Expression of the *rolC* gene in transgenic plants of *Salpiglossis sinuata* L. HortSci. 31:571

Leshem B (1986) Carnation plantlets from vitrified plants as a source of somaclonal variation. HortSci. 21:320-321

Leyser O (2003) Regulation of shoot branching by auxin. Trends Plant Sci. 8:541-545

Ljung K, Bhalerao RP, Sandberg G (2001) Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. Plant J. 28:465-474

Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD, Sandberg G (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol. Biol. 50:309-332

Lo KH, Giles KL, Sawhney VK (1997a) Acquisition of competence for shoot regeneration in leaf discs of *Saintpaulia ionantha* x *confusa* hybrid (African violet) cultured in vitro. Plant Cell Rep. 16:416-420

Lo KH, Giles KL, Sawhney VK (1997b) Histological changes associated with acquisition of competence for shoot regeneration in leaf discs of *Saintpaulia ionantha* x *confusa* hybrid (African violet) cultured in vitro. Plant Cell Rep. 16:421-425

Mackay WA, Kitto SL (1988) Factors affecting in vitro shoot proliferation of French tarragon. J. Amer. Soc. Hort. Sci. 113:282-287

Majada JP, Fal MA, Sánchez-Tamés R (1997) The effect of ventilation rate on proliferation and hyperhydricity of *Dianthus caryophyllus* L. In Vitro Cell. Dev. Biol. 33:62-69

Majada JP, Centeno ML, Feito I, Fernández B, Sánchez-Tamés R (1998) Stomatal and cuticular traits on carnation tissue culture under different ventilation conditions. Plant Growth Regul. 25:113-121

Majada JP, Tadeo F, Fal MA, Sánchez-Tamés R (2000) Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell Tiss. Org. Cult. 63:207-214

Majada JP, Sierra MI, Sánchez-Tamés R (2001) Air exchange rate affects the in vitro developed leaf cuticle of carnation. Sci. Hort. 87:121-130

Malik KA, Saxena PK (1992) Regeneration in *Phaseolus vulgaris* L.: High-frequency induction of direct shoot formation in intact seedlings by N^6-benzylaminopurine and thidiazuron. Planta 186:384-389

Masalles RM, Carreras J, Farràs A, Ninot JM, Camarasa JM (eds) (1988) Plantes Superiors. Història Natural dels Països Catalans, Vol. 6. Enciclopèdia Catalana S.A., Barcelona

Maurel C, Barbier-Brygoo H, Spena A, Tempé J, Guern J (1991) Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. Plant Physiol. 97:212-216

Maurel C, Leblanc N, Barbier-Brygoo H, Perrot-Rechenmann C, Bouvier-Durand M, Guern J (1994) Alterations of auxin perception in *rolB*-transformed tobacco protoplasts. Plant Physiol. 105:1209-1215

McGaw BA, Burch LR (1995) Cytokinin biosynthesis and metabolism. In: Davies PJ (ed) Plant Hormones: Physiology, Biochemistry and Molecular Biology (pp. 98-117). Kluwer Academic Publishers, Dordrecht, The Netherlands

Messeguer J, Arconada MC, Melé E (1993) Adventitious shoot regeneration in carnation (*Dianthus caryophyllus* L.). Sci. Hort. 54:153-163

Meyer AD, Tempé J, Costantino P (2000) Hairy root: A molecular overview. Functional analysis of *Agrobacterium rhizogenes* T-DNA genes. In: Stacey G, Keen NT (eds) Plant-Microbe Interactions (pp. 93-139). APS Press, St. Paul, MI, USA

Mii M, Buiatti M, Gimelli F (1990) Carnation. In: Ammirato PV, Evans DR, Sharp WR, Vajaj YPS (eds) Handbook of Plant Cell Culture (pp. 284-318). McGraw-Hill, New York, USA

Miller CO, Skoog F, Von Saltza MH, Strong FM (1955) Kinetin, a cell division factor from deoxyribonucleic acid. J. Amer. Chem. Soc. 77:1392-1393

Miller RM, Kaul V, Hutchinson JF, Maheswaran G, Richards D (1991a) Shoot regeneration from fragmented flower buds of carnation (*Dianthus caryophyllus* L.). Ann. Bot. 68:563-568

Miller RM, Kaul V, Hutchinson JF, Richards D (1991b) Adventitious shoot regeneration in carnation (*Dianthus caryophyllus* L.) from axillary bud explants. Ann. Bot. 67:35-42

Mills D, Tal M (2004) The effect of ventilation on *in vitro* responses of seedlings of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt stress. Plant Cell Tiss. Org. Cult. 78:209-216

Mitiouchkina TY, Dolgov SV (2000) Modification of chrysanthemum flower and plant architecture by *rolC* gene from *Agrobacterium rhizogenes* introduction. The 19th International Symposium on Improvement of Ornamental Plants. Acta Hort. 508:163-169

Miyawaki K, Matsumoto-Kitano M, Kakimoto T (2004) Expression of cytokinin biosynthetic isopentenyltransferase genes in *Arabidopsis*: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. 37:128-138

Mok MC, Mok DWS (1985) The metabolism of [^{14}C]-thidiazuron in callus tissues of *Phaseolus lunatus*. Physiol. Plant. 65:427-432

Mok DWS, Mok MC (2001) Cytokinin metabolism and action. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52:89-118

Mok MC, Mok DWS, Armstrong DJ, Shudo K, Isogai Y, Okamoto T (1982) Cytokinin activity of *N*-phenil-*N'*-1,2,3-thidiazol-5-ylurea (thidiazuron). Phytochemistry 21:1509-1511

Moncaleán P, Rodríguez A, Fernández B (2002) Plant growth regulators as putative physiological markers of developmental stage in *Prunus persica*. Plant Growth Regul. 36:27-29

Mundhara R, Rashid A (2002) Stimulation of shoot-bud regeneration on hypocotyl of *Linum* seedlings, on a transient withdrawal of calcium: effect of calcium, cytokinin and thidiazuron. Plant Sci. 162:211-214

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497

Murthy BNS, Murch SJ, Saxena PK (1995) Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): Endogenous growth regulator levels and significance of cotyledons. Physiol. Plant. 94:268-276

Murthy BNS, Murch SJ, Saxena PK (1998) Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. In Vitro Cell. Dev. Biol. 34:267-275

Nakano M, Mii M (1992) Protoplast culture and plant regeneration of several species in the genus *Dianthus*. Plant Cell Rep. 1:225-228

Nakano M, Mii M (1993a) Callus and root formation from a intergeneric somatic hybrid between *Dianthus caryophyllus* and *Gypsophila paniculata*. Sci. Hort. 53:13-19

Nakano M, Mii M (1993b) Somatic hybridization between *Dianthus chinensis* and *D. barbatus* through protoplast fusion. Theor. Appl. Genet. 86:1-5

Nakano M, Mii M (1995) Regeneration of plants from protoplasts of *Dianthus* species (carnation). In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 34. Plant Protoplasts and Genetic Engineering VI (pp. 33-42). Springer-Verlag, Berlin, Heidelberg, Germany

Nakano M, Hoshino Y, Mii M (1994) Adventitious shoot regeneration from cultured petal explants of carnation. Plant Cell Tiss. Org. Cult. 36:15-19

Nakano M, Hoshino Y, Mii M (1996) Intergeneric somatic hybrid plantlets between *Dianthus barbatus* and *Gypsophila paniculata* obtained by electrofusion. Theor. Appl. Genet. 92:170-172

Neill SJ, Horgan R (1987) Abscisic acid and related compounds. In: Rivier L, Crozier A (eds) Principles and Practice of Plant Hormone Analysis (pp. 111-162). Academic Press, London, UK

Nilsson O, Moritz T, Imbault N, Sandberg G, Olsson O (1993) Hormonal characterization of transgenic tobacco plants expressing the *rolC* gene of *Agrobacterium rhizogenes* TL-DNA. Plant Physiol. 102:363-371

Nilsson O, Moritz T, Sundberg B, Sandberg G, Olsson O (1996) Expression of the *Agrobacterium rhizogenes rolC* gene in a deciduous forest tree alters growth and development and leads to stem fasciation. Plant Physiol. 112:493-502

Nilsson O, Olsson O (1997) Getting to the root: The role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. Physiol. Plant. 100:463-473

Nordström A, Tarkowski P, Tarkowska D, Norbaek R, Astot C, Dolezal K, Sandberg G (2004) Auxin regulation of cytokinin biosynthesis in *Arabidopsis thaliana*: A factor of potential importance for auxin-cytokinin-regulated development. Proc. Natl. Acad. Sci. USA 101:8039-8044

Nugent G, Wardley-Richardson T, Lu CY (1991) Plant regeneration from stem and petal of carnation (*Dianthus caryophyllus* L.). Plant Cell Rep. 10:477-480

Olmos E, Hellín E (1998) Ultrastructural differences of hyperhydric and normal leaves from regenerated carnation plants. Sci. Hort. 75:91-101

Olmos E, Piqueras A, Martínez-Solano JR, Hellín E (1997) The subcellular localization of peroxidase and the implication of oxidative stress in hyperhydrated leaves of regenerated carnation plants. Plant Sci. 130:97-105

Oono Y, Kanaya K, Uchimiya H (1990) Early flowering in transgenic tobacco plants possessing the *rolC* gene of *Agrobacterium rhizogenes* Ri plasmid. Jpn. J. Genet. 65:7-16

Ovadis M, Zuker A, Tzfira T, Ahroni A, Shklarman E, Scovel G, Itzhaki H, Ben-Meir H, Vainstein A (1999) Generation of transgenic carnation plants with novel characteristics by combining microprojectile bombardment with *Agrobacterium tumefaciens* transformation. In: Altman A, Izhar S, Ziv M (eds) Plant Biotechnology and *In Vitro* Biology in the 21st Century (pp. 189-192). Kluwer Academic Publishers, Dordrecht, The Netherlands

Owens LD, Wozniak CA (1991) Measurement and effects of gel matric potential and expressibility on production of morphogenic callus by cultured sugarbeet leaf discs. Plant Cell Tiss. Org. Cult. 26:127-133

Ozawa S, Yasutani I, Fukuda H, Komamine A, Sugiyama M (1998) Organogenesis responses in tissue culture of *srd* mutants of *Arabidopsis thaliana*. Development 125:135-142

Pasqualetto P-L, Zimmerman R, Fordham I (1988) The influence of cation and gelling agent concentration on vitrification of apple cultivars in vitro. Plant Cell Tiss. Org. Cult. 14:31-40

Petru E, Landa Z (1974) Organogenesis in isolated carnation plant callus tissue cultivated *in vitro*. Biol. Plant. 16:450-453

Piqueras A, Cortina M, Serna MD, Casas JL (2002) Polyamines and hyperhydricity in micropropagated carnation plants. Plant Sci. 162:671-678

Redig P, Shaul O, Inzé D, van Montagu M, van Onckelen HA (1996) Levels of endogenous cytokinins, indole-3-acetic acid and abscisic acid during the cell cycle of synchronized tobacco BY-2 cells. FEBS Lett. 391:175-180

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. 17:208-212

Ricci A, Carra A, Torelli A, Maggiali CA, Vicini P, Zani F, Branca C (2001) Cytokinin-like activity of N'-substituted N-phenylureas. Plant Growth Regul. 34:167-172

Roest S, Bokelmann GS (1981) Vegetative propagation of carnation in vitro through multiple shoot development. Sci. Hort. 14:357-366

Sallanon H, Maziere Y (1992) Influence of growth room and vessel humidity on the *in vitro* development of rose plants. Plant Cell Tiss. Org. Cult. 30:121-125

Savin KW, Baunidette SC, Graham MW, Michael MZ, Nugent GD, Lu C-Y, Chandler SF, Cornish EC (1995) Antisense ACC oxidase RNA delays carnation petal senescence. HortSci. 30:970-972

Schmülling T (2001) CREam of cytokinin signaling: receptor identified. Trends Plant Sci. 6:281-284

Schmülling T, Schell J, Spena A (1988) Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J. 7:2621-2629

Schmülling T, Beinsberger S, De Greef J, Schell J, van Onckelen H, Spena A (1989) Construction of a heat-inducible chimaeric gene to increase the cytokinin content in transgenic plant tissue. FEBS Lett. 249:401-406

Schmülling T, Fladung M, Grossmann K, Schell J (1993) Hormonal content and sensitivity of transgenic tobacco and potato plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. Plant J. 3:371-382

Schmülling T, Schäfer S, Romanov GA (1997) Cytokinins as regulators of the gene expression. Physiol. Plant. 100:505-519

Scorza R, Zimmerman TW, Cordts JM, Footen KJ, Ravelonandro M (1994) Horticultural characteristics of transgenic tobacco expressing the *rolC* gene from *Agrobacterium rhizogenes*. J. Amer. Soc. Hort. Sci. 119:1091-1098

Serret MD, Trillas MI (2000) Effects of light and sucrose levels on the anatomy, ultrastructure, and photosynthesis of *Gardenia jasminoides* Ellis leaflets cultured *in vitro*. Int. J. Plant Sci. 161:281-289

Serret MD, Trillas MI, Matas J, Araus JL (1997) The effect of different closure types, light, and sucrose concentrations on carbon isotope composition and growth of *Gardenia jasminoides* plantlets during micropropagation and subsequent acclimation *ex vitro*. Plant Cell Tiss. Org. Cult. 47:217-230

Shabde M, Murashige T (1977) Hormonal requirements of excised *Dianthus caryophyllus* L. shoot apical meristem in vitro. Amer. J. Bot. 64:443-448

Sharma VK, Hänsch R, Mendel RR, Schluze J (2004) A highly efficient plant regeneration system through multiple shoot differentiation from commercial cultivars of barley (*Hordeum vulgare* L.) using meristematic shoot segments excised from germinated mature embryos. Plant Cell Rep. 23:9-16

Shen WH, Davioud E, David C, Barbier-Brygoo H, Tempé J, Guern J (1990) High sensitivity to auxin is a common feature of hairy root. Plant Physiol. 94:554-560

Simard MH, Michaux-Ferriere N, Silvy A (1992) Variants of carnation (*Dianthus caryophyllus* L.) obtained by organogenesis from irradiated petals. Plant Cell Tiss. Org. Cult. 29:37-42

Singh ND, Sahoo L, Sarin NB, Jaiwal PK (2003) The effect of TDZ on organogenesis and somatic embryogenesis in pigeonpea (*Cajanus cajan* L. Millsp). Plant Sci. 164:341-347

Singha S (1982) Influence of agar concentration on *in vitro* shoot proliferation of *Malus* sp. 'Almey' and *Pyrus communis* 'Seckel'. J. Amer. Soc. Hort. Sci. 107:657-660

Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G (1992) Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. Plant Physiol. 99:1062-1069

Skoog F, Miller CO (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. Symp. Soc. Exp. Biol. 11:118-131

Slightom JL, Durand-Tardif M, Jouanin L, Tepfer D (1986) Nucleotide sequence analysis of TL-DNA of *Agrobacterium rhizogenes* agropine type plasmid. J. Biol. Chem. 261:108-121

Smith MAL, Spomer LA (1995) Vessels, gels, liquid media, and support systems. In: Aitken-Christie J, Kozai T, Smith ML (eds) Automation and Environmental Control in Plant Tissue Culture (pp. 371-404). Kluwer Academic Publishers, Dordrecht, The Netherlands

Sossountzov L, Maldiney R, Sotta B, Sabbagh I, Habricot Y, Bonnet M, Miginiac E (1988) Immunocytochemical localization of cytokinins in Craigella tomato and a sideshootless mutant. Planta 175:291-304

Sotta B, Stroobants C, Sossountzov L, Maldiney R, Miginiac E (1992) Immunochemistry applied to cytokinins: Techniques and their validation. In: Kamínek M, Mok DWC, Zazímalová E (eds) Physiology and Biochemistry of Cytokinins in Plants (pp. 455-460). SPB Academic Puhlishing bv, The Hague, The Netherlands

Souq F, Coutos-Thevenot P, Yean H, Delbard G, Maziere Y, Barbe JP, Boulay M (1996) Genetic transformation of roses, 2 examples: one on morphogenesis, the other on anthocyanin biosynthetic pathway. Second International Symposium on Roses. Acta Hort. 424:381-388

Spanò L, Mariotti D, Cardarelli M, Branca C, Costantino P (1988) Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. Plant Physiol. 87:479-483

Spena A, Schmülling T, Koncz C, Schell J (1987) Independent and synergistic activity of *rolA*, *B* and *C* loci in stimulating abnormal growth in plants. EMBO J. 6:3891-3899

Spurr AR (1969) A low viscosity epoxy resin embedding medium for electronmicroscopy. J. Ultrasctruct. Res. 26:31-43

Stals H, Inzé D (2001) When plant cells decide to divide. Trends Plant Sci. 6:359-364

Steward FC, Mapes MO, Mears K (1958) Growth and organized development of cultured plant cells. II. Organization in cultures grown from freely suspended cells. Amer. J. Bot. 45:705-709

Strnad M (1997) The aromatic cytokinins. Physiol. Plant. 101:674-688

Suchomelová P, Velgová D, Masek T, Francis D, Rogers HJ, Marchbank AM, Lipavská H (2004) Expression of the fission yeast cell cycle regulator *cdc25* induces de novo shoot formation in tobacco: evidence of a cytokinin-like effect by this mitotic activator. Plant Physiol. Biochem. 42:49-55

Sugiyama M (1999) Organogenesis *in vitro*. Curr. Opin. Plant Biol. 2:61-64

Sun J, Niu Q-W, Tarkowski P, Zheng B, Tarkowska D, Sandberg G, Chua N-H, Zuo J (2003) The *Arabidopsis AtIPT8*/*PGA22* gene encodes an isopentenyl transferase that is involved in *de novo* cytokinin biosynthesis. Plant Physiol. 131:167-176

Susi P, Hohkuri M, Wahlroos T, Kilby NJ (2004) Characteristics of RNA silencing in plants: similarities and differences across kingdoms. Plant Mol. Biol. 54:157-174

Swarup R, Parry G, Graham H, Allen T, Bennett M (2002) Auxin cross-talk: integration of signaling pathways to control plant development. Plant Mol. Biol. 49:411-426

Swiatek A, Lenjou M, van Bockstaele D, Inzé D, van Onckelen H (2002) Differential effect of jasmonic acid and abscisic acid on cell cycle progression in tobacco BY-2 cells. Plant Physiol. 128:201-211

Takei K, Sakakibara H, Sugiyama T (2001) Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, in *Arabidopsis thaliana*. J. Biol. Chem. 276:26405-26410

Tepfer D (1984) Transformation of several species of higher plants by *Agrobacterium rhizogenes*: Sexual transmission of the transformed genotype and phenotype. Cell 37:959-967

Thomas C, Bronner R, Molinier J, Prinsen E, van Onckelen HA, Hahne G (2002) Immuno-cytochemical localization of indole-3-acetic acid during induction of somatic embryogenesis in cultured sunflower embryos. Planta 215:577-583

Thorpe TA (1994) Morphogenesis and regeneration. In: Vasil IK, Thorpe TA (eds) Plant Cell and Tissue Culture (pp. 17-36). Kluwer Academic Publishers, Dordrecht, The Netherlands

Torné JM, Moysset L, Santos M, Simón E (2001) Effects of light quality on somatic embryogenesis in *Araujia sericifera*. Physiol. Plant. 111:405-411

Tréhin C, Planchais S, Glab N, Perennes C, Tregear J, Bergounioux C (1998) Cell cycle regulation by plant growth regulators: involvement of auxin and cytokinin in the re-entry of *Petunia* protoplasts into the cell cycle. Planta 206:215-224

Trovato M, Linhares F (1999) Recent advances on *rol* genes research: a tool to study plant differentiation. Curr. Top. Plant Biol. 1:51-62

Turner SR, Singha S (1990) Vitrification of crabapple, pear, and geum on gellan gum-solidified culture medium. HortSci. 25:1648-1650

Vainstein A, Hillel J, Lavi U, Tzuri G (1991) Assessment of genetic relatedness in carnation by DNA fingerprints analysis. Euphytica 56:225-229

Vainstein A (ed) (2002) Breeding For Ornamentals: Classical and Molecular Approaches. Kluwer Academic Publishers, Dordrecht, The Netherlands

Valdés AE, Ordás R, Fernández B, Centeno ML (2001) Relationships between hormonal contents and the organogenic response in *Pinus pinea* cotyledons. Plant Physiol. Biochem. 39:377-384

Valdés AE, Centeno ML, Espinel S, Fernández B (2002) Could plant hormones be the basis of maturation indices in *Pinus radiata*? Plant Physiol. Biochem. 40:211-216

van Altvorst AC (1994) Shoot regeneration and *Agrobacterium*-mediated transformation of carnation. Doctoral Thesis. Catholic University Nijmegen, The Netherlands

van Altvorst AC, Bino RJ, van Dijk AJ, Lamers AMJ, Lindhout WH, van der Mark F, Dons JJM (1992a) Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. Plant Sci. 83:77-85

van Altvorst AC, Koehorst HJJ, Bruinsma T, Jansen J, Custers JBM, de Jong J, Dons JJM (1992b) Adventitious shoot formation from *in vitro* leaf explants of carnation (*Dianthus caryophyllus* L.). Sci. Hort. 51:223-235

van Altvorst AC, Koehorst HJJ, Bruinsma T, Dons JJM (1994) Improvement of adventitious shoot formation from carnation leaf explants. Plant Cell Tiss. Org. Cult. 37:87-90

van Altvorst AC, Yancheva S, Dons H (1995) Cells within the nodal region of carnation shoots exhibit a high potential for adventitious shoot formation. Plant Cell Tiss. Org. Cult. 40:151-157

van der Salm TPM, Hänish ten Cate CH, Dons HJM (1996) Prospects for applications of *rol* genes for crop improvement. Plant Mol. Biol. Rep. 14:207-228

van der Salm TPM, van der Toorn CJG, Bouwer R, Hänisch ten Cate CH, Dons HJM (1997) Production of *rol* gene transformed plants of *Rosa hybrida* L. and characterization of their rooting ability. Mol. Breed. 3:39-47

Vaucheret H, Béclin C, Fagard M (2001) Post-transcriptional gene silencing in plants. J. Cell Sci. 114:3083-3091

Victor JMR, Murthy BNS, Murch SJ, Krishnaraj S, Saxena PK (1999) Role of endogenous purine metabolism in thidiazuron-induced somatic embryogenesis of peanut (*Arachis hypogaea* L.). Plant Growth Regul. 28:41-47

Vikrant, Rashid A (2002) Induction of multiple shoots by thidiazuron from caryopsis cultures of minos millet (*Paspalum scrobiculatum* L.) and its effect on the regeneration of embryogenic callus cultures. Plant Cell Rep. 21:9-13

Villalobos V (1981) Floral differentiation in carnation (*Dianthus caryophyllus* L.) from anthers cultured in vitro. Phyton 41:71-75

Vogler H, Kuhlemeier C (2003) Simple hormones but complex signalling. Curr. Opin. Biotechnol. 6:51-56

Watad AA, Ahroni A, Zuker A, Shejtman H, Nissim A, Vainstein A (1996) Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. Sci. Hort. 65:313-320

Werker E, Leshem B (1987) Structural changes during vitrification of carnation plantlets. Ann. Bot. 59:377-385

Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc. Natl. Acad. Sci. USA 98:10487-10492

Werner T, Motyka V, Laucou V, Smets R, van Onckelen HA, Schmülling T (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. Plant Cell 15:2532-2550

Whealy A (1992) Carnations. In: Larson RA (ed) Introduction to floriculture (pp. 43-65). Academic Press, Inc., San Diego, California, USA

White FF, Taylor BH, Huffman GA, Gordon MP, Nester EW (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. J. Bacteriol. 164:33-44

White PR (1943) A Handbook of Plant Tissue Culture. The Ronald Press Company, New York, USA

Winefield C, Lewis D, Arathoon S, Deroles S (1999) Alterations of *Petunia* plant form through the introduction of the *rolC* gene from *Agrobacterium rhizogenes*. Mol. Breed. 5:543-551

Yadav MK, Gaur AK, Garg GK (2003) Development of suitable protocol to overcome hyperhydricity in carnation during micropropagation. Plant Cell Tiss. Org. Cult. 72:153-156

Yamada K, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. Plant Cell Physiol. 42:1017-1023

Yantcheva A, Vlahova M, Antanassov A (1998) Direct somatic embryogenesis and plant regeneration of carnation (*Dianthus caryophyllus* L.). Plant Cell Rep. 18:148-153

Zazímalová E, Kamínek M, Brezinová A, Motyka V (1999) Control of cytokinin biosynthesis and metabolism. In: Hooykaas PJJ, Hall MA, Libbenga KR (eds) Biochemistry and Molecular Biology of Plant Hormones (pp. 141-160). Elsevier Science B.V., Dordrecht, The Netherlands

Zazímalová E, Napier RM (2003) Points of regulation for auxin action. Plant Cell Rep. 21:625-634

Zhang R, Zhang X, Wang J, Letham DS, McKinney SA, Higgins TJV (1995) The effect of auxin on cytokinin levels and metabolism in transgenic tobacco tissue expressing an *ipt* gene. Planta 196:84-94

Zhao Q-H, Fisher R, Auer C (2002) Developmental phases and *STM* expression during *Arabidopsis* shoot organogenesis. Plant Growth Regul. 37:223-231

Ziv M (1991) Vitrification: morphological and physiological disorders of *in vitro* plants. In: Debergh PC, Zimmerman R (eds) Micropropagation. Technology and Application (pp. 45-69). Kluwer Academic Publishers, Dordrecht, The Netherlands

Ziv M, Meir G, Halevy AH (1983) Factors influencing the production of hardened glaucous carnation plantlets *in vitro*. Plant Cell Tiss. Org. Cult. 2:55-65

Zobayed SMA, Armstrong J, Armstrong W (1999) Cauliflower shoot-culture: effects of different types of ventilation on growth and physiology. Plant Sci. 141:209-217

Zuker A, Ahroni A, Tzfira T, Ben-Meir H, Vainstein A (1999) Wounding by bombardment yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Mol. Breed. 5:367-375

Zuker A, Tzfira T, Ben-Meir H, Ovadis M, Shklarman E, Itzhaki H, Forkmann G, Martens S, Neta-Sharir I, Weiss D, Vainstein A (2002) Modification of flower color and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. Mol. Breed. 9:33-41

Zuker A, Tzfira T, Scovel G, Ovadis M, Shklarman E, Itzhaki H, Vainstein A (2001) *rolC*-transgenic carnation with improved agronomic traits: Quantitative and qualitative analyses of greenhouse-grown plants. J. Amer. Soc. Hort. Sci. 126:13-18

Zuker A, Tzfira T, Vainstein A (1998) Genetic engineering for cut-flower improvement. Biotechnol. Adv. 16:33-79