Universitat de Girona

MOLECULAR GENETICS OF CORK FORMATION

Marçal SOLER del MONTE

ISBN: 978-84-691-5735-0
Dipòsit legal: GI-996-2008

DEPARTAMENT DE BIOLOGIA

ÀREA DE BIOLOGIA CEL·LULAR

LABORATORI DEL SURO

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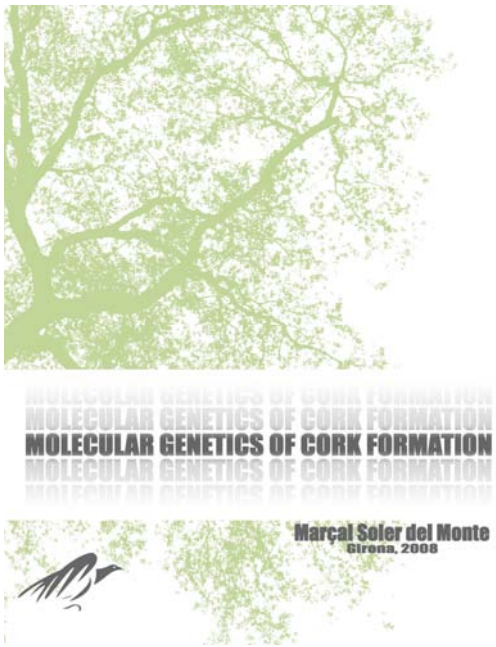

Marçal Soler del Monte

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Memòria presentada per adquirir el grau de
Doctor Europeu per la Universitat de Girona

El doctorand — **Marçal Soler del Monte**

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GIRONA, 2008

Aquesta tesi ha estat realitzada gràcies al suport econòmic del Fons Social Europeu i del Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (beques F.I. i B.E.), juntament amb el Ministerio de Ciencia y Tecnología y el Ministerio de Educación y Ciencia del Govern d'Espanya (projectes AGL2003-00416 i AGL2006-07342) i l'European Forest Genomic Network (beca STSM).

- No hauràs vingut a aquest conte encuriosit pel que t'hem dit unes pàgines abans? Encara que, després de tot, qui ha estat inventat per l'altre? Nosaltres per tu o tu per nosaltres? O potser pel futur lector que llegirà les pròximes pàgines? Arribats a aquest punt, tot pot ser invenció... Fins i tot el lector que ens conegui demà pot ser fruit de la nostra imaginació!

- Tot això és molt suggestiu, però ara jo vull anar-me'n d'aquest somni!

Hugo Pratt, Les Helvètiques

AGRAÏMENTS

Bé, ja hi som, la part de la tesi que tothom llegeix ja que, desenganyem-nos, poca gent llegirà més endavant d'aquest apartat (bé, alguns potser es miraran una mica els "sants", però poca cosa més). O sigui que em tocaria esforçar-me per fer els agraïments, llàstima que els he deixat pel final i ja n'estic fart d'escriure aquesta maleïda tesi. En altres paraules, que no m'hi penso esforçar gaire (encara que els faré amb molt d'amor eh!).

Vinga, comencem pel principi, i què hi ha més al principi que els meus pares (que serien com el meu BigBang particular) i la meva germana, que m'han ajudat i m'han fet costat des de que tinc memòria (i probablement abans)? Bé, potser també la meva àvia, que sempre que tenia un examen es passava la tarda resant per mi (tot i que he de dir que si no hagués resat per mi hagués resat per un altre motiu, encara que això no li treu mèrit). I encara que no els esmenti, gràcies a la resta de la meva família, ja siguin oncles, avis o cosins.

Apa, que això ja va avançant. I tot i ser obligatori, no treu que la Mercè, la meva directora, no es mereixi unes bones gràcies, i gegants, per ajudar-me en tot moment i recolzar-me sempre que no em sortissin les coses (que malauradament era més sovint del que m'hagués agradat). També vull donar les gràcies a l'Olga, la millor companya de "curro" que podia tenir, que m'ha ajudat tant durant aquest anys i sense la qual aquesta tesi no existiria (o com a mínim seria molt diferent de com és ara, però cap a pitjor). I juntament amb elles dues, he de donar les gràcies a la resta del grup, l'Elisabet, la Gemma, en Lluís, la Gisela i l'Eduard, i també a l'Albert i en Pau, ah, i a la "jefa" grossa, la Marisa, que també s'ho mereix i molt.

Avall que fa baixada. Ara toca sortir del grup de recerca, i les dues primeres persones a qui vull agrair són l'Imma i la Sara, que m'han fet la feina molt més fàcil, i també la Laia, que en època de baixes laborals ens va treure les castanyes del foc. També vull donar les gràcies a en Vicenç, l'Antònia i l'Emili, cada un dels quals m'ha ajudat en alguna part de la meva investigació que sol no hagués pogut fer. I encara dins la UdG, tot i que a un altre nivell, he de donar les gràcies a la Laia i a en Jesús, per permetre'm escriure tesi en hores que no tocarien. I encara que no siguin de l'esmentada universitat, també he donar les gràcies a en Tomàs i a en Josep, sense els quals no sé com haguéssim mostrejat les sureres.

Molt bé, ara sortim de la província. I per ordre cronològic, he de donar les gràcies a en Lauro i en Juanjo, del CRG, unes màquines manejant dades estadístiques. I de l'IBMB, vull donar les gràcies a l'Eva, l'Elena i la Marta, i a tota la gent del *blau* que em van ajudar uns dies que vaig estar remenant per allà. I ja a fora del país, al CNB de Madrid, he de donar les gràcies a la Salo pels consells que ens ha donat en tot moment, i he de dir que no van ser pocs.

I ara ja en "plan" internacional, agrair el suport a tota la gent que vaig conèixer a Àustria. En concret vull agrair l'ajuda que vaig rebre en tot moment dels companys de feina i de pis, en especial d'en Christian i l'Edward, com també de l'Eduard i d'en Carles.

I de fora, tornem cap a casa. I del meu poble (o ciutat), vull donar les gràcies als meus amics de tota la vida: en Xavi, l'altre Xavi, en David, l'altre David, en Jordi, en Francesc, l'Arcadi, en Ferran, en Marc, l'Alba, i la Mariona. Probablement els importi molt poc el tema d'aquesta tesi, i segur que seran dels que no passaran dels agraïments, però són únics, els meus amics que m'han fet costat molts anys; i segur que ho faran molts anys més.

Tornant a la UdG, comencem pels amics de la "promo" de biologia: l'Eduard, l'altre Eduard, la Maria, l'altra Maria, i també l'altra Maria, en Joan Marc, en Joel, l'Òscar, en Víctor, en Jordi, l'Amanda, l'Agnès, l'Olga, la Irene, en Gerard, en Miquel, l'Anneta, la Merche, l'Eva, l'Anna, la Mireia, la Carol, l'Estela, la Judith, la Natàlia... i tots els altres que m'he deixat per no seguir pensant més noms. Una de les millors coses d'estudiar biologia va ser coneixe'ls (va, ara ens posem tendres).

I ara, cap als companys de despatx (si és que d'allò se'n pot dir despatx, ja que modestament crec que antre s'adequa millor a les característiques de l'espai en qüestió) i del passadís, sense els quals hagués estat molt avorrit fer el doctorat i que tant m'han recolzat i ajudat tots aquests anys. La part trista d'acabar el doctorat és que segurament ja no els veuré tant sovint: Gaxi (sí, ja sé que l'Anna és l'única a qui dic pel mot, però va, un tracte especial per ajudar-me amb els "encabezados" i "pies de página"), Pere (insubstituïble, em va fer la portada i em va ajudar en qualsevol problema informàtic que tingués), Ariadna (responsable de la foto de la portada), Mireia, Alexandra, Jess, Montse, Vicky, Arantxa, Marc, l'altre Marc, Joan, Eva, l'altra Eva, Judit, Isabel, Marta, Anna, l'altra Anna, i encara l'altra Anna, Elena, Sílvia, Sònia, Dolors, Magalí, Clara, Xevi, Gerard, Roger, Mariona, Pep, l'altra Ariadna, Marga, Tere, Olga (sí, ja sé que la torno a nombrar, però es mereix molts agraïments ella)... i a tots els altres del passadís i de la facultat a qui no nombro per no fer-me encara més pesat, com els membres d'UdG.doc i els del passadís de dalt. Però això encara no s'acaba, que per no repetir més noms he deixat pel final els que van començar biologia amb mi i que també han estat companys de doctorat, hem passat molts anys junts però encara no m'he cansat d'ells, i probablement no me'n cansaré mai: Olaya, Núria i Àlex.

Gràcies a tots, de veritat.

I això ja està. Perdó per si m'he deixat algú (espero que no, em sabria molt de greu), però fent els agraïments l'últim dia pot passar de tot.

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SUMMARY / RESUM / RESUMEN / RÉSUMÉ

SUMMARY

The periderm is a complex structure that protects plant mature (secondary) organs and wounded tissues from water loss, injuries and pathogens. This barrier capacity is accomplished by the cork layer of the periderm, a tissue made of dead cells with suberin deposited into cell walls. Although cork and suberin are critical for the survival of land plants, very few is known about the molecular processes involved in their biosynthesis and differentiation, probably due to the lack of appropriate plant models. Here we developed a strategy to identify and characterize cork candidate genes using a combination of two model plants for periderm studies. The bark of cork oak (*Quercus suber*) was used to identify candidate genes and to analyze the seasonal behaviour of some of these genes. The potato (*Solanum tuberosum*) tuber was used to demonstrate the role of some selected candidates in the regulation of cork by reverse genetic analyses.

We isolated and identified 142 cork candidate genes, mainly up-regulated in the cork of cork oak in comparison to wood and somatic embryo, which suggest a specific role in the periderm. Most of these candidate genes, such as cytochromes P450, ABC transporters, acyltransferases and fatty acid elongases, encode enzymes which have been previously hypothesized as possible members of the suberin biosynthetic pathway. In addition, other candidate genes encode stress responsive and regulatory proteins, which are related to cork and suberin for the first time. Of note that transcript accumulation in cork of genes encoding NAM, MYB, HD-ZIPIII, KNOX and KANADI transcription factors suggests that some regulatory mechanisms may be conserved between the formation of wood and cork.

To contribute to a better understanding of cork formation and phenology, we analyzed the expression pattern during the growing season of twelve selected candidate genes in the cork of cork oak. Our results showed that many genes, such as the enzymes CYP86A1, GPAT and HCBT and the transcription factors NAM and WRKY, showed their highest expression in June, the month when cork growth rate is maximal. In contrast, the expression of genes encoding the enzymes F5H and FAT and the stress proteins HSP17.4 and ANN was correlated with temperature and relative humidity.

To shed some light on the role of the regulatory proteins NAM, RIK, DEM2 and REV in the cork tissue we performed a reverse genetic approach in potato periderm, obtaining in all cases kanamycin resistant lines indicating correct insertion of foreign DNA. Up- and down-regulated lines have been confirmed for the NAM transcription factor, and it is planned to confirm the rest of the lines and to perform phenotypic analyses in brief. If differences are detected between transgenic and wild-type plants, this will be, as far as we know, the first demonstration of the function of regulatory genes in the periderm.

RESUM

La peridermis és una estructura complexa que protegeix els òrgans vegetals madurs (secundaris) i les zones que han sofert ferides de la pèrdua d'aigua i dels patògens. Aquesta funció barrera que realitza la peridermis és deguda al fel·lema o súber, un teixit format per cèl·lules que dipositen suberina a les seves parets. Tant el fel·lema com la suberina són crucials per la vida de les plantes terrestres però, malgrat la seva importància, pràcticament no es coneix res dels processos moleculars que regulen la seva formació, la qual cosa probablement és deguda a la manca de models adequats. En aquesta tesi hem desenvolupat una estratègia per identificar i caracteritzar gens induïts al fel·lema mitjançant la combinació de dues plantes models pel seu estudi. L'escorça d'alzina surera (*Quercus suber*) l'hem utilitzat per aïllar gens candidats de la formació del fel·lema i per investigar el comportament d'alguns d'aquests gens durant l'estació de creixement, mentre que la pela de la patata (*Solanum tuberosum*) l'hem utilitzat per realitzar estudis de genètica reversa per demostrar la funció d'alguns gens reguladors al fel·lema.

Hem aïllat i identificat 142 gens candidats, majoritàriament induïts al fel·lema d'alzina surera en comparació amb el xilema i l'embrió somàtic, cosa que en suggereix un paper específic a la peridermis. Alguns dels gens identificats, com per exemple citocroms P450, transportadors ABC, aciltransferases i elongases d'àcids grassos, codifiquen per enzims que podrien estar implicats en la ruta biosintètica de la suberina segons estudis basats en la seva composició química. D'altres, en canvi, corresponen a gens reguladors i de resposta a estrés, relacionats amb el fel·lema i la suberina per primera vegada. Cal destacar, a més a més, que l'acumulació al fel·lema de transcrits dels gens NAM, MYB, HD-ZIPIII, KNOX i KANADI suggereix que alguns mecanismes reguladors poden estar conservats entre la formació dels teixits vasculars i la peridermis.

Amb la finalitat de contribuir a un millor coneixement de la formació i fenologia del fel·lema, es va estudiar el patró d'expressió de 12 gens durant l'estació de creixement al fel·lema de l'alzina surera. Els resultats mostren que la majoria dels gens, com per exemple els que codifiquen pels enzims CYP86A1, GPAT i HCBT, així com els factors de transcripció NAM i WRKY, tenen un màxim d'expressió al juny, el mes on la taxa de creixement del fel·lema és màxima. Per contra, l'expressió dels gens que codifiquen pels enzims F5H i FAT, juntament amb les proteïnes d'estrès HSP17.4 i ANN, està correlacionada amb la temperatura i la humitat relativa.

Per estudiar el paper d'algunes proteïnes reguladores al fel·lema es van realitzar estudis de genètica reversa a la peridermis de la patata pels gens NAM, RIK, DEM2 i REV, obtenint per tots ells línies resistents a la kanamicina, cosa que indica la correcta inserció del DNA exogen a les plantes. S'ha confirmat l'obtenció de línies induïdes i inhibides pel factor de transcripció NAM, i està previst en breu confirmar la resta de línies, així com la realització d'anàlisis fenotípiques. Si es detecten diferències entre les plantes transgèniques i les controls serà, probablement, la primera vegada que es demostra la funció molecular d'un gen regulador a la peridermis.

RESUMEN

La peridermis es una estructura compleja que protege los órganos vegetales maduros (secundarios) y las zonas heridas de la deshidratación y los patógenos. Esta función barrera que realiza la peridermis es debida al felema o súber, un tejido formado por células que depositan suberina en sus paredes. El felema y la suberina son cruciales para la vida de las plantas terrestres pero, pese a su importancia, apenas se conocen los procesos moleculares que regulan su formación, probablemente debido a la falta de modelos adecuados. En esta tesis hemos desarrollado una estrategia para identificar y caracterizar genes inducidos en el felema mediante la combinación de dos plantas modelo para su estudio. La corteza del alcornoque (*Quercus suber*) la hemos utilizado para aislar genes candidatos de la formación del felema y para investigar el comportamiento de algunos de estos genes durante la estación de crecimiento, mientras que la piel de la patata (*Solanum tuberosum*) la hemos utilizado para realizar estudios de genética reversa con el fin de demostrar la función de algunos genes reguladores en el felema.

Hemos aislado y caracterizado 142 genes candidatos, mayoritariamente inducidos en el felema de alcornoque en comparación con el xilema y el embrión somático, lo cual sugiere un papel específico en la peridermis. Algunos de estos genes, como por ejemplo citocromos P450, transportadores ABC, aciltransferasas y elongasas de ácidos grasos, codifican para enzimas que podrían estar implicadas en la ruta biosintética de la suberina según estudios basados en su composición química. Otros, por el contrario, corresponden a genes reguladores y de respuesta a estrés, relacionados con el felema y la suberina por primera vez. Cabe destacar, además, que la acumulación en el felema de transcritos de los genes NAM, MYB, HD-ZIPIII, KNOX y WRKY sugiere que algunos mecanismos reguladores pueden estar conservados entre la formación de los tejidos vasculares y la peridermis.

Con el fin de contribuir a un mejor conocimiento de la formación y fenología del felema, se estudió el patrón de expresión de 12 genes durante la estación de crecimiento en el felema de alcornoque. Los resultados muestran que la mayoría de genes, como por ejemplo los que codifican para las enzimas CYP86A1, GPAT y HCBT, así como los factores de transcripción NAM y WRKY, tienen un máximo de expresión en junio, el mes en el cual la tasa de crecimiento del felema es máxima. Por el contrario, la expresión de los genes que codifican para las enzimas F5H y FAT, junto con las proteínas de estrés HSP17.4 i ANN, está correlacionada con la temperatura y la humedad relativa.

Para estudiar el papel de algunas proteínas reguladoras en el felema se realizaron estudios de genética reversa en la peridermis de patata de los genes NAM, RIK, DEM2 y REV, obteniendo para todos ellos líneas resistentes a la kanamicina, cosa que indica la correcta inserción del DNA exógeno en las plantas. Se ha confirmado la obtención de líneas inducidas e inhibidas para el factor de transcripción NAM, y está previsto en breve confirmar el resto de líneas, así como la realización de análisis fenotípicos. Si se detectan diferencias entre las plantas transgénicas y las controles será, probablemente, la primera vez que se demuestra la función molecular de un gen regulador en la peridermis.

RÉSUMÉ

Le périderme est une structure complexe qui protége les organes végétales matures (secondaires) et les zones blessées des plantes de la perte d'eau et d'infections de pathogènes. Cette fonction de barrière que réalise le périderme est due au liège, un tissu formé par des cellules qui déposent de la subérine sur ses parois. Le liège et la subérine sont cruciaux pour la vie des plantes terrestres, mais malgré leur importance, on ne connaît pratiquement rien sur les processus moléculaires qui régulent leur formation, probablement due au manque de modèles adéquats. Dans cette thèse nous avons développé une stratégie pour l'identification et la caractérisation des gènes induits au liège grâce à la combinaison de deux plantes modèles pour l'étude du périderme. L'écorce du chêne-liège (*Quercus suber*) a été utilisé pour isoler les gènes candidats pour la formation du liège et pour réaliser un travail de recherche sur le comportement de quelques-uns de ces gènes candidats pendant la saison de croissance, tandis que la peau de la pomme de terre (*Solanum tuberosum*) a été utilisée pour des études de génétique reverse pour démontrer la fonction de quelques protéines régulatrices au liège.

Nous avons isolé et identifié 142 gènes, principalement induits au liège du chêne-liège en comparaison avec le xylème et l'embryon somatique. Cela suggère un rôle spécifique au périderme. Beaucoup d'enzymes identifiés, parmi lesquels des cytochromes P450, des transporteurs ABC, des acyltransferases et des élongases d'acides grasses, ont un rôle hypothétique pour la synthèse de subérine selon des études antérieures basées sur la composition chimique. Nous avons également isolé d'autres gènes, incluant des protéines régulatrices et de réponse au stress, mises en relation avec le liège et la subérine pour la première fois. De plus, l'accumulation au liège des transcrits des gènes NAM, MYB, HD-ZIPIII, KNOX et KANADI suggère que quelques mécanismes régulateurs peuvent être conservés entre la formation des tissus vasculaires et le périderme.

Pour contribuer à connaître mieux de la formation et phénologie du liège, nous avons étudié le profil d'expression de 12 gènes pendant la saison de croissance au liège du chêne-liège. Les résultats montrent que la majorité des gènes, comme les enzymes CYP86A1, GPAT et HCBT et les facteurs de transcription NAM et WRKY, ont un maximum d'expression en juin, le mois où le taux de croissance du liège a la plus haute activité. Par contre, l'expression de deux gènes mis en relation avec le stress (HSP17.4 et ANN) et de deux gènes qui codent pour enzymes (F5H et FAT) sont associés à la température et à l'humidité relative.

Pour étudier le rôle de quelques protéines régulatrices au liège, nous avons réalisé des études de génétique reverse des gènes NAM, RIK, DEM2 et REV. Nous avons identifié des lignes de plantes résistantes à la kanamicine, qui indique la correcte insertion du DNA étranger dans les plantes. L'obtention de lignes induites et inhibés a été confirmée pour le facteur de transcription NAM. Nous prévoyons avoir bientôt la confirmation des autres gènes et commencer des analyses phénotypiques. Si on observe des différences entre les plantes transgéniques et les contrôles, ce sera, probablement, la première fois que sera démontré le rôle moléculaire des gènes régulateurs au périderme.

INTRODUCTION

1. The periderm: a protective structure of plants

About 450 million years ago the first land plants appeared. Their evolution from an aquatic media to a terrestrial one required several structural adaptations, such as the development of efficient fluid-conducting systems (xylem and phloem), organs for anchorage and absorption of water and minerals (roots) and the emergence of support tissues (collenchyma and sclerenchyma). Moreover, the water potential gradient between the plant body and the atmosphere could produce lethal dehydration, so a protective barrier against water loss which permitted the exchange of CO_2 and O_2 for photosynthesis and respiration was vital (Lendzian, 2006). All the primary aerial surfaces of vascular plants (leaves, primary stems, flowers, petioles, trichomes and many fruits) are covered by the cuticle, a continuous layer secreted by the epidermis which consists of cutin and associated waxes. The cuticle-epidermis complex protects the plant from dehydration, while gas exchanges are facilitated by the stomata. However, the cuticle-epidermis complex is not regenerated when damaged or destroyed by wounding or during secondary growth. Instead, it is replaced by a complex structure referred to as the periderm, which protects the inner tissues from water loss, injury and pathogens (Lendzian, 2006).

The periderm is found in woody stems, roots and tubers. It consists of three tissues: the phellogen or cork cambium, which is the meristematic layer; the phellem or cork, produced toward the outside, which is responsible for the barrier function; and the phelloderm, a tissue often resembling cortical or phloem parenchyma, consisting of the inner derivatives of the phellogen (Figure 1). The periderm works as an effective barrier because the cork layer is a tissue made of cells whose walls are impregnated with suberin, a lipid polyester analogous to cutin. The phelloderm, although it can be relatively thick in some tropical trees, is nearly vestigial in most temperate plants. The role of stomata is performed in the periderm by the lenticels, aerenchymatous areas used as paths for the exchange of water vapour, oxygen and carbon dioxide between the inside and outside.

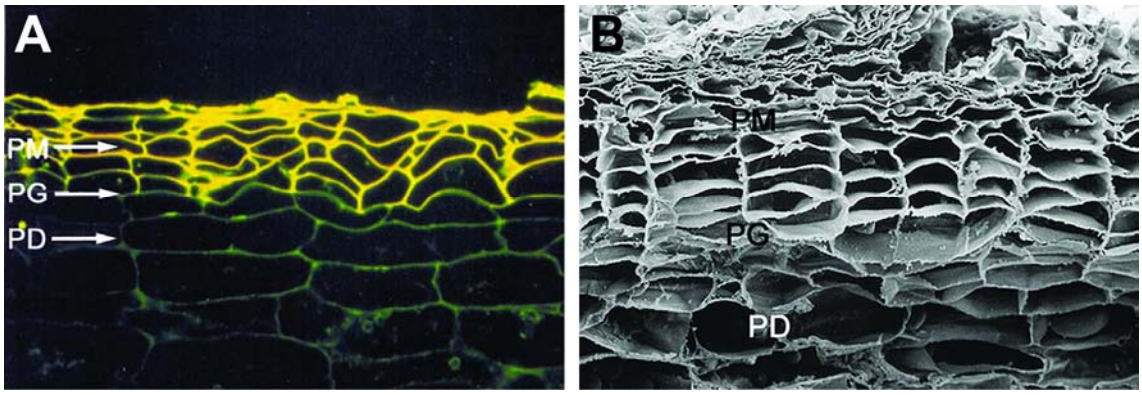

Figure 1. Detail of the three tissues of the potato tuber periderm: cork or phellem (PM), phellogen or cork cambium (PG) and phelloderm (PD), extracted from Lulai and Freeman (2001) **A.** Optic fluorescence micrograph of periderm treated with Sudan III/IV to stain cork cells. **B.** Scanning Electron Microscopy (SEM) micrograph of periderm.

1.1. Ontogeny of the periderm in stems, roots and wounding tissues

The phellogen is a plant's secondary meristem formed by cellular dedifferentiation. The first phellogen may arise at different depths outside the vascular cambium, depending on the organ and species. In stems, the first phellogen usually dedifferentiates in the subepidermis or in the subjacent layer of cells. In most roots, however, the first periderm originates deep in the axis, usually in the pericycle. Phellogen cells divide periclinally, giving rise to cork cells on the outside and phelloderm cells on the inside. At the beginning of the season, when the phellogen is highly active, thin-walled active cells are produced, but towards the end of the season, cell divisions become rare and thick-walled cells are formed (Waisel, 1995).

The first periderm commonly develops during the first year of stem and root growth, but with the continuation of secondary thickening, it is replaced by new periderms formed deeper in the living tissue of the plant axis. As a result, tissues exterior to the new periderm become cut off from water and nutrient supplies and so die, forming the outer bark or rhytidome. In species such as *Betula, Fagus, Abies* or *Quercus,* the periderm may be retained for life or for many years. In these species there are seasonal differences in the types of cork cells produced and, as a result, bands which can apparently be regarded as annual cork growth rings develop. Monocotyledonous rarely produce a periderm similar to that produced by other angiosperms. In most woody monocots, including palms, a special type of protective tissue is formed by the repeated division of cortical parenchyma cells. This tissue develops without an initial layer or phellogen being formed, and is referred to as storied cork (Evert, 2006).

Generally, in those places where living plant tissue is exposed to the air as a result of wounding, a necrophylactic or wound periderm develops. This periderm is preceded by an impervious layer of cells commonly called the boundary layer, which develops immediately below the necrosed cells on the surface of the wound. Below the boundary layer, a phellogen may be produced in the undamaged living layers, and produce cork and phelloderm in the usual manner.

1.2. Cork is a boundary layer that protects internal tissue

Cork, which forms the external or boundary tissue of the periderm, is a multilayered tissue made of cells with suberized walls. Cork cells are usually arranged compactly, meaning that the tissue lacks intercellular spaces (Evert, 2006). Immature cork cells are parenchymatous with thin primary walls, but, during maturation, a thick layer of suberin is deposited on the walls, finally resulting in the death of the cells. The cell protoplasm is lost and the cell lumen becomes filled, sometimes with air and sometimes with resiniferous or tanniniferous substances (Fahn, 1969).

Suberin is responsible for the isolating properties of the cork. It is deposited internally in their primary walls, forming a continuous lipid layer called suberin lamella. Under the Transmission Electron Microscope (TEM) the suberin lamella has a layered appearance because it consists of alternating electron-dense and electron-translucent layers oriented in parallel to cell wall (Thomson et al., 1995) (Figure 2). Suberin is not totally impermeable to water, but cork cells often contain considerable amounts of soluble lipids (waxes) which are though to reduce water loss through cell walls (Soliday et al., 1979; Vogt et al., 1983).

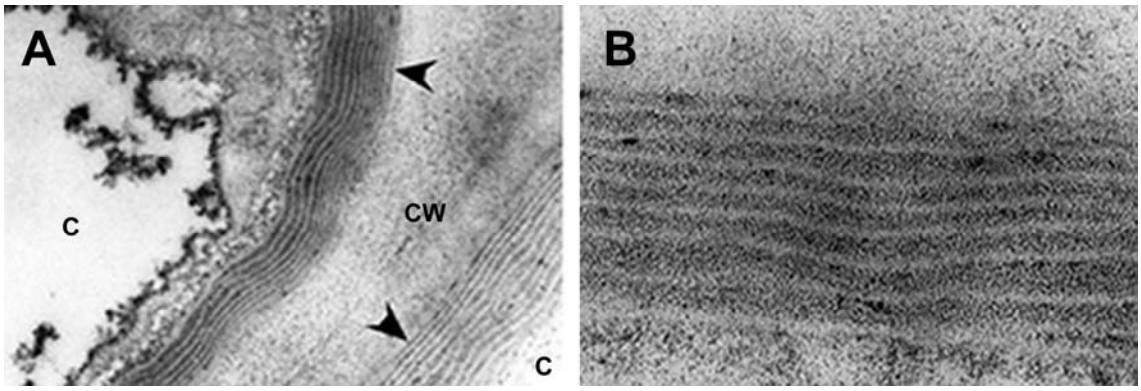

Figure 2. TEM micrographs of suberin lamella from suberized root tissues of wild-type *Arabidopsis* plants at the beginning of secondary thickening of the root, extracted from Nawrath (2002). **A.** Suberin lamellas from two cells (indicated by arrows), located between the cytosol (C) and the cell walls (CW). **B.** Detail of suberin lamella.

1.3. Plant models for periderm studies

Although all higher plants form a periderm, most of what is known about suberin and periderm has been studied in the outer bark of cork oak (*Quercus suber*) and in the potato (*Solanum tuberosum*) tuber periderm.

Cork used commercially as stoppers for bottles comes from cork oak. The bark of this tree is stripped off (about every 10 years) and then processed as commercial cork. It consists of small thin-walled cells with air-filled lumen, highly impervious to water and gases. In addition, it is light in weight and has thermal insulating characteristics. The chemical composition of cork tree bark has been widely analyzed (Silva et al., 2005). Although the amounts of different compounds can vary significantly, on average it contains a 63% suberin and a 15% waxes and tannins.

The periderm of the potato tuber (also called skin) is an effective barrier that protects the tuber from dehydration and pathogens. The cellular structure, maturation process and chemical composition of the potato periderm have been characterized (Kolattukudy and Agrawal, 1974; Graça and Pereira, 2000a; Lulai and Freeman, 2001; Sabba and Lulai, 2002; Schreiber et al., 2005).

2. Suberin: a glycerol-based cell wall polyester

Suberin is defined in the literature as a complex lipid biopolymer found in cell walls. It consists of an aliphatic domain cross-linked with an aromatic lignin-like domain (Bernards, 2002). The aliphatic domain is a cutin-like glycerol-bridged polyester esterified with phenolics (Moire et al., 1999; Graça and Santos, 2006). The aromatic domain is a polyphenolic substance presumably involved in linking the aliphatic domain to the cell wall (Kolattukudy, 1980; Bernards and Lewis, 1998). Ultrastructural and cytochemical evidence indicates that suberin-type polymers are not only present in cork, but also in other plant tissues such as the endodermis (Casparian bands), exodermis and hypodermis of roots (Kolattukudy, 1980).

Aliphatic suberin consists mainly of long- and very long-chain ω-hydroxyacids and fatty α,ω-diacids (C_{16} to C_{30}) esterified to glycerol, although minor quantities of alcohols may also be present (Schreiber et al., 1999; Graça and Pereira, 2000a). However, some variations exist among the suberin model species, for while *Q.suber* bark is characterized by in-chain epoxides and vicinal diols (Graça and Pereira, 2000b), potato tuber periderm and *Arabidopsis thaliana* roots are characterized by monounsaturates (Graça and Pereira, 2000a; Franke et al., 2005; Schreiber et al., 2005). As concerns to aromatic suberin, some studies have shown that it is a hydroxycinnamic acid-derived polymer primarily comprised of ferulic acid and N-feruloyl tyramine (Négrel et al., 1996; Bernard and Razem, 2001), while other researchers have reported a significant amount of monolignols (Yan and Stark, 2000). It may be, therefore, as hypothesized by Graça and Santos (2007), that two types of polyaromatics are present in suberized cell walls: one, associated with the polysaccharides in the primary cell wall, a polymer similar to lignin and based on monolignols; the other, associated with aliphatic suberin, a polymer based on ferulic acid or a yet to be elucidated structure.

The three-dimensional structure of suberin is still not clear, although several tentative models have been proposed (Kolattukudy, 1981, 2001; Bernards, 2002; Graça and Santos, 2007). It is believed that long-chain and very long-chain α,ω-diacids esterified to glycerol at both ends are the core of the suberin macromolecule, and that the suberin polymer grows two- and three-dimensionally by the formation of ester linkages to additional α,ω-diacids and ω-hydroxyacids. At the interface of the glycerol-based polymer, ferulates attach the aliphatic polyester to the polyaromatics, which themselves are thought to form the link to cell wall carbohydrates (Franke and Schreiber, 2007). Thus, appearance of alternating opaque and translucent lamellar bands with TEM would be achieved by successive layers of aliphatic compounds alternating with polyaromatics (Figure 3).

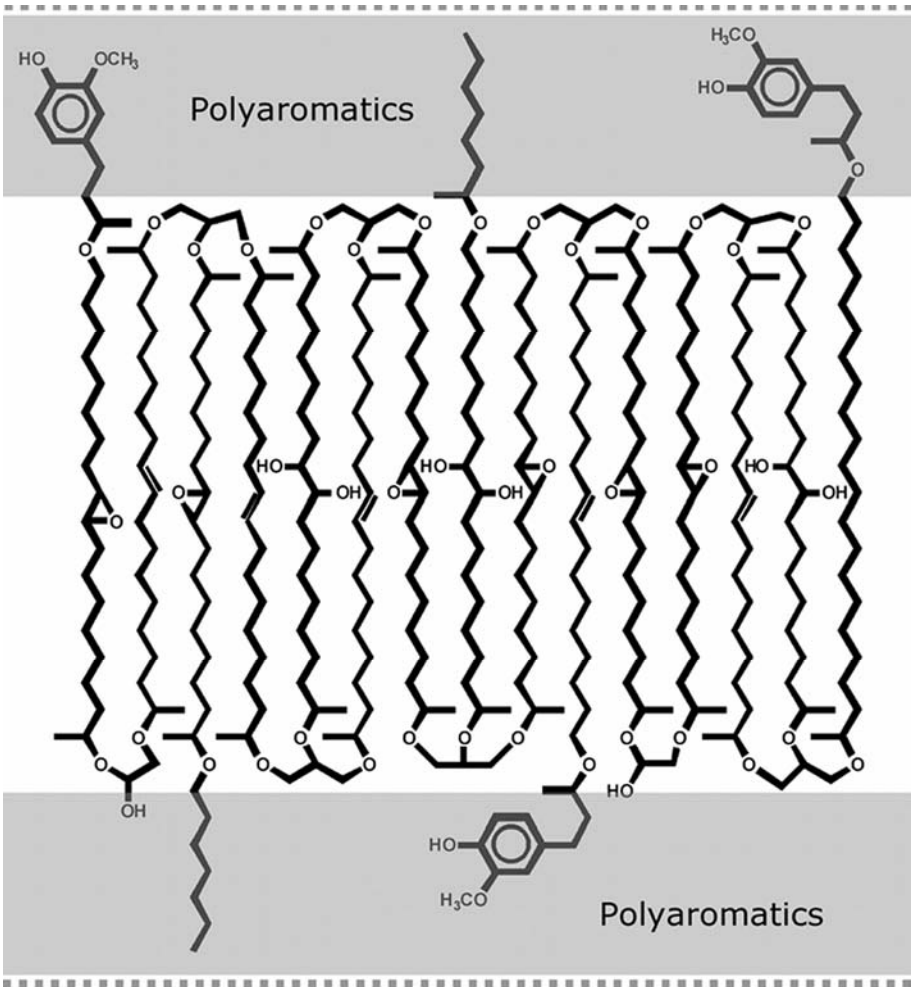

Figure 3. Proposed model of suberin structure, extracted from Graça and Santos (2007). The predicted three-dimensional structure of suberin is based on the esterification of α,ω-diacids and ω-hydroxyacids to glycerol and ferulic acid respectively. Accordingly, successive layers of aliphatic compounds are alternated with hydroxycinnamic acids forming the translucent and opaque lamellar bands visible in TEM. It has also been suggested that ω-hydroxyacids could cross-link aromatic layers to aliphatic layers.

2.1. Biosynthesis, polymerization and cross-linking of suberin

Many of the enzymatic activities necessary for suberin biosynthesis have been inferred from its chemical composition. The biosynthetic pathway for suberin monomers has been hypothesized by Kolattukudy (2001), Bernards (2002) and Franke and Schreiber (2007). However, practically nothing is known about the cellular site of suberin biosynthesis, or how the suberin monomers are transported to the cell wall and incorporated into the polymer, thereby forming the highly regular lamellar structure visible under TEM.

The biosynthetic pathway of aliphatic monomers

It is generally accepted that this begins with the general fatty acid (FA) pathway. Fatty acid elongases (FAEs) catalyze the elongation of the carbon chain of fatty acids to different lengths, giving rise to very long-chain fatty acids. The hydroxylation of fatty acids in the ω-position is typically catalyzed by NADPH-dependent cytochrome P450 monooxygenases (Duan and Schuler, 2005; Kandel et al., 2006). Recently, the role of *Arabidopsis* CYP86A1 in the fatty acid ω-hydroxylation of suberin was reported (Li et al., 2007). The formation of α,ω-diacids from ω-hydroxyacids may be catalyzed by a two-step mechanism (Kolattukudy, 2001; Kurdyukov et al., 2006) or by a single enzyme (LeBouquin et al., 2001), although neither of them has been shown to be directly involved in suberin biosynthesis. Mid-chain modifications in aliphatic suberin monomers may be produced by a cytochrome P450 or by a peroxygenase-dependent pathway (Kolattukudy, 2001; Kandel et al., 2006), while the unsaturations may be formed by fatty acid desaturases (FADs) (Graça and Pereira, 2000a; Schreiber et al., 2005). Glycerol is likely to enter the suberin biosynthetic pathway as phosphoglycerol (Nawrath, 2002).

The biosynthetic pathway of aromatic monomers

The biosynthesis of the aromatic suberin domain is based on phenylpropanoid metabolism, starting with the deamination of phenylalanine by the phenylalanine ammonia-lyase (PAL) enzyme (Kolattukudy, 1981). The phenylpropanoid pathway is followed by a set of enzymatic reactions, including hydroxylation, methylation, coenzyme A-ligation and reduction, leading to the formation of hydroxycinnamic acids (ferulic, sinapic, caffeic and p-coumaric acids) and monolignols (p-coumaryl, coniferyl and sinapyl alcohols) (Boerjan et al., 2003; Boudet et al., 2003). The hydroxycinnamic acids may be activated by a 4CL (4-coumarate: coenzyem A ligase) and incorporated into suberin by the action of hydroxycinnamoyl: coenzyme A transferases (Bernards and Lewis, 1998; Bernards, 2002).

Transport, polymerization and cross-linking

Traditionally, building units for cell wall polymers were thought to be exported to their extracellular destination by a vesicular pathway via Golgi-derived vesicles from the endoplasmic reticulum. Recently, however, some authors have proposed a direct plasma membrane pumping by ABC transporters (Samuels, 2002; Pighin, 2004; Franke and Schreiber, 2007). For polymerization, it is generally assumed that the assembly of the aromatic moiety occurs through a peroxidase/H_2O_2 mediated process termed radical coupling, analogous to lignin formation (Nawrath, 2002), although the precise identity of the peroxidases involved remains unproven (Bernards, 2002). The polymerization of the suberin glycerol polyester is poorly understood, but is thought to take place in the apoplast by esterification of the carboxyl groups of ω-hydroxyacids and α,ω-diacids with the alcohol groups of either glycerol or ω- and mid-chain hydroxy fatty acids (Kolattukudy, 2001). The linkage with glycerol was confirmed by analyzing a suberin-defective glycerol-3-phosphate acyltransferase (GPAT) mutant in *Arabidopsis* (Beisson et al., 2007). Enzymes involved in linkages between aliphatic and aromatic suberin units are mostly unknown, although esterified ferulates to fatty acids are very important suberin monomers (Adamovics et al., 1977; Bernards

and Lewis, 1992; Lotfy et al., 1994; Graça and Santos, 2007). Ferulic-acid-dependent acyltransferases and other members of the BAHD superfamily may catalyze the esterification of hydroxycinnamates to fatty acids (Lotfy et al., 1995; Franke and Schreiber, 2007), although no genes encoding these proteins have been identified to date.

3. Regulatory mechanism for suberin biosynthesis and cork differentiation

Several factors seem to be involved in the induction and control of phellogen. Apparently, the first divisions of an initiating phellogen are located immediately below live trichomes, and the mechanical pressures exerted on the bark by the growing xylem are thought to be the fundamental cause of phellogen initiation, although many other factors such as tissue water potential, oxygen pressure, photoperiod, thermoperiod, growth substances and wound effects have been also reported (Waisel, 1995). In particular, it has been demonstrated that abscisic acid (ABA) induces suberization in potato wound periderm (Soliday et al., 1978). Presumably, ABA triggers a series of biochemical events which induce the synthesis of enzymes involved in suberization (Kolattukudy, 1980). However, no regulatory molecular mechanisms acting on periderm formation and suberin deposition have been described to date.

3.1. The regulation of suberin biosynthesis and deposition

The genetic control of suberin biosynthesis, polymerization and deposition in the cell walls is unknown. However, some regulatory mechanisms similar to those described for lignin and cutin may be active in suberin.

It seems that most of the genes postulated as being directly involved in lignin biosynthesis in *Arabidopsis* contain at least one copy of a cis-regulatory element named AC element within 1000 bp of 5′ non-coding sequence upstream of the start codon. These common AC elements may provide a mechanism by which the different stages of lignin biogenesis are coordinately regulated and expressed in vascular tissue. In addition, AC elements are recognized by the DNA binding domain of some plant R2R3-MYB transcription factors, which suggests that these transcription factors have the potential to coordinately regulate the expression of genes related to lignin (Rogers and Campbell, 2004). Several R2R3-MYB factors have been described as regulators of lignification (Tamagnone et al., 1998; Patzlaff et al., 2003; Karpinska et al., 2004; Goicoechea et al., 2005; Fornalé et al., 2006). But apart from MYB genes, a member of the LIM family of transcriptional regulators in *Nicotiana tabacum* (NtLIM1), which is also able to bind to an AC element, was found to have the capacity to regulate the expression of some lignin biosynthetic genes (Kawaoka et al., 2000). In addition, mutation in many other genes affecting lignin deposition, directly or indirectly by impairing vascular and fibre differentiation, has been reviewed by Rogers and Campbell

(2004). As regards to cutin biosynthesis, very few approaches focusing on regulatory proteins have been performed. Two interesting studies identified a clade of AP2/EREBP called SHINE (SHN) playing a role in the regulation of the lipid biosynthesis of plant surfaces (Aharoni et al., 2004) and a MYB transcription factor involved in cuticle metabolism (Cominelli et al., 2008).

3.2. Regulatory mechanisms of periderm initiation and growth

The vascular cambium and the phellogen are the two secondary meristems that contribute to the radial growth of woody species. Cork and phelloderm differentiate asymmetrically from phellogen, analogous to the way that the radial patterning of wood (xylem) and phloem differentiates from vascular cambium. The similarity of both processes suggests that some regulatory mechanisms acting on vascular differentiation may also be present in the periderm. However, the identification of these genes and the demonstration of their function are still lacking.

Vascular cambium formation is driven by a polar auxin transport (PAT), itself driven by PINOID genes (Frimi et al., 2004; Carlsbecker and Helariutta, 2005) probably triggered by the weight supported by the plant body (Ko et al., 2004). The maintenance of meristematic cells in the *Arabidopsis* shoot apical meristem (SAM) is regulated by CLAVATA (CLV1, 2, 3), WUSCHEL (WUS), KNOTTED-1 LIKE HOMEBOX GENES (KNOX) and ASYMETRIC LEAVES 1 (AS1) genes (Traas and Vernoux, 2002). Some orthologs of these genes, but not all, have been found in the cambial region of *Populus*, demonstrating that similar regulatory mechanisms are active in cambium and apical meristems (Schrader et al., 2004; Groover et al., 2006).

It is well known that the establishment and maintenance of leaf adaxial and abaxial identity depends on the HD-ZIP/KANADI genetic system (Emery et al., 2003). The same is thought to occur in the radial patterning of stems and roots (Hawker and Bowman, 2004; Schrader et al., 2004; Groover, 2005; Zhao et al., 2005; Demura and Fukuda, 2007). Other players in the establishment of adaxial/abaxial identity and vascular patterning might include the genes ASYMMETRIC LEAVES 1 and 2 (AS1, 2) and brassinosteroid (BR) receptors, although their intersection with the HD-ZIP/KANADI pathway is unclear (Carlsbecker and Helariutta, 2005). Some members of the NAC gene family, including NAM, ATAF and CUC transcription factors, deserve separate mention. They are preferentially expressed in developing vascular tissue and related to the transdifferentiation of various cells into cells with lignified secondary walls (Kubo et al., 2005; Mitsuda et al., 2005; Demura and Fukuda, 2007; Ko et al., 2007; Mitsuda et al., 2007; Zhong et al., 2007). However, it is still unknown whether the initiation, maintenance and differentiation of meristematic phellogenic cells and the radial patterning of the periderm depend on similar regulatory genes described in vascular patterning.

4. Molecular strategies for studying suberin and cork

Very little is known about the molecular processes that govern the suberin biosynthesis and the cork formation, and this lack of knowledge can be attributed to the difficulties of working with appropriate model plants. *Arabidopsis*, the model for molecular studies in dicotyledonous, is an herbaceous short-lived plant which can present secondary growth in certain advanced stages of development. However, technical difficulties involved in obtaining and isolating its periderm, together with the lack of mutants with an obvious phenotype, have hindered the analysis of molecular mechanisms acting on suberin and periderm. To date, only three mutants with altered suberin have been described: *elongation defective1*, a pleiotropic mutant showing ectopic suberin deposition (Cheng et al., 2000), a knock-out mutant for the GPAT5 gene, which alters suberin composition (Beisson et al., 2007) and the double mutant with ectopic over-expression of GPAT5 and CYP86A1, which results in the accumulation of monomers typical of suberin in the epidermal polyesters (Li et al., 2007).

Alternative models for periderm and suberin research should be plants with a widely studied periderm, like cork oak bark and potato tuber skin. Cork oak is the paradigm for cork and suberin because each year it produces about 60 layers of almost pure cork cells containing high amounts of suberin. Besides this, the chemical and physical properties of commercial cork are fairly well known (Silva et al., 2005). However, due to its low transformation efficiency and its very long regeneration times, functional genetic studies using this tree are unachievable. On the other hand, potato periderm has also been extensively studied (Sabba and Lulai, 2002; Kolattukudy and Agrawal, 1974; Graça and Pereira, 2000a; Schreiber et al., 2005) and it can be easily transformed by *Agrobacterium tumefaciens* and grown in the laboratory.

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OBJECTIVES

The **main objective** of the present work is to better understand cork formation and regulation by the identification of candidate genes and the characterization of some regulatory genes. The specific objectives are the following:

1. Identification of candidate genes for cork and suberin formation in cork oak.

1.1. Construction of a suppression subtractive hybridization library of cork tissue using somatic embryo as control tissue (Chapter 1).

1.2. Highlighting the best candidates by a comparison between cork and wood transcriptomes (Chapter 1).

1.3. Identification of cork regulatory genes using primers designed based on *Populus* databases (Annex Chapter 1).

2. Studying the regulatory processes which take place in cork.

2.1. Investigation of gene expression during the cork oak growing season to better understand the environmental factors influencing gene expression in cork (Chapter 2).

2.2. Obtaining of genetic modified potato plants for functional characterization of some regulatory genes in cork (Chapter 3).

RESULTS

Chapter 1

A Genomic Approach to Suberin Biosynthesis and Cork Differentiation[1][C][W][OA]

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Cork (phellem) is a multilayered dead tissue protecting plant mature stems and roots and plant healing tissues from water loss and injuries. Cork cells are made impervious by the deposition of suberin onto cell walls. Although suberin deposition and cork formation are essential for survival of land plants, molecular studies have rarely been conducted on this tissue. Here, we address this question by combining suppression subtractive hybridization together with cDNA microarrays, using as a model the external bark of the cork tree (*Quercus suber*), from which bottle cork is obtained. A suppression subtractive hybridization library from cork tree bark was prepared containing 236 independent sequences; 69% showed significant homology to database sequences and they corresponded to 135 unique genes. Out of these genes, 43.5% were classified as the main pathways needed for cork biosynthesis. Furthermore, 19% could be related to regulatory functions. To identify genes more specifically required for suberin biosynthesis, cork expressed sequence tags were printed on a microarray and subsequently used to compare cork (phellem) to a non-suberin-producing tissue such as wood (xylem). Based on the results, a list of candidate genes relevant for cork was obtained. This list includes genes for the synthesis, transport, and polymerization of suberin monomers such as components of the fatty acid elongase complexes, ATP-binding cassette transporters, and acyltransferases, among others. Moreover, a number of regulatory genes induced in cork have been identified, including MYB, No-Apical-Meristem, and WRKY transcription factors with putative functions in meristem identity and cork differentiation.

Land plants have evolved lipophilic barriers that protect the internal living tissues from dehydration, injuries, and pathogens, and have evolved regulatory networks to adjust the barriers to the changing physiological and environmental conditions of the plant. Plant primary organs, such as young stems and leaves, are protected by the cuticle, a lipophilic extracellular polymer membrane composed of cutin and waxes. Secondary (mature) stems and roots, tubers, and healing tissues are protected by cork, a tissue with multiple layers of cells that are dead at maturity. Key compounds for cork impermeability are suberin, a complex polymer comprising both aliphatic and aromatic domains, and associated waxes. Cork is part of the plant constitutive defense system and contains secondary compounds such as triterpenoids and soluble phenylpropanoids that act on herbivores, microbes, and fungi.

Cork, or phellem, which is the technical term for cork, is formed by the phellogen (cork cambium). Cork formation involves proliferation and commitment of the phellogen derivatives, cell expansion and extensive deposition of suberin and waxes, and an irreversible program of senescence ending in cell death. The two best known and most studied examples of cork are the suberized skin of potato (*Solanum tuberosum*) tuber (Sabba and Lulai, 2002) and the bark of the cork tree (*Quercus suber*), from which bottle cork is obtained (Silva et al., 2005). In the cork tree, the phellogen forms a continuous layer of cells that envelops the tree trunk and produces, each year, a 2- to 3-mm thick layer of almost pure cork that adheres to that of the previous year (Caritat et al., 2000). The chemical composition of the cork tree bark has been widely analyzed by chemical fractionation (for review, see Silva et al., 2005). Although the amounts of the different components can show significant variations (Pereira, 1988; Lopes et al., 2001), on average it contains 15% extractives (7.5% waxes and 7.5% tannins), 41% aliphatic suberin (referred to as suberin in cork tree literature), 22% aromatic suberin (referred to as lignin in cork tree literature), 20% polysaccharides, and 2% ashes (Pereira, 1988). The monomeric composition of the aliphatic (suberin) fraction has been analyzed by Holloway (1983) and by

[1] This work was supported by the Spanish Ministerio de Ciencia y Tecnología (grant no. AGL2003–00416), by Ministerio de Educación y Ciencia (FPI grant to O.S.), by the European Social Funds and the Departament d'Universitats, Investigació i Societat de la Informació of Catalonia (FI and BE grants to M.S.), and by the European Forest Genomic Network (STSM to M.S. and O.S.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.106.094227

Bento et al. (1998) and that of the aromatic (lignin) fraction by Marques et al. (1999) and Lopes et al. (2000), among others. Waxes have been analyzed by Castola et al. (2005) and mainly consist of terpenes and sterols. Tannins, mostly ellagitannins, and other soluble polyphenols have been analyzed by Cadahia et al. (1998) and Varea et al. (2001). Many of the enzymatic activities necessary for cork biosynthesis may be inferred from the chemical composition. Cork is a site for four main secondary metabolic pathways: acyl-lipids, phenylpropanoids, isoprenoids, and flavonoids. The acyl-lipids pathway is required for the biosynthesis of the linear long-chain compounds forming the aliphatic suberin domain, which share upstream reactions with cutin biosynthesis (Kolattukudy, 1981; Nawrath, 2002; Heredia, 2003; Kunst and Samuels, 2003). The phenylpropanoids pathway is needed for the biosynthesis of the cork aromatic components, which share the same basic reactions with wood lignin (Dixon et al., 2002; Boerjan et al., 2003). The isoprenoids pathway is needed for wax terpenes (Laule et al., 2003) and sterols (Benveniste, 2004), and the flavonoids pathway for tannins (Koes et al., 2005).

Suberin, the main cork component, is defined in literature as a complex biopolymer found in suberized cells that comprises an aliphatic cutin-like and an aromatic lignin-like domain (Bernards, 2002). The aliphatic domain is a glycerol-bridged polyester with associated esterified phenolics (Moire et al., 1999; Graca and Santos, 2006). The aromatic domain is a polyphenolic substance mostly composed of hydroxycinnamic acid derivatives and is presumably involved in linking the aliphatic domain to cell wall (Kolattukudy, 1980; Bernards and Lewis, 1998). The three-dimensional structure of suberin is not yet clear, although several tentative models have been presented (Kolattukudy, 1981, 2001; Lopes et al., 2000; Bernards, 2002). These models discuss possible linkages within the suberin and postulate linkages between suberin and the lignin/carbohydrate cell wall matrix. Only a few studies report enzymatic activities involved in suberization, and most of them deal with the aromatic metabolism. Cottle and Kolattukudy (1982) and Bernards and Razem (2001) demonstrated the induction in suberizing tissues of key enzymes capable of generating hydroxycinnamic acids and proposed a biosynthetic pathway for the aromatic domain based in lignin biosynthesis. Peroxidase activity associated with suberization was detected in potato tuber (Espelie et al., 1986; Bernards et al., 1999) and in tomato (*Solanum lycopersicum*) root tissue (Quiroga et al., 2000). The presence of a hydrogen peroxide (H_2O_2) generating system necessary for the peroxidase activity was indirectly demonstrated by Razem and Bernards (2003). Experimental evidence for enzymes involved in the metabolism of the aliphatic domain is limited to the in vitro demonstration of an ω-hydroxyacid oxidation (Agrawal and Kolattukudy, 1977), the demonstration of an elongase activity in maize (*Zea mays*) roots (Schreiber et al., 2005), and the isolation from potato tuber discs of a ferulate acyltransferase possibly involved in linking the aromatic monomer ferulate to the aliphatic domain (Lotfy et al., 1994, 1995). The biosynthetic pathway for the aliphatic monomers has been hypothesized (Kolattukudy, 2001; Bernards, 2002; Franke et al., 2005). It is accepted that the synthesis begins with the general fatty acid (FA) synthesis pathway giving rise to long-chain FAs (LCFAs), that the condensation of very LCFAs (VLCFAs) takes place in the endoplasmic reticulum, and that P450 monooxygenases catalyze most FA oxidation reactions. The three-dimensional polyester is thought to be achieved by esterification between FA, with glycerol acting as an important small cross-linker (Kolattukudy, 2001). However, transport of the aliphatic monomers and polymerization in the apoplast are still unknown.

Although cork and suberin are critical to the life of both herbaceous and woody plants, molecular genetic approaches are still lacking (Yephremov and Schreiber, 2005). Today, analysis of suberin biosynthesis and function should be conducted using suberin-defective mutants, which cannot be easily obtained with cork tree. A much better choice would be Arabidopsis (*Arabidopsis thaliana*); however, despite the fact that Arabidopsis synthesizes suberin and develops a phellem, only two mutants with altered suberin or defective phellem have been identified to date: *elongation defective1*, a pleiotropic mutant showing ectopic suberin deposition (Cheng et al., 2000), and, very recently, a knockout mutant for the glycerol-3-P acyltransferase 5 gene (GPAT5; Beisson et al., 2007).

Molecular genetic approaches to suberin are limited to the cloning and characterization of suberin-associated peroxidases in potato (Roberts and Kolattukudy, 1989), tomato (Quiroga et al., 2000), and muskmelon (*Cucumis melo*; Keren-Keiserman et al., 2004), but their role has not clearly been proven (Sherf et al., 1993; Lucena et al., 2003). Cuticle mutants have been identified for Arabidopsis, a fact that allowed researchers to identify a number of genes necessary for the synthesis and transport of cutin and wax (Jenks et al., 2002; Kunst and Samuels, 2003; Yephremov and Schreiber, 2005). Recently, a genome-wide study of the shoot epidermis of Arabidopsis (Suh et al., 2005) highlighted a series of new candidate genes relevant in cutin and wax synthesis. In lignin research, molecular genetic approaches have been widely used in the past. Global xylem transcript profiling has been reported for Arabidopsis (Ko et al., 2004; Ehlting et al., 2005) and for several tree species (Whetten et al., 2001; Kirst et al., 2004; Andersson-Gunneras et al., 2006). For a number of genes, involvement in lignin biosynthesis was demonstrated by forward and reverse genetic approaches (Anterola and Lewis, 2002; Sibout et al., 2005; Abdulrazzak et al., 2006). An excellent review (Carlsbecker and Helariutta, 2005) summarizes this knowledge of the molecular genetics of regulatory networks in xylem.

To reveal the genetic repertoire of cork cells and to identify genes likely to be related to suberin synthesis, we used a two-step strategy. First, by means of

suppression subtractive hybridization (SSH), a library of ESTs preferentially induced in cork was obtained. Then, these ESTs were printed on a microarray and subsequently used for a global comparison between a suberin-producing (cork/phellem) and a non-suberin-producing (wood/xylem) tissue. Isolation of suberin genes in the cork tree is particularly attractive because of its exceptional capacity to produce suberin. Peeling of the external bark from the cork tree trunk allowed the harvesting of differentiating cork layers (Fig. 1A) and provided a highly enriched material for molecular investigations. In the following pages, we present an initial analysis of the genomics of cork cells in cork tree bark; as far as we know, this is the first global approach to cork and suberin molecular biology.

RESULTS

Cork Subtractive Library

Suberin is a product of the secondary metabolism that is regulated in a tissue-specific manner. It was our intention to find candidate genes for cork and suberin biosynthesis; therefore, we chose as driver tissue for the SSH a fully undifferentiated tissue consisting of the proliferative mass obtained from cork tree somatic embryo cultures (Fig. 1B). The proliferative mass is a translucent, fully undifferentiated, nonvascularized tissue that develops in the hypocotyls of the recurrent somatic embryos. Cork tree somatic embryogenesis has been carefully characterized at anatomical and ultrastructural levels in previous works (Puigderrajols et al., 1996, 2001). Neither suberin deposition nor multilamellated cell walls could be detected in somatic embryos using optical and fluorescent microscopy techniques or by electron microscopy. The extraction of RNA from cork is difficult due to its high proportion of dead cells and phenols; however, using the protocol of Chang et al. (1993) that prevents oxidation of phenols, good quality RNA was obtained. A further mRNA purification step was added to reduce rRNA contamination and to increase SSH efficiency. Finally, the SSH products were cloned into pCR4-TOPO vector, resulting in a library of 975 cDNA clones. Subtraction efficiency was checked by cohybridizing tester (cork) and driver (embryo) RNAs on the cork tree microarray described in the "Materials and Methods" section. This control experiment confirmed a high yield of subtraction efficiency; 96% of reliable ESTs (coefficient of variation < 0.3) were up-regulated in cork (fold change [FC] > 1.5), and 75% of reliable ESTs showed signal intensity not significantly higher than background in embryo (data are shown in Supplemental Table S2).

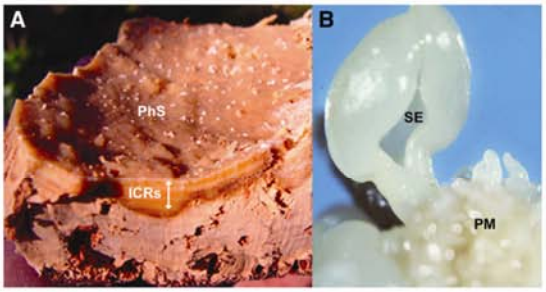

Figure 1. Cork (tester) and somatic embryo (driver) tissues used for SSH. A, Piece of cork showing the internal phellodermic surface (PhS) and, in the transversal section, the inner cork rings (ICRs) consisting of cells in different stages of suberization and dead cells that still contain a high amount of water. RNA used in our experiments was extracted from scrapings taken from the phellogenic surface comprising the inner cork rings. B, Proliferative mass (PM) obtained from cork tree somatic embryo (SE) cultures. [See online article for color version of this figure.]

Single-run sequencing of the library yielded 694 readable sequences longer than 100 bp. Of these, 579 grouped into 121 contiguous sequences (contigs) and 115 were single sequences (singletons). Thus, in total, 236 independent sequences were obtained. Sequence redundancy ($100 \times [1 - \{\text{contigs} + \text{singletons}/\text{readable sequences}\}]$) was of 66%. BLASTX analysis (Altschul et al., 1990) showed that 69% of the independent sequences (71 singletons and 92 contigs) showed high sequence homology at the amino acid level to database sequences (e value $< e^{-21}$) and that 31% (44 singletons and 29 contigs) could not be assigned to any gene ontology and were classified as no hits (e value $> e^{-10}$). Sequences with the same GenBank entry were assumed to represent the same gene, and, thus, the library was found to contain 135 unique genes (121 with assigned function and 14 with unknown function). The genes with assigned function were manually grouped into functional categories using National Center for Biotechnology Information, The Arabidopsis Information Resource (TAIR), and published data. Categories were established taking into account the main metabolic and cellular processes leading to cork biosynthesis. Table I shows a selected list of genes with putative functions thought to be important for suberin biosynthesis and cork regulation grouped in functional categories. The complete list is given in Supplemental Table S1.

The relative contribution of the genes to the different categories is shown in Figure 2. Acyl lipids, isoprenoids, phenylpropanoids, and flavonoids, the four categories that represent the major pathways for the synthesis of cork chemical components, amounted to 43.5% of the genes. The regulatory proteins category, which includes transcriptional regulation, signal transduction, and regulated proteolysis-related genes, amounted to 19% of the genes. The category stress, which combines genes related to detoxifying enzymes and cell wall strengthening, amounted to 9.5%; and the category unknown, which groups those genes with no assigned biological function, amounted to 10%. The genes not fitting into any of the above classes were grouped according to their annotations into two different categories named miscellaneous and others. The miscellaneous category, which includes genes compatible with

Table I. *Selected list of the more relevant candidate genes for suberin biosynthesis and cork regulation grouped in functional categories*

The complete list of genes is reported on Supplemental Table S1. For each gene, the EST GenBank accession numbers and the putative molecular function are given. The putative functions were assigned based on the highest BLASTX score match (e value $< 10^{-20}$) and not always are supported by biochemical data in plants. The cork to wood expression ratio is given as FC for genes with good evidence of being differential expressed (B > 3). *N* is the number of ESTs in the library.

Functional Categories	EST GenBank Accession No.	Description	Best TAIR BLASTX	FC	*N*
Acyl lipids					
FA synthesis	EE743836	Dihydrolipoamide *S*-acetyltransferase, putative	At3g25860	6	2
FA synthesis	EE743871	Biotin carboxyl carrier protein subunit	At5g15530	45	1
FA elongation	EE743843	PATE/FAT	At1g08510	21	5
FA elongation	EE743810, EE743811	LACS, putative	At1g49430	41	21
FA elongation	EE743856	ACL, putative	At5g49460	7	5
FA elongation	EE743839	KCS, putative	At5g43760	46	5
FA elongation	EE743779, EE743679	KCR, putative	At1g67730	21	2
FA hydroxylation	EE743846, EE745211, EE743708	FA ω-hydroxylase, CYP86A1	At5g58860	38	6
Lipid oxidation	EE743788, EE743799, EE743803	LOX-1	At3g22400	24	8
Lipid oxidation	EE743678	LOX-1	At1g55020	–	1
Lipid metabolism (putative)	EE743851	GDSL-motif lipase/hydrolase, putative	At2g23540	49	14
Lipid metabolism (putative)	EE743823, EE743824, EE743825	GDSL-motif lipase/ hydrolase-like protein	At5g37690	35	33
Lipid metabolism (putative)	EE743815, EE743814	GDSL-motif lipase/ hydrolase-like protein	At5g22810	29	8
Lipid metabolism (putative)	EE743858, EE743859	GDSL-motif lipase/hydrolase family protein, similar to family II lipases	At1g74460	24	8
Lipid metabolism (putative)	EE743686, EE743687	GDSL-motif lipase/hydrolase family protein, putative APG protein	At4g26790	11	5
Lipid metabolism (putative)	EE743698	GDSL-motif lipase/hydrolase family protein, putative nodulin	At1g54790	3	1
Lipid metabolism (putative)	EE743672	Esterase/lipase/thioesterase family protein	At3g62860	33	1
Lipid metabolism (putative)	EE743671	Lipase class 3 family protein	At4g00500	5	1
Glycerol ester synthesis	EE743864, EE743865	GPAT	At3g11430	41	7
Glycerol ester synthesis	EE743668	GPAT	At1g01610	9	4
Lipid transfer	EE743694	ABC transporter protein (WBC subfamily), putative	At5g13580	32	1
Phenylpropanoids					
Phenylpropanoid pathway	EE743744	PAL, putative	At2g37040	19	3
Phenylpropanoid pathway	EE743705, EE743706	PAL	At3g53260/ At2g37040	8	7
Phenylpropanoid pathway	EE743662	Cinnamate 4-hydroxylase, CYP73	At2g30490	2	1
Phenylpropanoid pathway	EE743863	4CL	At3g21240	3	1
Phenylpropanoid pathway	EE743677	4CL	At1g51680	–	1
Phenylpropanoid pathway	EE743816	Cinnamoyl CoA reductase	At1g15950	–	3
Phenylpropanoid derivatives	EE743676, EE743847	F5H, CYP84A1, putative	At4g36220	11	2
Acyltransferase	EE743861	HCBT	At5g41040	32	27
Acyltransferase	EE743848, EE745210, EE743849	HCBT, putative	At5g41040	36	13
Cross-linking/oxidase	EE743777, EE743766	Diphenol oxidase laccase	At5g05390	8	16
Cross-linking/oxidase	EE743857	Laccase	At2g40370	9	6

(*Table continues on following page.*)

Table I. (*Continued from previous page.*)

Functional Categories	EST GenBank Accession No.	Description	Best TAIR BLASTX	FC	*N*
Cross-linking/oxidase	EE743784, EE743807	Multicopper oxidase, putative	At5g05390	6	4
Cross-linking/oxidase	EE743710	Copper-containing amine oxidase	At4g12290	2	1
Miscellaneous					
Monoxygenase	EE743874, EE745209, EE745208	Cytochrome P450 protein, CYP72A14	At3g14680	65	16
Monoxygenase	EE743711, EE743700	Cytochrome P450 protein, CYP72A15	At3g14690	50	32
Monoxygenase	EE743852, EE743841	Cytochrome P450 protein, CYP72A7	At3g14610	61	21
Monoxygenase	EE743868	Cytochrome P450-like protein, CYP86B2	At5g08250	23	2
Monoxygenase	EE743831	Cytochrome P450 family protein, CYP87A2	At1g12740	30	10
Monoxygenase	EE743830	Cytochrome P450, CYP72A59	At3g14680	49	6
Acyltransferase	EE743818, EE743866	BAHD acyltransferase, putative	At1g24430	48	28
Transfer protein	EE743658	ABC transporter like protein (ATH subfamily)	At2g40090	79	1
Stress					
Reactive oxygen species scavenging	EE743659	Ascorbate peroxidase, putative	At3g09640	67	1
Regulatory Proteins					
Transcription factor	EE743870	MYB-related transcription factor	At5g52660	60	1
Transcription factor	EE743680	R2R3-MYB transcription factor	At3g49690	3	1
Transcription factor	EE743809	WRKY transcription factor	At2g46130	28	4
Transcription factor	EE743827	NAM-like protein	At3g18400	16	5
Transcription factor	EE743667	NAM-like protein	At5g13180	3	1
Transcription factor	EE743828	SQUAMOSA promoter-binding protein like	At2g47070	6	3
Transcription regulator	EE743879	AS1-interacting KH protein	At3g29390	5	1
Peptide receptor	EE743817	PSKR, Leu-rich repeat receptor kinase	At2g02220	7	2
Phosphatase activity	EE743872	Protein phosphatase 2C, putative	At4g31750	4	1
Signal transduction	EE743878	Annexin, chain A	At1g35720	27	1
Signal transduction	EE743733	Calmodulin-binding family protein	At3g59690	–	2
Ethylene forming	EE743812	ACC oxidase	At1g05010	–	2
Ethylene forming	EE743887	ACC oxidase, putative	At2g19590	–	1
Regulated proteolysis	EE743670	Major surface glycoprotein like	At5g42620	6	1
Regulated proteolysis	EE743819	Cys proteinase	At4g39090	81	1
Regulated proteolysis	EE743884	26S proteasome regulatory subunit, putative	At5g09900	91	1
Regulated proteolysis	EE743682	26S proteasome regulatory particle non-ATPase subunit12	At1g64520	4	1
Regulated proteolysis	EE743666	Ubiquitin-fusion degradation protein like	At5g15400	4	1
Regulated proteolysis	EE743674	RING-H2 zinc finger protein, ubiquitin ligase, putative	At1g72220	–	1

the main pathways leading to cork biosynthesis but whose substrates have not been characterized, amounted to 9%. The genes in others are not further discussed in this article. On the other hand, as can be observed in Table I and Supplemental Table S1, the number of ESTs (*N*, redundancy) showed remarkable differences among the genes. In SSH libraries, although SSH should, in principle, decrease the frequency of abundant transcripts while increasing the probability of rare transcripts, genes both differentially and strongly expressed become overrepresented (Ranjan et al., 2004). Therefore, with all precautions, we can hypothesize that genes showing a high redundancy are preferentially and strongly expressed in cork. This is the case of genes encoding long-chain acyl CoA synthase (LACS), hydroxycinnamoyl-CoA/benzoyl transferase (HCBT), and cytochromes of the CYP72A subfamily, among others.

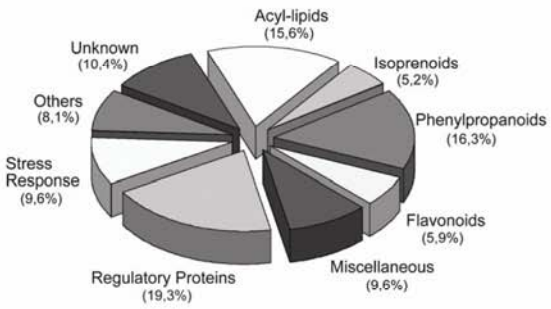

Figure 2. Relative contribution of the cork library genes to the different functional categories used in this work.

Differential Screening between Phellem and Xylem by Microarray Hybridization

Cork (phellem) and wood (xylem) tissues have common features: both originate from secondary (cambial) meristems and both synthesize aromatic polymers. However, only cork tissue produces suberin and associated waxes. Therefore, to identify genes mostly related to suberin synthesis, the cork ESTs from our library were printed on a microarray (for details, see "Materials and Methods") and hybridized to cork and wood tissue. For hybridization, cork and wood RNA was obtained from field-grown cork trees during the vegetative season when both cambial layers are in full activity. Three independent cork trees (biological replicates) were sampled and a dye swap for each biological sample (technical replicates) was performed. Microarray data were lowess normalized to account for intensity-dependent differences between channels. After normalization, dye swap replicates showed no strong deviations from linearity (Fig. 3A), proving low dye bias. The comparison between the biological and technical replicates showed a high degree of interarray reproducibility, with Pearson's correlation coefficients ranging from 0.95 to 0.98 (Supplemental Fig. S1). To select those genes with good evidence of being differentially expressed, we used a Volcano plot (B/M, odds versus ratio; Fig. 3B) and established a cutoff of $B > 3$. FC values for genes with $B > 3$ are given in Table I and Supplemental Table S1; all data can be found in Supplemental Table S2.

The great majority of the library genes were up-regulated in cork ($B > 3$, FC > 2). Regarding the main cork biosynthetic pathways, genes within the acyl lipids and isoprenoid categories, more relevant for suberin and wax biosynthesis, showed much higher FC values than genes within the phenylpropanoids and flavonoids categories, more relevant for aromatic compounds biosynthesis. The fact that most genes in the phenylpropanoids category were up-regulated in cork could indicate that specific paralogs are induced in this tissue. This hypothesis is supported, for instance, by two 4-coumarate: CoA ligase 1 (4CL)-coding genes, with only one paralog differentially expressed in cork. Quite the opposite, the two paralogs coding HCBT are both strongly cork up-regulated (FC = 37 and FC = 32, respectively). Because such a strong cork induction suggests a specific role in cork synthesis, HCBT could be a key enzyme for synthesis of phenylpropane derivatives characteristic of suberin, such as feruloyltyramine. Most genes of the acyl-lipids category were strongly up-regulated in cork. This applies to genes possibly involved in the synthesis of suberin monomers, such as the ω-hydroxylase CYP86A1 or the β-ketoacyl-CoA synthase (KCS), and enzymes that catalyze ester bonds, such as GPAT. The putative lipases/esterases, including GDSL-motif putative lipases, were highly cork up-regulated, and, although the lipase function of these proteins has not been proven in plants, a possible lipase role in cork cannot be discarded. Moreover, interestingly, the highest FC values within the functional categories were shown by genes of the miscellaneous category. This is a remarkable result taking into account that the miscellaneous category contains genes encoding enzymes, such as cytochrome P450s, transporters, and one putative acyltransferase, which may catalyze reactions important in the biosynthesis of suberin or other cork chemical components.

With regard to the stress, regulatory proteins, and others categories, changes of the FC within each category showed diverse behaviors. Two genes involved in regulated proteolysis (ubiquitin/26S proteasome regulatory subunit, FC = 91; Cys proteinase, FC = 81) were the two most phellem up-regulated genes in the library. It should also be noted that some transcription factors (MYB, FC = 60; WRKY, FC = 28; and No-Apical-Meristem [NAM], FC = 16) and some signal

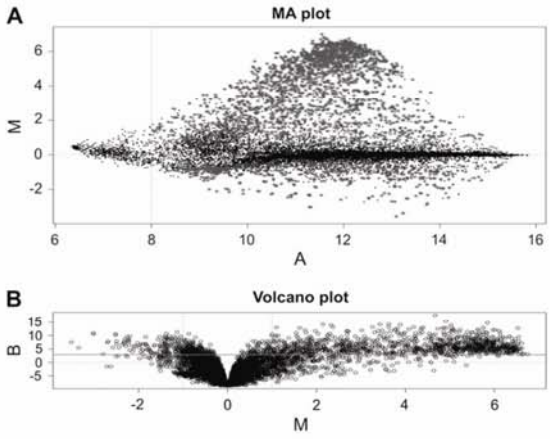

Figure 3. Plots illustrating the quality criteria applied in microarray hybridization. Average data over six hybridizations of cork versus wood are shown. A, MA plot: expression ratio versus intensity. Bigger dots correspond to ESTs differentially expressed. No dye bias can be observed in this plot. B, Volcano (BM) plot: odds of differential expression versus ratio. Genes with log odds greater than 3 (over the solid horizontal line) are considered as differentially expressed. Note that most spots are cork up-regulated.

transduction genes (protein kinase, FC = 31; calcium-binding annexin, FC = 27) exhibited high FC values. On the other hand, all genes of the unknown category showed strong cork up-regulation, a fact pointing to possible phellem-specific functions for these genes.

Validation of Differential Expression of Genes by Reverse Transcription-PCR

We used the reverse transcription (RT)-PCR with incremental cycle numbers to validate the cork-to-wood gene expression ratios measured by the microarray. The transcript abundance was analyzed for six relevant candidate genes having moderate to high FC values. The genes selected for validation were: HCBT, ferulate 5-hydroxylase (F5H), LACS, palmitoyl-acyl carrier protein thioesterase (PATE/FAT), WRKY transcription factor (WRKY), and phytosulfokine receptor (PSKR). As control, the transcript levels of three constitutive genes (actin, elongation factor, and cap-binding protein) were measured to verify that equal amounts of cDNA were used for both tissues (Fig. 4A). Gene-specific oligonucleotides (Supplemental Table S3) were used in PCR reactions containing equal amounts of both cork and wood cDNAs as templates. Products of incremental cycle numbers were subsequently analyzed. The difference in cycle numbers required for equal amplification of the corresponding PCR product in cork and wood, respectively, was used to estimate levels of differences in expression within the two tissues. The cDNA was obtained from the three biological replicates used in the microarray hybridization. The possible contamination by genomic DNA was excluded using actin primers specifically designed to differentiate genomic DNA from cDNA.

Results of RT-PCR with incremental cycle numbers (Fig. 4B) confirmed the differential expression of all six selected genes. Amplification products of their transcripts exhibited differences of three to nine cycles, which correspond to the cork-to-wood gene expression ratios measured by the microarray.

DISCUSSION

We report a collection of cork genes potentially important for cork biosynthesis and differentiation based on sequence homology and microarray comparison. This list includes a set of genes possibly involved in the biosynthesis, transport, and polymerization of suberin that, in general, agrees with the biosynthetic pathway suggested by Kolattukudy (2001), Bernards (2002), and Franke et al. (2005). The list also contains a number of putative cork regulatory genes that might be of particular interest, considering the lack of knowledge in this field. Finally, a number of genes with unknown function strongly induced in cork appeared in this study. Although this work does not prove the involvement of the candidate genes in cork differentiation, on the basis of this study, direct experimental approaches can be designed.

In the two following sections, we discuss the putative roles of a set genes potentially relevant for suberin biosynthesis and the regulation of cork differentiation, considering available data on homologous (best BLASTX hit) and related genes.

Candidate Genes for Suberin

Synthesis of Aliphatic Monomers

Dihydrolipoamide *S*-acetyltransferase and biotin carboxyl carrier protein are enzymes involved in de novo FA biosynthesis, a step necessary for the synthesis of LCFA and VLCFA. VLCFAs are precursors of waxes and some suberin monomers. Their up-regulation in cork may be due to a higher demand for acyl chains in this tissue. PATE/FAT and LACS are enzymes involved in the export of FA and LCFA from the chloroplast, a required step for the synthesis of VLCFA. Genes encoding FAT and LACS enzymes are required for normal wax and cuticle development (Bonaventure et al., 2003; Schnurr et al., 2004). Cytochromes P450 catalyze several key reactions in the synthesis of the aliphatic monomers. CYP86A1 is a

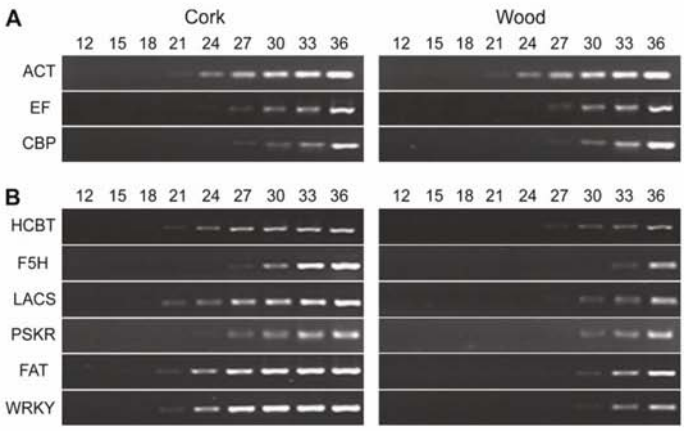

Figure 4. RT-PCR analysis of transcripts differentially expressed between cork and wood after incremental PCR cycles. Equal amounts of cork and wood cDNA were used as template for each PCR reaction. PCR products were analyzed at each cycle number indicated. Note that amplification products of housekeeping genes (A) show no differences between both tissues, whereas target genes (B) exhibit moderate to more pronounced differences.

hydroxylase that has capacity for ω-hydroxylation of C12-C18 FA (Benveniste et al., 1998). Members of the CYP86A family, LACERATA and ATT1, are involved in cuticle and cutin synthesis (Wellesen et al., 2001; Xiao et al., 2004). Lipoxygenases (LOXs) catalyze the addition of molecular oxygen to polyunsaturated FA and have been related to epoxydation of cutin monomers by the LOX/peroxygenase pathway (Blee and Schuber, 1993; Lequeu et al., 2003). FA elongase complexes in the endoplasmic reticulum catalyze chain elongation required for the biosynthesis of VLCFA. Two genes encoding condensing enzymes of these complexes are present in our library, a KCS and a β-ketoacyl CoA reductase (KCR). These two genes show, respectively, high similarity to At5g43760 (91% similarity) and At1g67730 (79% similarity), which have been identified as possible candidates for cuticular wax biosynthesis (Costaglioli et al., 2005). Mutations in KCS (KCS1 [Todd et al., 1999]; FIDDLEHEAD [Yephremov et al., 1999; Pruitt et al., 2000]; CUT1 [Millar et al., 1999]; CER6 [Fiebig et al., 2000]) and KCR (Dietrich et al., 2005) have a pronounced effect on cuticular wax deposition. ATP-citrate-lyase (ACL) produces the acetyl-CoA needed for chain elongation. Down-regulation of cytosolic ACL reduces cuticular wax deposition (Fatland et al., 2005).

In a transcriptome approach, Suh et al. (2005) identified different members of the FAT, LACS, KCS, KCR, and CYP86A gene families as candidates for waxes and cutin biosynthesis in Arabidopsis.

Transport of Aliphatic Materials

ATP-binding cassette (ABC) transporters are membrane proteins known for their function of translocating a broad range of substances across biological membranes, including lipids, sterols, and drugs. The library contains a cork up-regulated ABC transporter showing 78% similarity to AtWBC6. This transporter was put within the category acyl-lipids metabolism, because close members of this family are related to lipid transfer (Otsu et al., 2004) and the export of cuticular wax (CER5/AtWBC12; Pighin et al., 2004). Transporters of this subfamily are up-regulated in the stem epidermis of Arabidopsis (Suh et al., 2005). The library also contains a highly cork up-regulated ABC transporter, classed in "Miscellaneous," which belongs to the ATH subfamily. AtABC1, the only member of this subfamily investigated in plants, encodes a chloroplast protein putatively involved in light signaling (Moller et al., 2001). However, in mammals, members of this subfamily translocate a wide range of substrates, including terpenes (Sun et al., 1999; Sanchez-Fernandez et al., 2001).

Assembly of the Aliphatic Polyester

The polymerization of the suberin glycerol polyester is poorly understood but is thought to take place in the apoplast by esterification of the carboxyl groups of α,ω-diacids and ω-hydroxyacids with the alcohol groups of either glycerol or ω- and midchain hydroxy FA (Kolattukudy, 2001). Our library contains two putative GPATs that catalyze the formation of ester bonds. Although the known plant GPATs were previously thought to be involved in membrane and storage lipid synthesis, the analysis of the transcriptome of the stem epidermis suggested a role of these enzymes in the synthesis of aliphatic polyesters (Suh et al., 2005), which has now been confirmed by analysis of the gpat5 mutant (Beisson et al., 2007).

Synthesis of Aromatic Monomers

Genes encoding phenylpropanoid-related enzymes that act upstream in the synthesis of aromatic compounds are represented in the library. Most of them catalyze biosynthetic reactions, leading to the phenylpropanoid precursors of the aromatic part of suberin (see Bernards, 2002). Some of these enzymes, such as Phe ammonia-lyase (PAL) and cinnamate 4-hydroxylase, are key enzymes for the regulation of the phenylpropanoids pathway. Other enzymes, such as F5H, are thought to be important for the synthesis of the hydroxycinnamic acids typical of suberin (Bernards and Lewis, 1998). Two genes, both strongly up-regulated in phellem, code for HCBTs, a family of enzymes capable of catalyzing the synthesis of *N*-hydroxycinnamoyl amides such as feruloyltyramine (Yang et al., 1997). Feruloyltyramine is found in potato wound suberin (Negrel et al., 1996) and some evidence supports the hypothesis that it is also a component of the aromatic suberin in cork tree (Marques et al., 1999). HCBTs belong to the BAHD acyltransferase superfamily (D'Auria, 2006), discussed below. On the other hand, the biosynthesis of hydroxycinnamic acid amides and their subsequent polymerization in the plant cell wall is generally accepted as a plant defense response (Facchini et al., 2002).

Assembly of the Aromatic Polymer

Despite the fact that peroxidase-mediated coupling was proposed for the assembly of the aromatic monomers in suberizing cells and that several suberin-associated class III peroxidases have been described (Kolattukudy, 2001; Bernards, 2002), no class III peroxidase was found in our cork library. However, cell wall peroxidase activity could be developed by other proteins present in the library, like the apoplastic annexin (classed in the regulatory proteins category), which has peroxidase activity (Gorecka et al., 2005). The extracellular H_2O_2 needed for the peroxidase catalytic activity could be provided by the copper-containing amine oxidase (Rea et al., 2004). Conversely, this study underscores three genes coding for laccases, which are extracellular oxidases with capacity for coupling phenylpropanoids. A role for laccases in lignin synthesis has been proposed (Kiefer-Meyer et al., 1996; LaFayette et al., 1999; Ranocha et al., 2002; Ehlting

et al., 2005) and a similar function in the synthesis of the aromatic part of suberin can be hypothesized (Liang et al., 2006).

Linkages between Aliphatic and Aromatic Units

Esterified ferulates are very important suberin monomers (Adamovics et al., 1977; Bernards and Lewis, 1992; Lotfy et al., 1994). Some members of the BAHD acyltransferase superfamily catalyze the esterification of hydroxycinnamates with FAs (Lotfy et al., 1995). BAHD acyltransferases are a large superfamily of enzymes showing in vitro high catalytic versatility and wide substrate specificity (D'Auria, 2006). The phellem up-regulated transferase classed in miscellaneous shows high homology at the amino acid level with several members of the BAHD superfamily (*e* value $< e^{-30}$). Interestingly, CER2/Glossy2, a gene whose knockout leads to a wax mutant, encodes a BAHD member (Kunst and Samuels, 2003; D'Auria, 2006). Conversely, as previously discussed, the HCBT genes that also belong to this superfamily are more probably related to synthesis of aromatic amides.

In summary, our cork library contains a set of structural enzymes that are probably good candidates for the synthesis of the aliphatic and aromatic monomers of the suberin and also provides putative candidates for the assembly of the polymer. One interesting observation is the relative importance of the cytochrome P450 superfamily in the cork library. The abundance of P450 monooxygenases and of oxidases reflects the high complexity of synthesizing the cork polymeric matrix and indicates that cork cell metabolism must generate reactive oxygen species in high amounts. This corresponds to previous observations showing that cork cells suffer from fairly high oxidative stress (Pla et al., 1998, 2000). Here, we found that ascorbate peroxidase, a crucial enzyme for H_2O_2 detoxifying in plant cells (Davletova et al., 2005), was strongly up-regulated in cork.

Candidate Genes for Regulation of Cork Formation

Only very limited knowledge is available about the hormonal control of cork formation. The ethylene-forming enzyme aminocyclopropane-carboxylate (ACC) oxidase was highly expressed in cork and wood, without showing significant differences between both tissues. Although the possible role of ethylene in these tissues is unclear (Andersson-Gunneras et al., 2003; Lulai and Suttle, 2004), it could be a common regulator in cork and wood. Phytosulfokines (PSKs) are peptide hormones that induce cell dedifferentiation and reentrance into the cell cycle (Matsubayashi et al., 2002). The presence of a cork up-regulated PSK receptor kinase suggests a possible role of PSKs in phellem regulation. On the other hand, enzymes acting on lipid catabolism such as LOX1 and putative lipases (GDSL-motif proteins) could be involved in the synthesis of wound hormones, such as jasmonic acid (Wasternack et al., 2006). Interestingly, the library contains a highly phellem up-regulated annexin. This gene shows 89% similarity to At1g35720, an Arabidopsis annexin that senses the Ca^{2+} signal elicited by ABA and transmits it downstream in the signaling pathway (Lee et al., 2004).

Phellogen derivatives undergo very rapidly phases of cell division, cell expansion, bulk suberin deposition, and cell death marked by the complete autolysis of the cells. Regulated proteolysis is required during programmed cell death and for the switch from one developmental phase to another, a process that requires removing preexisting regulatory networks (Sullivan et al., 2003; Vierstra, 2003). Genes encoding regulated proteolysis, such as a Cys protease and a Ub/26S proteasome system, were highly induced in cork. Cys proteases have been involved in programmed cell death (Rojo et al., 2004). Moreover, Cys proteases and Ub/26S proteasome genes are among the most expressed genes during fiber cell death (Moreau et al., 2005).

We have found five transcription factors related to meristem identity that could play a key role in the maintenance of the phellogenic identity of cells or promote their differentiation into phellem cells. One of them is a R2R3 MYB transcription factor involved in axillary meristem identity in Arabidopsis (Muller et al., 2006). Another one is an asymmetric leaves 1-interacting protein, which could be important for establishing cell fate in leaf development (Phelps-Durr et al., 2005). Two ESTs encode proteins of the NAM family (Souer et al., 1996). Finally, a SQUAMOSA/APETALA1 transcription factor, which is a floral meristem identity gene (Mandel and Yanofsky, 1995), was also highly up-regulated. On the other hand, the library also contains a WRKY transcription factor that is highly induced in cork. WRKY transcription factors are a large family of plant-specific regulators that mainly control senescence, stress, and defense responses (Eulgem et al., 2000). WRKY factors modulate gene expression by binding to W boxes of some stress-induced genes, including P450s (Mahalingam et al., 2003; Narusaka et al., 2004).

In conclusion, a number of interesting regulatory candidate genes for cork regulation have been identified, although much more work is needed to elucidate their function.

MATERIALS AND METHODS

Plant Material and Tissue Harvesting

Cork (phellem) and wood (xylem) tissues were harvested from 15- to 20-year-old field-grown cork trees (*Quercus suber*) at Peratallada (Girona, Spain) during the growing season. External bark (cork bark) was removed and, using sterile scalpels, the exposed phellem tissue was harvested. Thus, fractions rich in differentiating phellem were obtained (Fig. 1A). Wood was obtained after removing the internal bark (secondary phloem) and fractions enriched in differentiating xylem were harvested as described above. Harvested samples of cork and wood were immediately frozen in liquid nitrogen and stored at −80°C. To prevent genetic and environmental variability, both

cork and wood samples cohybridized in the array were obtained from the same tree specimens.

As a source of somatic embryos, a cork tree recurrent embryogenic line maintained in a medium free of plant growth regulators was used (Puigderrajols et al., 1996). Macronutrients were those from Schenk and Hildebrant medium (Schenk and Hildebrant, 1972), and micronutrients and vitamins were from Murashige and Skoog medium (Murashige and Skoog, 1962), including 3% (w/v) Suc. The culture was solidified with 0.6% (w/v) agar, and the pH was adjusted to 5.7. The cultures were incubated in a growth chamber at 25°C and a 16-/8-h photoperiod at 50 μmol m^{-2} s^{-1} was provided by cool-white plus Grolux fluorescent lamps. Subcultured cotyledonary stage embryos showing signs of secondary embryogenesis were picked, their hypocotyledonary region dissected, immediately frozen in liquid nitrogen, and stored at −80°C.

RNA Extraction and Double-Stranded cDNA Synthesis

Total RNA was extracted from cork and wood tissue as described by Chang et al. (1993) and from somatic embryo as described by Martin et al. (1993). Remaining traces of genomic DNA were removed by TURBO DNAse treatment (Ambion) and a further purification step was performed with the CleanUp protocol of RNeasy Plant Mini kit (Qiagen). RNA quality and purity were checked by formamide-formaldehyde denaturing agarose gel electrophoresis spectrophotometry using Nanodrop and Bioanalyzer 2100 (Agilent). To obtain poly(A^+) RNA, 150 μg of total RNA was purified using Oligotex mRNA kit (Qiagen). The quality and yield of the mRNA was checked by denaturing agarose gel electrophoresis and RiboGreen RNA quantitation reagent (Molecular Probes). Double-stranded cDNA was synthesized from 300 ng of mRNA using primers from the PCR-Select cDNA Subtraction kit (CLONTECH), Superscript II, *Escherichia coli* DNA ligase, *E. coli* DNA polymerase, RNase H, and T4 DNA polymerase from Invitrogen, all according to manufacturer's recommendations.

cDNA Subtractive Library Construction (SSH)

The cork tree phellem subtractive library was made applying the SSH technique (Diatchenko et al., 1996, 1999) and using the PCR-Select cDNA Subtraction kit (CLONTECH). To isolate ESTs preferentially induced in phellem, cDNA from cork tissue as tester and cDNA from somatic embryo as driver were used. Two rounds of subtractive hybridization and PCR amplification according to CLONTECH instructions were performed. To improve the yield of long ESTs, products from five secondary PCRs were pooled and size selected on an agarose gel electrophoresis in three fractions: 150 to 800 bp, 800 to 1,200 bp, and 1,200 to 2,000 bp. Each cDNA fraction was concentrated and purified using Minelute PCR Purification kit (Qiagen), cloned into the pCR4-TOPO T/A cloning vector, and transformed in TOP10 competent cells (Invitrogen). Kanamycin-resistant colonies were picked and grown overnight in liquid Luria-Bertani medium containing kanamycin at 37°C and 320 rpm. Finally, glycerol was added to grown cultures to 15% final concentration and clones were stored at −80°C in 96-deep-well plates.

Sequencing and EST Analysis

Plasmids were isolated from overnight-grown bacterial cultures using a standard alkaline lysis protocol with SDS in 96-well format. Inserted fragments were amplified by PCR using M13 oligonucleotides. Reactions were carried out in a final volume of 100 μL containing 100 μM dNTPs mix, 4 μM M13 forward primer (GTAAAACGACGGCCAG), 4 μM M13 reverse primer (CAGGAAACAGCTATGAC), 0.02 units μL^{-1} DyNAzyme (Finnzymes), 2.5 mM $MgCl_2$, and 50 to 100 μg plasmid template and using PTC220 Multicycler (Dyad). PCR was done for 1 cycle at 95°C for 2 min, 35 cycles at 95°C for 45 s, 50°C for 2 min, 72°C for 1 min, and an additional cycle at 72°C for 6 min. Liquid handling steps in plasmid preparation as well as for PCR set up were carried out using a liquid handler robot RSP 200 (Tecan). All PCR products were separated on agarose gels. Gel images were analyzed to verify the amplicon length and quality using GelMaster software (Bajla et al., 2005). Single-run sequencing was carried out by MWG-Biotech AG using ABI capillary sequencers. Raw sequences and base confidence scores were obtained from chromatogram files using the base-calling program Phred (Ewing and Green, 1998; Ewing et al., 1998). Poly-A tails were removed with Trimest, a tool from the EMBOSS package. Vector masking and trimming as well as adaptor removal were performed with cross match (http://www.phrap.org). Sequences with less than 100 bases after trimming were discarded. Remaining sequences were assembled using StackPack (SANBI, http://www.sanbi.ac.za/Dbases.html) with the standard configuration (for initial clustering, a similarity cutoff value of 96 matching bases over a window size of 100 bp compared in words of length 6). Assembled sequences were annotated by using the StackPack Annotation Module (Universite Bordeaux 2, http://cbi.labri.fr). With StackPack Annotation Module, the consensus sequences and singletons were blasted locally against the following databases: SWISSPROT (ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/swissprot.gz), NR (nonredundant protein sequence database; ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz), and NT (nucleotide sequence database; ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nt.gz). EST sequences with an e value $> 10^{-10}$ were considered as no hit and excluded from further analysis. Functional assignment was conducted manually by analyzing the BLASTX- and BLASTN-annotated ESTs, where SWISSPROT hits were favored over NR hits and NR hits were favored over NT hits.

Construction of cDNA Microarray

The cDNA inserts of 975 clones from the cork oak (*Quercus* spp.) phellem SSH library genes were amplified as described above and printed on a microarray. Along with cork ESTs, 2,298 poplar (*Populus* spp.) ESTs (Dejardin et al., 2004), 2,096 oak ESTs, and 1,167 potato (*Solanum tuberosum*) ESTs from the PICME repository (http://www.picme.at) were added and used to normalize intensity data (Supplemental Table S2). For orientation, 48 Cy3 and 48 Cy5 labeled oligos were spotted as guide dots. Spotting buffer and human clones were used as negative controls. Controls and amplified ESTs were spotted twice side by side on Corning GAPS II glass slides using a contact spotter (Omnigrid/Gene-Machines). The spotting was performed with 48 SMP3 pins obtaining 17 × 18 spots per block. During spotting, the relative humidity was set to 45%. After printing, slides were scanned, cross-linked at 65 mJ, and stored at room temperature in the dark.

Differential Screening by Microarray Hybridization

For hybridization, 20 μg of total RNA of each tissue were reverse transcribed using Superscript II (Invitrogen) and dNTP mix containing aminoallyl-dUTP (CLONTECH). Purification of cDNA, coupling of fluorescent dye, and probes purification were performed using Atlas Glass Fluorescent Labeling kit (CLONTECH) following manufacturer's recommendations. We used Cy3 and Cy5 Mono-Reactive Dye packs (Amersham). After labeling, the probes were quantified using a Nanodrop spectrophotometer. Microarray hybridization was performed using a humidity hybridization chamber (InSlide-Out, Boeckel Scientific). Briefly, slides were prehybridized with 5× SSC, 0.1% (w/v) SDS, 0.1 mg/mL bovine serum albumin for 1 h at 42°C, followed by a wash in 0.1× SSC for 5 min at 25°C, two washes in water for 30 s, and dried by centrifugation at 1,600 rpm for 2 min. Then, slides were hybridized overnight (18 h) at 37°C in DIG Easy Hyb (Roche) containing labeled probes, yeast (*Saccharomyces cerevisiae*) tRNA (0.1 mg/mL), and salmon sperm DNA (0.1 mg/mL). After hybridization, slides were washed three times in 0.1% (w/v) SDS, 1× SSC for 10 min at 50°C, and washed four to six times in 1× SSC. Then, slides were dried by centrifuging at 500 rpm for 5 min and immediately scanned using the DNA Microarray Scanner (Agilent) at 10-μm resolution and 100% laser intensity and photomultiplier tube settings.

Microarray images were quantified using GenePix 6.0 (Axon) software. Only spots with signal intensities twice above the local background, not saturated and not flagged by GenePix, were considered reliable and used for subsequent analysis. Extracted intensities were subtracted from the local background and the $\log_2$ ratios were normalized in an intensity-dependent fashion by print-tip lowess. For phellem to xylem hybridization, normalized $\log_2$ ratios were scaled between arrays to make all data comparable. Statistically significant differences in gene expression were determined by computing a Bayesian statistic using all $\log_2$ ratios from replicate hybridizations. ESTs were considered as differentially expressed when their Bayesian statistic B was higher than 3. All quantitative and statistical analyses were performed using MMARGE tool, a web implementation of the Limma package in the *R* environment. Relative fold-change data were recalculated using xylem as the reference tissue. For phellem to embryo hybridization, ESTs showing $\log_2$ ratio coefficient of variation lower than 0.3 (calculated among duplicated spots) were considered as reliable.

RT-PCR with Incremental Cycle Numbers

To verify the difference of expression levels between cork and wood, equal amounts of cDNA were used for PCR with gene-specific oligonucleotides in

100-μL reactions. For each tissue sample, single-stranded cDNA was synthesized from 1 μg total RNA using the Superscript II (Invitrogen) in a 20-μL reaction. Then, cDNA was 2.5-fold diluted and 1 μL of diluted cDNA was used as template. Primers were designed with Primer3 software (Rozen and Skaletsky, 2000); sequences are shown on Supplemental Table S3. PCR conditions were 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 μM each forward and reverse oligonucleotide, and 0.05 units μL^{-1} Eurotaq polymerase (Euroclone). Thermal cycling parameters were as follows: 1 cycle at 95°C for 3 min and 36 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, using a T-Gradient thermocycler (Biometra). Aliquots of 10 μL were taken every three cycles from cycle 12 to 36 and analyzed by agarose gel electrophoresis stained with ethidium bromide as described (Lê et al., 2005). Control amplifications were performed with specific primers to actin, elongation factor, and cap-binding protein. Target amplifications were performed with specific primers to HCBT, WRKY, LACS, F5H, PSKR, and FAT. To discard possible genomic DNA contaminations we designed the actin primers complementary to two exons separated by an intron of 94 bp using *Populus* genomic DNA sequence.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers EE743887, EE743657 to EE743884, and EE745207 to EE745213.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Comparison between biological and technical replicates.

Supplemental Table S1. List of unigenes from the cork subtractive library classified into functional categories and their expression pattern in the cork to wood microarray comparison.

Supplemental Table S2. Experimental data for all ESTs represented on the microarray together with the detailed results of the hybridization experiments.

Supplemental Table S3. Primers used for semiquantitative PCR.

ACKNOWLEDGMENTS

The authors thank Drs. L. Sumoy and J. Lozano for the efficiency of its microarray service as well as their kind suggestions along this work (Microarray Laboratory, Centre de Regulació Genòmica, Barcelona). The authors also thank Prof. L. Schreiber and Dr. R. Franke (University of Bonn) for discussions and very helpful comments on the manuscript, and Prof. S. Prat (Centro Nacional de Biotecnología, Madrid), Dr. E. Domínguez (Institut de Biologia Molecular de Barcelona), and Dr. G. Mir (Universitat de Girona) for their support and fruitful experimental advice. The authors are also very grateful to Dr. M. Toribio (IMIDRA, Madrid) for providing somatic embryos and Mr. J. Casellas (Germans Casellas S.A.) and E. Juliol (Universitat de Girona) for providing cork material. S.F. performed the sequencing, annotation, and printing of ESTs. This work is part of the PhD thesis of M.S. and O.S.

Received October 19, 2006; accepted March 2, 2007; published March 9, 2007.

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Annex Chapter 1

A TARGETED APPROACH TO PERIDERM PATTERNING

Introduction

Regulatory mechanisms are essential for understanding how enzymatic processes take place in living cells. The periderm is a structure crucial to the protection of land plants but, despite its importance, nothing is known about the regulatory mechanisms acting on its formation. However, taking into account the similarities between the formation of the periderm and the vascular tissues, it is logical to suppose that some regulators may be common in both processes. Vascular system differentiation, meanwhile, has been studied to a greater extent and it has been shown that transcription factors such as MYB, NAC, KNOX, KANADI, class III homeodomain-leucine zippers (HD-ZIPIII) and brassinosteroid (BR) receptors are required in its formation (Hawker and Bowman, 2004; Schrader et al., 2004; Carlsbecker and Helariutta, 2005; Groover, 2005; Zhao et al., 2005; Demura and Fukuda, 2007).

In a previous work entitled "A Genomic Approach to Suberin Biosynthesis and Cork Differentiation" (Soler et al., 2007) we performed a Suppression Subtractive Hybridization (SSH) library in cork oak (*Quercus suber*) using a comparison between cork and somatic embryo cDNAs in order to discover candidate genes for suberin biosynthesis and cork formation. The genes identified comprise mainly secondary metabolism enzymes, but also stress-responsive and regulatory proteins of the NAC and MYB families, among others. However, regulatory genes related to meristematic activity and organ patterning, such as KNOX, KANADI, HD-ZIPIII and BR receptors, are lost during SSH, since the use of meristematic somatic embryo to subtract common genes can impair their finding. In consequence, we performed a targeted screening in cork cells to isolate some putative regulatory genes using primers designed and based on *Populus* databases. We also carried out a preliminary analysis in order to make a first attempt at a tissue transcript profile.

Results and discussion

We looked for transcripts of the genes KNOX, KANADI, HD-ZIP III and BR receptors in the cork of the cork oak using primers designed from *Populus* sequences complementary to regions conserved with their *Arabidopsis* putative orthologs. Using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) we obtained seven Expressed Sequence Tags (ESTs) from different transcription factors. These ESTs were sequenced and annotated by TBLASTX (Altschul et al., 1997) analysis using GenBank and TAIR databases. TBLASTX results showed that the ESTs isolated were highly homologous to the expected genes (*e* values < $e^{-40}$and similarity values >82%) as can be seen in Table A1 and Supplemental Table A1.

Table A1. List of the regulatory ESTs found in the cork of cork oak by targeted screening. For each EST, the primers used and the amplicon length are given. GenBank and TAIR accession numbers, a description of the most similar sequence and similarity values are also provided.

			Best TBLASTX GenBank			Best TBLASTX TAIR		
Name	**Primers**	**Length**	Acc. number	Specie	Similarity	Description	Acc. number	Similarity	Description
QsKNOX	F:TGGCTCATCCTCACTACCATC R:GCATCCATCACCACAAACTG	673bp	GI:54042994	*Populus alba x Populus tremula*	95%	homeodomain protein ARBORKNOX1	At1g62360	90%	BUM1, SHL, WAM1, STM SHOOT MERISTEMLESS transcription factor
QsHD-ZIPIII(1)	F:GGTTTCAAAATCGCAGGTGT R:GACACGGAAACCAGAGGGTA	1502bp	GI:63115353	*Gossypium barbadense*	94%	class III HD-ZIP protein	At5g60690	91%	IFL, IFL1, REV REVOLUTA transcription factor
QsHD-ZIPIII(2)	F:TGTCCTGCAAGGATGGTAAG R:ATTGAACCACCACCTTCACA	991bp	GI:60327628	*Populus trichocarpa*	98%	class III HD-ZIP protein 5 (HB5)	At1g52150	91%	ATHB15, CNA, ICU4 INCURVATA 4 transcription factor
QsHD-ZIPIII(3)	F:TTCCTTCTGGTTTCCGTATCA R:GCAACCAAAGTTGTCTCAAGC	530bp	GI:60327634	*Populus trichocarpa*	95%	class III HD-Zip protein 8 (HB8)	At1g52150	82%	ATHB15, CNA, ICU4 INCURVATA 4 transcription factor
QsHD-ZIPIII(4)	F:AAACCCAACACCTCAGCAAC R:AACAAGCAAAGCAGGAGGAA	976bp	GI:60327626	*Populus trichocarpa*	96%	class III HD-ZIP protein 4 (HB4)	At2g34710	91%	ATHB14, PHB PHABULOSA transcription factor
QsKAN	F:TCAGATCAAGATTTATGCCAAA R:GTCAGTGCTCTTAACAGTTCTATACAT	227bp	GI:84778267	*Ipomoea nil*	98%	KANADI-like transcription factor FEATHERED	At5g16560	100%	KAN1 KANADI transcription factor
QsBRL	F:CCAGCAGAAGGAAGAACAGA R:AAGAAGCCTCTCCTCTCCAA	385bp	GI:47679009	*Daucus carota*	95%	LRR-S/T-RLK putative leucine rich repeat-type serine/threonine kinase	At5g13380	94%	BRL3 BRI1-LIKE 3 protein kinase

For these seven genes, a preliminary expression pattern study was performed on cork, xylem, leaf and somatic embryo using RT-PCR (Figure A1). The expression levels of the actin (ACT) housekeeping gene were also studied to check that the cDNA levels of all tissues were similar. Although this experiment is not conclusive, it should be noted that the expression levels of all genes in cork are approximately similar to those in other tissues where their function has been demonstrated, suggesting a putative role of these genes in periderm.

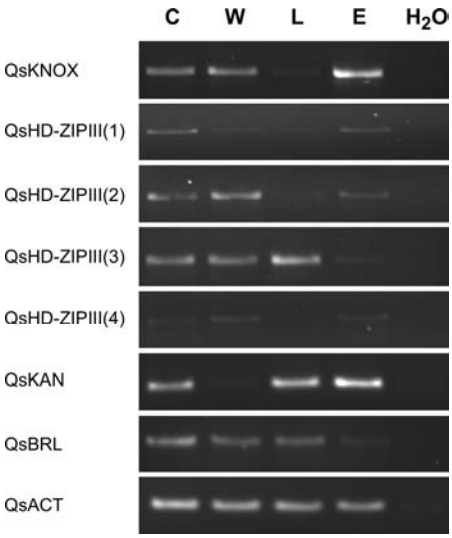

Figure A1. Transcript profile of the regulatory genes isolated. RT-PCR analysis of transcript accumulation in four tissues: cork (C), wood (W), leaf (L) and the somatic embryo (E) of cork oak. Equal amounts of each sample were used as a template for each PCR reaction. Note that control amplification products of the actin (ACT) housekeeping gene show no differences between the tissues. No amplification products could be observed in the samples where water was used instead of the template, indicating the absence of cross or environmental contamination.

Cork oak KNOX gene is the putative ortholog of the *Arabidopsis* SHOOT-MERISTEMLESS (STM), which encodes a class-I KNOX homeodomain protein necessary for the stem cell maintenance of the shoot apical meristem (Long et al., 1996). The *Arabidopsis* STM and its poplar ortholog ARBORKNOX1 (ARK1) are also expressed in stems during secondary growth, thereby promoting stem cell fate in cambium (Ko et al., 2004; Schrader et al., 2004; Groover, 2005; Groover et al., 2006). The expression of the cork oak putative ortholog of STM and ARK1 in cork suggests that this gene could be involved in maintaining the stem cell activity of phellogen, the meristem which forms the periderm.

HD-ZIPIII transcription factors direct the development of at least three tissues, the adaxial domains of lateral organs, the apical meristems and the vascular bundles. In each case, the activity of the HD-ZIPIII genes is opposed by the activity of the KANADI transcription factors (Emery et al., 2003). Interestingly, these two families of transcription factors are under the genetic control of microRNA165 (miR165) and microRNA166 (miR166) (Carlsbecker and Helariutta, 2005; Groover, 2005). Thus, a common genetic mechanism based on miRNAs directs the adaxial/abaxial pattern of leaves and the radial patterning of stems by the activities of the HD-ZIPIII and the KANADI transcription factors. The finding that several members of the HD-ZIPIII and one member of KANADI are induced in the cork of cork oak points to a putative function of this genetic mechanism as controlling the radial patterning of the periderm, characterized by the formation of cork towards the outside and phelloderm towards the inside.

Brassinosteroids are a group of steroidal plant hormones thought to be implicated in vascular differentiation (Carlsbecker and Helariutta 2005). *Arabidopsis* BRASSINOSTEROID INSENSITIVE3 (BRL3) is a leucine-rich receptor kinase that has been shown to bind brassinosteroids, which together with BRL1 act specifically on vascular differentiation (Caño-Delgado et al., 2004). Interestingly, some HD-ZIPIII genes are induced by brassinosteroids during vascular differentiation (Carlsbecker and Helariutta, 2005). The expression of a cork oak putative ortholog to BRL3 in cork indicates a hypothetical role of brassinosteroids in the formation of periderm, which in turn may also affect the expression of HD-ZIPIII genes.

In conclusion, this study permitted to enlarge the list of candidate genes for suberin and cork obtained by our laboratory (Soler et al., 2007), thereby providing new candidate genes for meristem maintenance and periderm differentiation. Moreover, preliminary expression analysis of these genes points to a role in the formation of the periderm. Thus, on the basis of this approach, functional studies can be carried out to determine the specific role that these genes perform in periderm.

Material and methods

Plant material and tissue harvesting

Cork (phellem), wood (xylem) and leaves were harvested from 15- to 20-year-old field grown cork oak trees (*Q. suber*) at Peratallada (Girona, Spain) during the growing season. Cork and wood harvesting and the obtaining of somatic embryos were described in Soler et al. (2007). Samples were frozen in liquid nitrogen and stored at -80ºC.

Total RNA extraction

Total RNA was extracted from cork, wood and leaf tissues as described by Chang et al. (1993) and from somatic embryos as described by Martin et al. (1993). Remaining traces of genomic DNA were removed by TURBO DNase treatment (Ambion) and a further purification step was performed with the RNeasy MinElute CleanUp kit (Qiagen). RNA quality, quantity and purity were checked by formamide-formaldehyde denaturing agarose gel electrophoresis and by the Nanodrop spectrophotometer.

Single-stranded cDNA synthesis and RT-PCR

Single-stranded cDNA synthesis and RT-PCR were performed in accordance with Soler et al. (2007). Thermal cycling parameters were as follows: 1 cycle at 95ºC for 3 minutes; 37 cycles at 95ºC for 20 seconds, annealing for 30 seconds and 72ºC for 1 minute; 1 cycle at 72ºC for 10 minutes. The annealing temperatures for each primer pair were the following: 60ºC for QsKNOX, 58ºC for QsHDZIPIII(1) and QsHDZIPIII(2), 55ºC for QsKAN, 54ºC for QsHDZIPIII(3), and 52ºC for QsHDZIPIII(4) and QsBRL. Control amplifications were performed with specific primers to actin (QsACT) described in Soler et al. (2007) at an annealing temperature of 52ºC. The polymerase chain reaction was conducted using a T-Gradient thermocycler (Biometra). Aliquots of 5 μL were taken after the amplification and analysed by agarose gel electrophoresis stained with ethidium bromide. To detect possible environmental and cross-contamination, amplifications were also carried out using water instead of cDNA. Actin primers complementary to two exons separated by an intron of 94 bp were used to detect possible genomic DNA contamination.

Cloning, sequencing and annotation

PCR products were cloned into the pCR4-TOPO T/A cloning vector and transformed into TOP10 competent cells (Invitrogen) in accordance with the manufacturer's instructions. When PCR gave rise to more than one amplicon, the band showing the predicted correct size according to *Populus* sequences was excised and purified using QIAquick Gel Extraction (Qiagen). Direct sequencing of the transformed

plasmids was performed using a BigDye Terminator 3.1 kit (Applied Biosystems). Sequences were manually checked and annotated by TBLASTX analysis using GenBank and TAIR databases.

Supplemental Table A1. Sequence of the ESTs found by a targeted screening approach in the bark of cork oak.

QsKNOX
TGGCTCATCCTCACTACCATCGTCTCCTGGCTGCCTATGCCAACTGTCAAAAGGTTGGAGCACCGCCTGAAGTGGTGGCAAGACTAGA
ACAAGCTTGTTCAACAGCGGCTAATATGGGCCACTCAGCTTCCACAGGCTGTATTGGTGAAGATCCGGCGTTAGATCAGTTCATGGAG
GCCTACTGTGAGATGCTGACCAAGTATGAGCAAGAACTTTCAAAACCCTTCAAGGAAGCCATGCTTTTCCTCCAAAGGATCGAGTGCC
AATTCAAAGCACTCGCAGTTTCTTCTTCAGATTCTGCCTGTGTCGAGGCTGTGGATAGGAATGTATCTTCTGAAGAAGAGGTTGATGT
GAATAACAACTTCATTGATCCCCAAGCAGAGGACCGAGAATTAAAAGGTCAGCTTTTGCGCAAGTACAGTGGATATTTAGGCAGTCTG
AAGCAGGAATTCATGAAGAAAAGAAAGAAAGGGAAACTGCCTAAAGAAGCCAGGCAACAGTTGCTGGATTGGTGGAGCAGGCATTACA
AATGGCCTTACCCATCGGAATCGCAGAAGCTGGCTCTTGCAGAATCCACAGGCTTGGATCAGAAGCAAATAAACAACTGGTTCATGAA
TCAAAGAAAACGTCACTGGAAGCCTTCAGAAGATATGCAGTTTGTGGTGATGGATGC
QsHD-ZIPIII(1)
AAATCGCAGGTGTCGAGAGAAGCAGAGAAAAGAGTCTTCACGGCTCCAGACTGTGAACAGGAAATTGACAGCGATGAACAAGCTGTTG
ATGGAGGAGAATGATCGCCTGCAGAAACAGGTTTCGCAGCTGGTATGCGAAAATGGGTATATGCGGCAACAACTGCATACTACATCAG
CGGCAACTACTGATGCAAGTTGTGACTCTGTGGTTACCACTCCTCAGCATTCTCTGAGAGATGCTAATAACCCTGCTGGACTCCTCTC
CATTGCAGAGGAGACCTTGGCAGAGTTCCTTTCAAAGGCTACAGGAACTGCTGTCGATTGGGTCCAGATGCCTGGGATGAAGCCTGGT
CCGGATTCGGTTGGGATCTTTGCCATTTCCCAAAGTTGCAATGGAGTGGCAGCTCGAGCTTGCGGTCTTGTAAGTTTGGAACCTACAA
AGATTGCAGAGATCCTTAAAGATCGTCCATCTTGGTTTCGGGAATGTCGGAGCCTTGAAGTTTTCACCATGTTTCCAGCTGGTAATGG
AGGGACAATTGAACTTGTTTACACACAGACATATGCTCCAACTACACTGGCTCCTGCACGGGATTTTTGGACTTTAAGATACACTACA
AGTTTAGACAATGGCAGTCTTGTGGTTTGTGAGAGATCTCTGTCTGGTTCTGGTGCTGGCCCAAATGCAGCTGCTGCTGCCCAGTTTG
TGAGAGCTGAAATGCTTCCCTGTGGCTATCTGATTCGGCCATGTGAGGGTGGAGGGTCAATCATCCATATTGTTGACCACCTGAATCT
TGAGGCATGGAGTGTGCCAGAAGTGCTGCGACCCCTTTATGAATCATCAAAAGTAGTGGCCCAGAAAATGACTATTGCGGCCCTGCGC
TATATAAGGCAAATAGCTCAGGAGACAAGTGGTGAGGTTGTGTATGGTTTGGGCAGGCAGCCGGCTGTTCTGAGGACTTTTAGCCAAA
GATTGAGCAGAGGCTTCAATGATGCCGTCAATGGATTCAATGACGATGGCTGGTCGTTGATCAACAGTGATGGTGCTGAAGATGTGAT
TATTGCAGTTAATTCAACCAAGAACTTGAGTGCCACCTCTAATCCTGCAAATTCCCTCTCATTTGTTGGAGGAATTCTGTGTGCAAAG
GCTTCCATGCTACTCCAAAATGTCCCTCCTGCTGTCCTGGTGCGCTTCCTGAGGGAGCACCGCTCAGAGTGGGCTGATTTCAATGTTG
ATGCGTATTCAGCTGCATCATTGAAAGCGGGCTCATATGCTTATCCAGGGATGAGGCCTACAAGGTTTACTGGGAGCCAAATTATCAT
GCCTCTTGGCCACACAATAGAACATGAAGAATTGCTTGAAGTTATTCGACTTGAAGGACATTCTCTTCCTCAAGAAGATGCTTTTGCA
TCAAGGGACATTCATCTATTGCAGATATGTAGTGGAGTTGACGAGAATGCTGTGGGAGCTTGTTCTGAACTTGTATTTGCTCCAATTG
ATGAAA
QsHD-ZIPIII(2)
ATGGTAAGCAGAGTGCGATTGACAGTGGCAAGTATGTCCGGTACACACCTGAGCAGGTTGAAGCCCTCGAGAGGCTCTATCATGAGTA
CCCAAAGCCGAGTTCGATTCGGCGTCAGCAGCTTATTCGTGAGTGTCCAATTCTCTCCAACATCGAGCCGAAACAGATCAAGGTCTGG
TTCCAGAACAGAAGATGCAGAGAGGTCTGGTTCCAGAACAGAAGATGCAGAGAAAAACAGAGGAAAGAGGCTTCGCGACTTCAAGCTG
TGAATAGGAAGCTGACAGCCATGAACAAACTTCTGATGGAGGAGAATGATAGGTTGCAGAAGCAAGTATCACAGCTGGTGTATGAGAA
TGGTTACTTCCGCCAGCATACCCAGAATACAACTCTTGCAACCAAAGACACAAGTTGTGAATCGGTGGTGACTAGTGGTCAACACCAT
TTGACGCCTCAGCATCCGCCAAGGGATGCTAGCCCTGCAGGGCTTTTGTCCATTGCAGAAGAGACTTTAGCAGAGTTTCTTTCAAAGG
CCACTGGAACTGCCGTGGAGTGGGTCCAAATGCCTGGAATGAAGCCTGGTCCGGATTCCATTGGAATCGTTGCTATTTCTCATGGTTG
CACTGGAGTGGCAGCACGAGCCTGTGGCCTAGTGGGTCTAGAACCTACAAGGGTTGCAGAAATCCTCAAAGATCGGCCTTCGTGGTTC
CGCGATTGCCGAGCTGTGGATGTTCTGAATGTGCTCCCCACTGCAAACAGTGGAACCATCGAGCTGCTTTACATGCAGCTCTATGCTC
CGACTACATTGGCGCCTGCTCGCGACTTTTGGTTGTTGCGCTACACTTCTGTTTTAGAAGATGGCAGCCTTGTGGTCTGTGAGAGGTC
ACTTAAAAATACTCAAAATGGTCCAAGCATGCCACCAGTGCAGCATTTTGTGAGAGCAAAAATGCTGCCTAGCGGGTATCTCATACGG
CCCTGTGAAGGTGGTGGTTCAAT
QsHD-ZIPIII(3)
TTCCTTCTGGTTTCCGTATCATTCCTCTTGATTCTGGAATTGATTCCTCTAGTCCAAACCGTACACTTGACCTTGCCTCTGCTCCTGA
AGTTGGACCCACTGGAAATAGAGCATCCGGTGAAAATTCTGGTCAGTGTGGGAGCACCAAATCTGTGATGACAATAGCATTCCAGTTT
GCATTTGAAATCCACCTTCAAGACAATGTAGCAGCCATGGCTAGACAATACATCCGGAGCATTATAGCATCTGTGCAGAGGGTGGCAT
TGGTGCTCTCTCCTTCTCATTTTGGTTCTCATGCTGGCATTCGGCCACCACCTGGCACTCCTGAAGCTCACACGCTTGCTCGGTGGAT
CTGTCAAAGCTATAGGTGCTATCTAGGGGTTGAACTACTTAAAAGTGAAGGAAGTGAATCCATTCTCAAAACTCTTTGGCATCACTCA
GATGCAGTCATGTGCTGCTCTTTGAAGGCACTGCCAGTTTTTACATTTGCAAATCAGGCAGGTCTTGATATGCTTGAGACAACTTGGT
GC
QsHD-ZIPIII(4)
AACCCAACACCTCAGCAACCCCAAAGGGATGCTAACAACCCAGCTGGTCTTCTCGCGATTGCTGAGGAGACCCTGGCAGAGTTCCTTT
CTAAGGCTACTGGAACTGCTGTCGACTGGGTCCAGATGATTGGGATGAAGCCTGGTCCGGATTCTATTGGAATCGTTGCTGTTTCCCG
CGACTGTAGTGGGGTAGCAGCACGAGCCTGCGGTCTTGTGAGTCTAGAGCCCACGAAGGTCGCTGAAATCCTTAAAGAACGTCTATCA
TGGTTTCGTGATTGTCGAAGCATTGATGTTTTGAGTGCAATCCCTACTGGGAATGGAGGAACAATTGAGCTGATATACATGCAGACTT
ATGCACCTACAACATTGGCTGCGGCACGTGACTTCTGGACGTTGAGATATACTACAAGTTTGGAAGATGGAAGTCTTGTGATATGTGA
GAGATCATTGACCTCTTCTACTGGTGGCCCAGCAGGGCCTCCCACTACTAGTTTTGTAAGAGCTGAAGTGCTTCCCAGTGGCTATCTA
ATACGGCCTTGTGAGGGTGGTGGCTCCATAATTCACATTGTTGATCATGTCGATTTAGATGCATGGAGTGTTCCTGAAGTTCTCAGGC
CACTTTATGAATCATCAAAGATTCTTGCTCAGAAAATGACTGTCGCTGCCTTGCGACACATACGACAAATTGCACAAGAGAGTAGCGG
AGAAATTCAGTATGGTGGGGGCCGCCAACCTGCTGTTTTAAGGACATTCAGTCAGAGACTTTGTAGGGGTTTCAATGATGCTGTTAAC
GGGTTTGCGGATGATGGTTGGTCACTAATGGGAAGTGATGGTGTGGAAGATGTGACCATCATGATAAACTCATCTCCAAACAAATTTC
TTGGCTCCCAGTATAGCACATCAATGTTCTCAACTTTTGGAGGAGGGGTGCTGTGTGCCAAGGCATCAATGCTGCTGCAGAATGTTCC
TCCTGCTT
QsKAN
TTAGATCAAGATTTATGCCAAAGCTACCAACAAAGAGGAGTATGAGAGCACCAAGAATGCGCTGGACAAGCACTCTCCATGCTAGATT
TGTTCACGCTGTTGAGCTTCTTGGTGGCCATGAAAGAGCTACACCAAAATCAGTTCTTGAGCTCATGGATGTGAAGGATCTCACATTA
GCGCATGTAAAAAGCCATTTACAGATGTATAGAACTGTTAAGAGCACTGAC

QsBRL
CCAGCAGAAGGAAGAACAGAGAGAGAAATACATTGAAAGTCTTCCAACTTAAAGTAGGAGCAGCTGGAAACTTTCCAGTGTTCCTGAA
CCCCTTAGTATCAATGTTGCTACATTTGAAAAACCTCTGCGGAAATTGACCTTCGCTCATCTACTCGAAGCTACAAATGGTTTCAGTG
CTGACAGCTTAATTGGTTCTGGAGGTTTTGGTGAGGTCTACAAAGCTCAATTGGGAGATGGCTGTGTTGTTGCAATTAAGAAGCTTAT
TCATGTCACTGGTCAGGGAGACAGGGAGTTTATGGCAGAGATGGAAACTATTGGGAAGATCAAGCACCGAAACCTTGTTCCGTTGATG
GGTTACTGTAAGATTGGAGAGGAGAGGCTTCTT

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Chapter 2

Marçal Soler , Olga Serra , Marisa Molinas, Emili García-Berthou, Antònia Carita and Mercè Figueras. “Seasonal variation in transcript abundance in cork tissue analyzed by real time RT-PCR”. *Tree physiology*. vol. 28, issue 5 : p. 419-431

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Received August 21, 2007; accepted October 26, 2007; published online March 3, 2008

Abstract

The molecular processes underlying cork biosynthesis and differentiation are mostly unknown. Recently, a list of candidate genes for cork biosynthesis and regulation was made available opening new possibilities for molecular studies in cork oak (*Quercus suber* L.). Based on this list, we analyzed the seasonal variation in mRNA abundance in cork tissue of selected genes by real time reverse-transcriptase polymerase chain reaction (RT-PCR). Relative transcript abundance was evaluated by principal component analysis and genes were clustered in several functional subgroups. Structural genes of suberin pathways such as *CYP86A1*, *GPAT* and *HCBT*, and regulatory genes of the *NAM* and *WRKY* families showed highest transcript accumulation in June, a crucial month for cork development. Other cork structural genes, such as *FAT* and *F5H*, were significantly correlated with temperature and relative humidity. The stress genes *HSP17.4* and *ANN* were strongly positively correlated to temperature, in accord with their protective role.

Keywords

cork cambium, cork oak, periderm, phellem, phenology, *Quercus suber*, suberin biosynthesis.

Chapter 3

FUNCTIONAL CHARACTERIZATION OF CORK REGULATORY GENES

Introduction

Land plants have evolved barriers that protect their internal living tissues from dehydration, injuries, and pathogens. This has been achieved by means of regulatory networks that adapt the barriers to the physiological requirements of the plants. Secondary (mature) organs and healing tissues are protected by the periderm, a complex structure that replaces the epidermis in plant secondary growth. The periderm is composed of three distinct tissues from the exterior to the interior: the cork or phellem, a stratified tissue responsible of the barrier properties; the phellogen or cork cambium, which is the mother layer; and the phelloderm, a parenchymatous internal tissue. Cork formation involves the proliferation of phellogen derivatives, cell expansion and an extensive deposition of suberin and waxes into the walls, which makes the tissue impervious and resistant, and ends with the programmed death of the cells.

Plant regulatory networks respond to the external environment by optimising the functions to ensure plant survival. These regulatory networks comprise hormones, signalling pathways and transcription factors. However, despite the importance of the cork tissue for the survival of higher plants, almost nothing is known about the mechanisms that regulate its formation and differentiation. In contrast, regulatory factors acting on lignin deposition and wood differentiation have been the subject of a number of studies (Rogers and Campbell, 2004; Carlsbecker and Helariutta, 2005; Groover, 2005; Demura and Fukuda 2007). These studies have highlighted the importance of auxin for wood formation and have also identified several regulatory proteins, such as the members of the KNOX, MYB, NAM, class III homeodomain-leucine zipper (HD-ZIPIII) and KANADI families among others.

To address the lack of knowledge concerning suberin and cork regulation we carried out a transcriptomic approach to cork formation in the bark of cork oak (*Quercus suber*). This provided a list of Expressed Sequence Tags (ESTs) from cork candidate genes, including regulators like hormone-related proteins, signal transductors and transcriptional regulators, which were related to cork for the first time (Soler et al., 2007). This list includes members of the MYB, NAM and WRKY families, and other regulators such as the AS1-interacting KH protein (RIK). In addition, using a targeted approach, we also identified members of the KNOX, KANADI and HD-ZIPIII families in cork (Annex Chapter 1). The expression of these genes in cork and the function attributed to putative orthologs in the literature suggests their involvement in periderm regulation. However, functional genetic studies are required to confirm their role, identify their targets and better understand the regulatory networks operating in cork.

With the aim of shedding some light on cork regulation processes, we selected four putative transcription regulators to demonstrate their role in suberin deposition and cork differentiation by reverse genetics in potato (*Solanum tuberosum*). The potato genes selected, chosen according to their putative role in the literature and the relative abundance of their transcripts in cork, encode a member of the NON APICAL MERISTEM (NAM) family of transcription factors, a DEFECTIVE EMBRYO MERISTEM2 (DEM2) protein and the putative orthologs of *Arabidopsis* AS1-interacting KH protein (RIK) and REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV) HD-ZIPIII transcription factor. Transcripts of NAM and RIK were isolated using a Suppression Subtractive Hybridization (SSH) library of cork oak bark against somatic embryo (Soler et al., 2007), whereas transcripts of REV were found using a targeted approach (Annex Chapter 1). On the other hand, DEM2 was obtained from a SSH library of potato periderm against potato parenchyma (data not shown).

We used the potato as a model because its tuber skin (periderm) is a good model for molecular studies, since it can be easily isolated for physiological and chemical analysis (Schreiber et al., 2005). Recently, reverse genetic approaches in potato have been used to demonstrate the role of the CYP86A33, an ortholog of *Arabidopsis* CYP86A1, in suberin chemical composition and periderm permeability properties (Serra et al., submitted).

Regulatory genes selected for reverse genetics in the potato

NAM

NAM is a subgroup of the NAC superfamily of transcription factors, involved in various plant developmental and morphogenic systems and characterized by the presence of the consensus sequence known as the NAC domain (Aida et al., 1997; Ooka et al., 2003). Some members of the NAC superfamily have been related to cell wall thickening in xylem (Kubo et al., 2005; Mitsuda et al., 2005; Ko et al., 2007; Mitsuda et al., 2007; Zhong et al., 2007). However, some members of the subgroup NAM are related to the shoot apical meristem (SAM) and cotyledon formation (Souer et al., 1996; Aida et al., 1999; Ishida et al., 2000), and it is predicted that other proteins in the subgroup also have important roles in development and morphogenesis (Ooka et al., 2003).

RIK

RIK is a predicted RNA binding protein that forms protein complexes with *Arabidopsis* ASYMMETRIC LEAVES 1, 2 (AS1, 2) and an ortholog of the histone chaperone HIRA. It has been suggested that these complexes repress class I KNOX genes (Phelps-Durr et al., 2005), which are involved in SAM maintenance. The ectopic expression of KNOX genes in leaves affects organ determination resulting in cell over-proliferation and patterning defects (Smith et al., 1992; Sinha et al., 1993; Chuck et al., 1996). This phenotype is similar to that obtained in *as1* mutants as well as their orthologs from maize (*Zea mays*, *rough sheath2*) and *Antirrhinum majus/Nicotiana tabacum* (*PHANTASTICA*) (Schneeberger et al., 1998; Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Ori et al., 2000; McHale and Koning, 2004). Furthermore, partial loss of HIRA function in *Arabidopsis* results in developmental defects analogous to those of *as1* (Phelps-Durr et al., 2005).

REV

REV, together with PHABULOSA, PHAVOLUTA, CORONA/ATHB15 and ATHB8, are the HD-ZIPIII proteins found in *Arabidopsis*. These genes play overlapping, opposing and divergent roles in *Arabidopsis* development (Prigge et al., 2005) and establish the radial patterning of cambium-derived vascular tissues and the adaxial-abaxial polarity in lateral organs (Carlsbecker and Helariutta, 2005; Groover, 2005). However, due to function complementation among HD-ZIPIII family members, identification of mutant phenotypes is difficult. REV is the only member able to display an identifiable single mutant phenotype (Baima et al., 2001; Emery et al., 2003; Prigge et al., 2005).

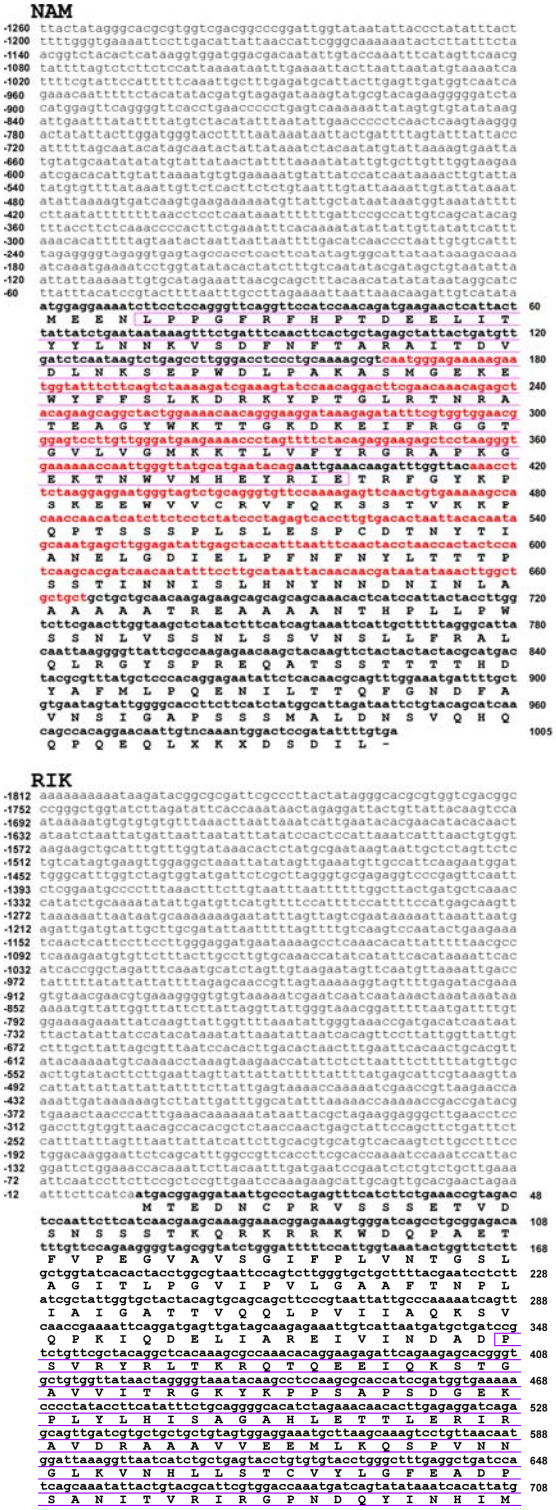
DEM2

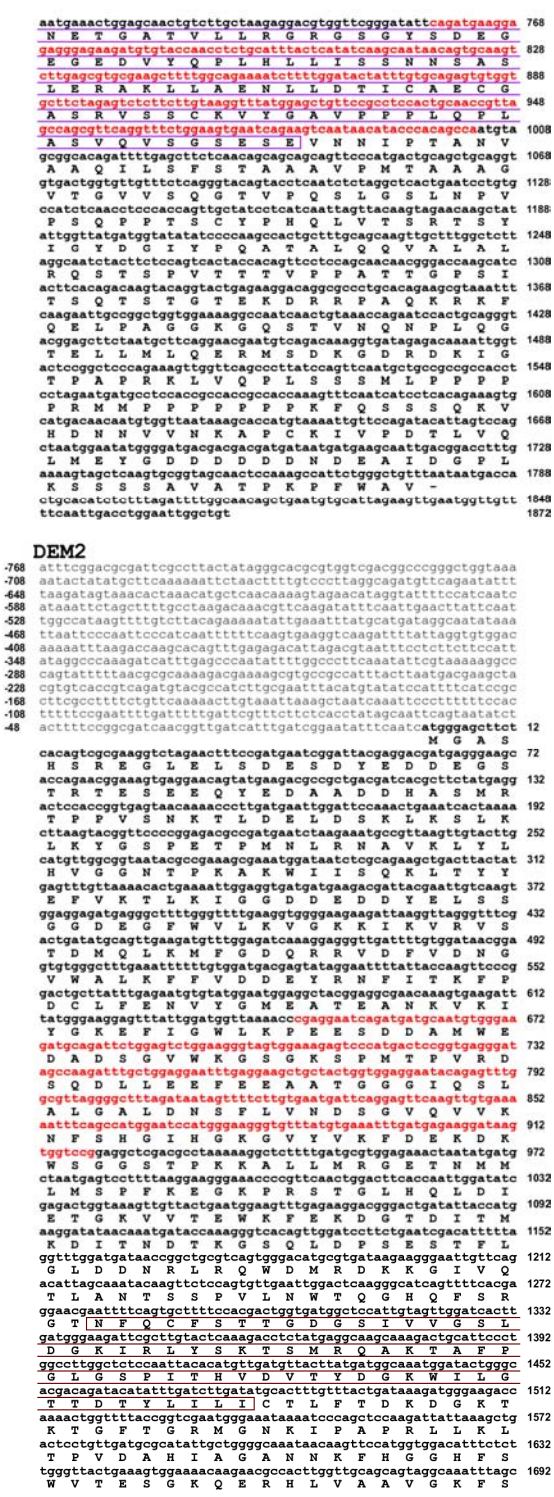
Although nothing is known about DEM2, a very similar protein named DEFECTIVE EMBRYO MERISTEM (DEM) has been studied in the tomato (*Lycopersicon esculentum*). As described by Keddie et al. (1998), *dem* mutant seedlings have a variable number of cotyledons and contain shoot and root apical meristems with disorganized cells. Therefore, it is clear that DEM is required for the proper organization of shoot and root apical meristems. Furthermore, in shoot apices DEM is coexpressed with the KNOTTED1-related homeodomain protein of the tomato, a member of the KNOX gene family, which is known to be required for SAM maintenance (Hareven et al., 1996; Keddie et al., 1998).

Results

For all the genes studied, we obtained the full-length coding sequence from potato tuber RNA and the regulatory region upstream from the start codon from leaf genomic DNA. Next, potato transgenic lines over-expressing or silenced by RNA interference (RNAi) were obtained. Silencing of the NAM transcription factor was done using two constructs: one contained one *specific region* to selectively down-regulate the member identified as a cork candidate (sNAM-RNAi from now on), whereas the other contained one *conserved region* in order to also silence putative family members more related to our candidate, and thus prevent putative function complementation by paralogs that can be expressed in cork (cNAM-RNAi from now on). The remaining genes were silenced using one construct targeted against a non-conserved region. Double insertion of these gene regions in inverse orientations into the pBIN19RNAi vector generated a construct able to produce a hairpin chimera to trigger the degradation of the target mRNA. The zones silenced are marked with red nucleotides in Figure 1. Lines ectopically over-expressing a NAM transcription factor under the genetic control of 35S CaMV promoter were also produced. Several kanamycin resistant lines that derived from independent transformation events were obtained for each construct. Kanamycin resistance indicates insertion of the T-DNA bearing neomycin phosphotransferase (nptII) gene, and therefore the recombinant construct, into the plant genome. However, confirmation by Northern blot analysis and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was only finished for cNAM-RNAi silencing and the over-expression of NAM.

Isolation of the full-length coding sequence and the promoter of regulatory genes NAM, RIK, DEM2 and REV





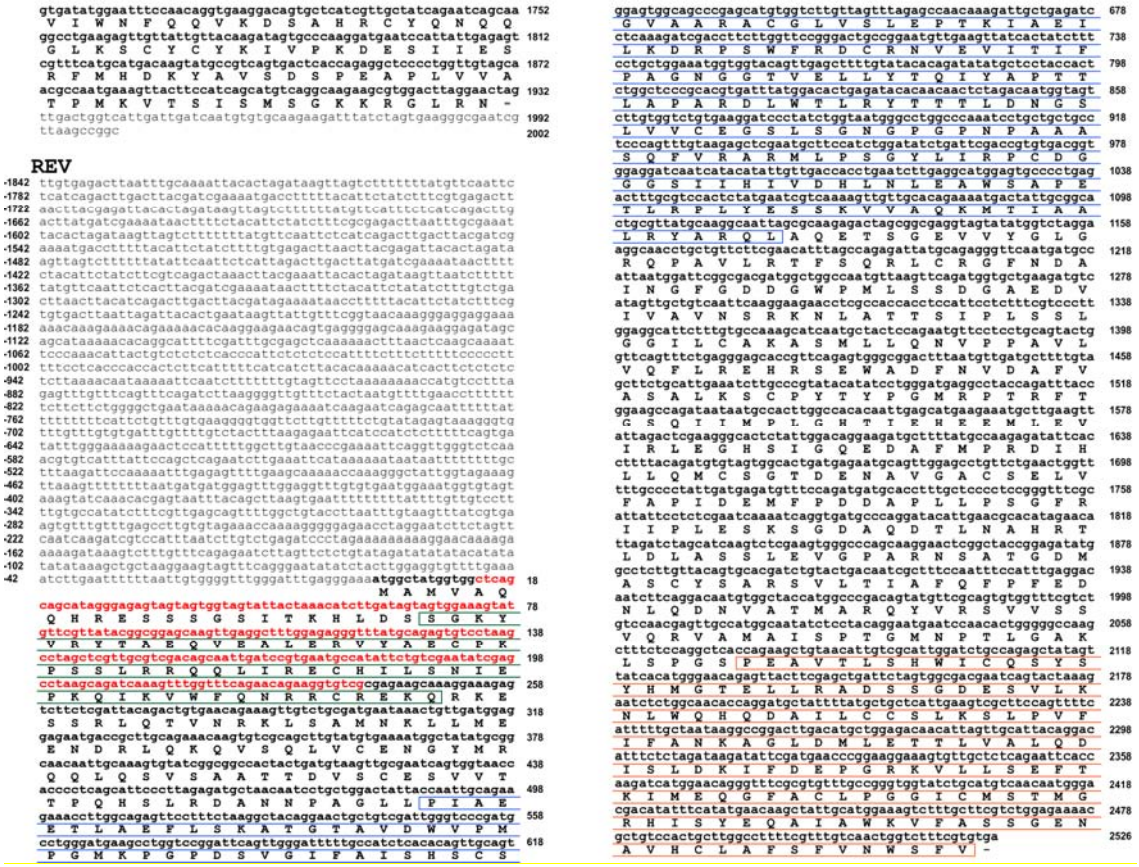


Figure 1. Promoter region linked to the coding sequence of the potato genes NAM, RIK, DEM2 and REV. Note that introns are not included and 3'UTR, if present, is not complete. The coding sequence is given in nucleotides and translated into amino acids, and the promoter region is given only in nucleotides. The numeration given corresponds to the nucleotides, with positives numbers corresponding to the coding region starting with the start codon, whereas the negatives numbers corresponding to the promoter region. As regards to protein domains, the best results detected using InterProScan are shown, with e-values ranging from e^{-15} to e^{-92}. Concerning to the NAM, a NAM domain (PF02365) was marked in pink. For the RIK, a BLOM7 domain (PTHR15744) was shown in purple. For the DEM2, a vacuolar import and degradation VID27 domain (PF08553) was shown in brown. Concerning to the REV, a HOMEOBOX domain (PF00046) in green, a lipid-binding START domain (PF01852) in blue and a MEKHLA domain (PF08670) in orange were marked. The zones silenced for each gene are marked with red nucleotides. Note that the NAM transcription factor contains two zones silenced, one zone inside the NAM domain corresponds to the construction cNAM-RNAi, whereas the zone outside the NAM domain corresponds to the construction sNAM-RNAi.

Complete coding sequences were achieved by 5'RACE and primers complementary to conserved regions from putative orthologous genes. Sequences obtained from cDNAs gave rise to proper open reading frames. Nucleotidic and amino acid sequences are given for the complete cDNA in Figure 1. The protein domains were searched for using InterProScan (Zdobnov and Apweiler, 2001; http://www.ebi.ac.uk/InterProScan/) to give some clues about the function of the genes NAM, RIK, DEM2 and REV. The isolation of promoter regions was accomplished by GenomeWalker, and these regulatory regions, include at least 750 bp upstream from the start codon (Figure 1). Putative cis-regulatory elements are searched for using PlantCare (Lescot et al., 2002; http://intra.psb.ugent.be:8080/PlantCARE), and only the elements found in promoter regions of both

potato and *Arabidopsis* are shown in Table 1. These putative cis-regulatory elements give more clues about the regulation of the genes, but this needs to be confirmed experimentally.

Table 1. Table of the cis-regulatory elements found in the promoter regions of potato using PlantCare. Only the elements found in the promoter regions of both potato and *Arabidopsis* are shown. The putative function of the recognized motifs is specified. The number below the gene indicates the number of times that the motif is repeated in the promoter. Promoter regions analyzed include 1200 bp for the NAM and REV, 768 bp for DEM2 and 431 bp for RIK.

Element	Putative function	NAM	RIK	DEM2	REV
5UTR Py-rich stretch	Confer high transcription levels			1	
AE box	Light response	1			
Box 4	Light response	5		1	
Box I	Light response				1
CAAT box	Common in promoters and enhancers	35	11	21	13
CCAAT box	MYB binding site			1	
CGTCA	Methyl jasmonate response			2	
GARE motif	Gibberellin response				3
GCN4 motif	Endosperm expression	2			
GT1 motif	Light response		1		
HSE	Heat stress response		1		3
SKn-1 motif	Endosperm expression	2		1	1
TATA box	Transcription start	104	7	17	35
TCA element	Salicylic acid response			1	3
TCT motif	Light response	1			1
TGACG motif	Methyl jasmonate response			2	
Circadian	Circadian control				1
Unnamed 4	?		3		10

Slencing and ectopic over-expression of the NAM transcription factor

Expression pattern

The expression pattern of the NAM transcription factor was studied in wild-type potato tuber skin, tuber parenchyma, leaf, stem and root (Figure 2). Note that transcripts are only detectable in tuber skin and root. These two tissues are characterized by their suberin content, since suberin is not only present in the periderm but also in the endodermis, exodermis and hypodermis of roots (Kolattukudy, 1980). Interestingly, the periderm corresponding to a wild-type freshly harvested tuber (FHT) shows much higher expression than a periderm belonging to a tuber stored for one week (1WST).

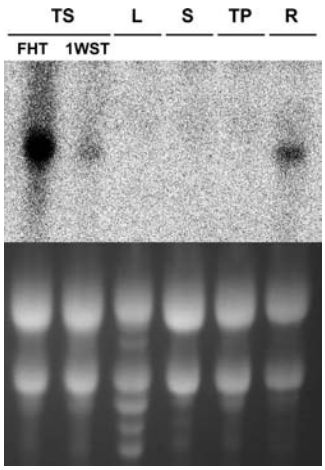


Figure 2. Expression pattern of the NAM transcription factor. The expression was analyzed by Northern blot in wild-type potato tuber skin (TS), leaf (L), stem (S), tuber parenchyma (TP) and root (R). Transcript is only detectable in the tuber skin and root. Note also that the periderm of a freshly harvested tuber (FHT) shows much higher expression than the periderm of a tuber stored for one week (1WST).

Gene silencing

Successful down-regulation of six cNAM-RNAi lines has been confirmed by Northern blot analysis and semi-quantitative RT-PCR (Figure 3). Figure 3A shows the Northern blot analysis of tuber skin from 30 kanamycin-resistant lines transformed with the construct cNAM-RNAi and from two wild-type tuber skins (FHT and 1WST). Tubers from transgenic plants were stored for four days before RNA extraction. Kanamycin resistant lines without a signal or with a signal of less intensity than the control 1WST (1, 2, 16, 31, 34 and 48) were selected and subsequently analyzed by RT-PCR with incremental cycle numbers, together with some apparently non-silenced (27, 30 and 46) and wild-type lines. Figure 3B shows the result of the RT-PCR analysis, evidencing that the lines 1, 2, 16, 31, 34 and 48 have a lower expression for the NAM than the wild-type and non-silenced lines. These silenced lines were therefore selected to carry out further studies aimed at obtaining better knowledge of gene function in cork.

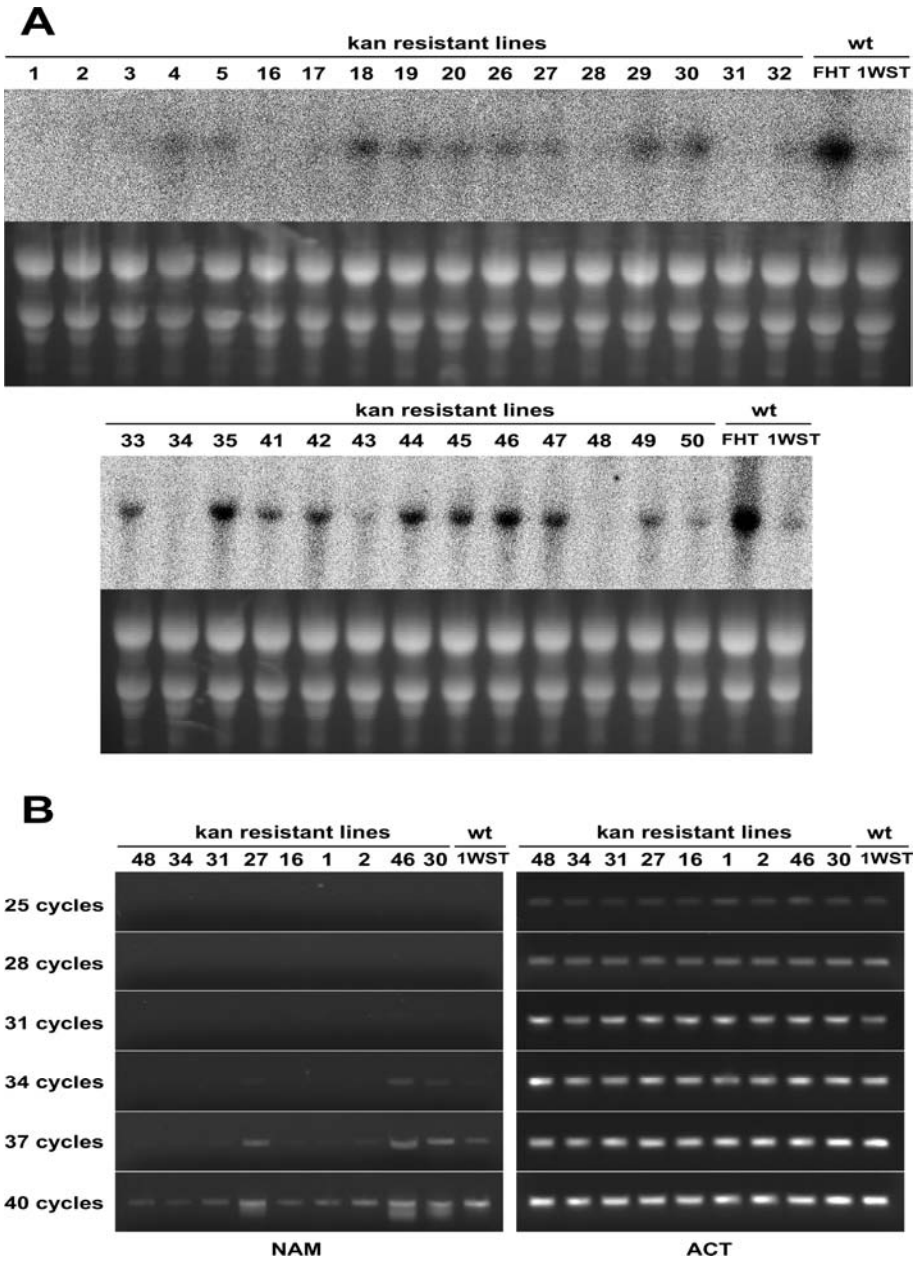


Figure 3. Gene silencing analysis of the cNAM-RNAi lines. **A.** Specific expression of the NAM transcription factor was analysed by Northern blot in tuber skin from 30 kanamycin resistant lines and two wild-type (wt) tubers: freshly harvested tuber (FHT) and one week stored tuber (1WST). Lines without a signal or with a less intense signal than that of the control 1WST were considered as silenced. **B.** Tuber skins from silenced (1, 2, 16, 31, 34 and 48) lines were subsequently analysed by RT-PCR with incremental cycle numbers together with some non-silenced (27, 30 and 46) and 1WST. Product accumulation of silenced lines appeared some cycles after non-silenced and wt lines, thus confirming the silencing. Actin (ACT) accumulation was used in RT-PCR as the control.

Ectopic over-expression

Fifteen lines of kanamycin resistant plants were obtained for the ectopic over-expression of the NAM transcription factor (Figure 4). Figure 4A shows the Northern blot analysis of leaf tissue from these lines. As a positive control, tuber skin RNA was included in the analysis. The kanamycin resistant lines with a stronger signal than that of wild-type leaf were selected and subsequently reconfirmed using RT-PCR (11, 15, 16, 17 and 19) together with some apparently non-over-expressed (14 and 18) and wild-type lines. As can be seen in Figure 4B, plant lines 11, 15, 16, 17 and 19 show higher amplicon accumulation than wild-type and non-over-expressed lines. These ectopically over-expressed lines were therefore selected for further studies.

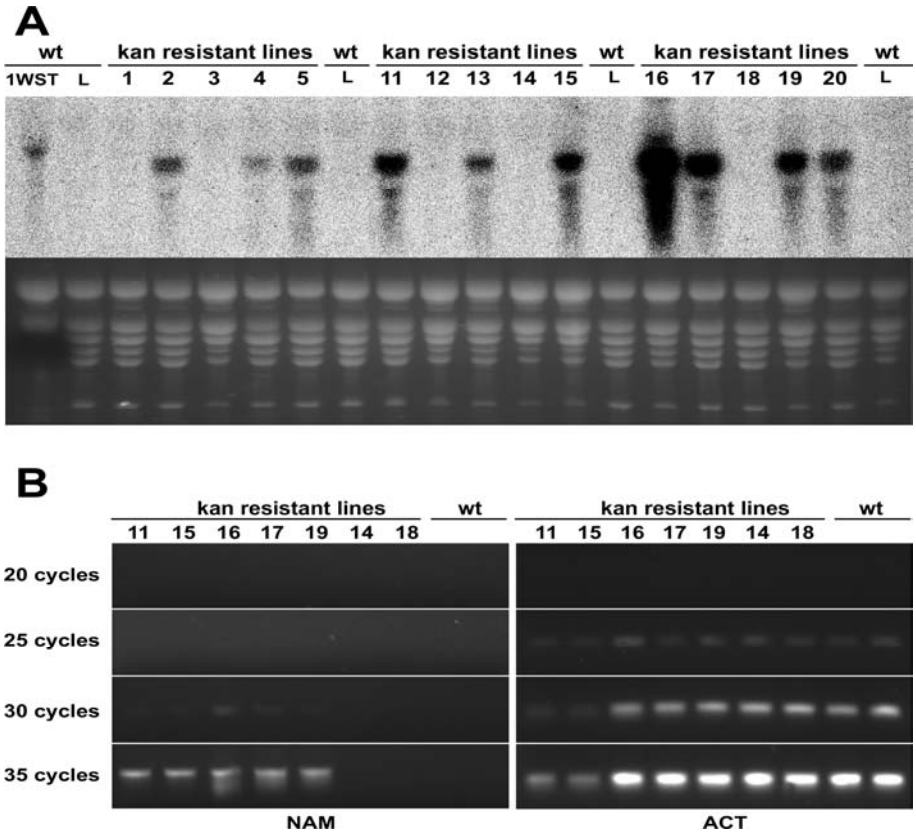


Figure 4. Ectopic over-expression analysis of the NAM transcription factor. **A.** Specific expression of the NAM transcription factor was analysed by Northern blot in leaves from 15 kanamycin resistant lines and wild-type (wt). In addition, a wild-type one-week stored tuber (1WST) was added as the positive control.

Lines with a stronger signal in the leaf than wild-types are considered as over-expressed. **B.** Leaves from over-expressed (11, 15, 16, 17 and 19) lines were subsequently analysed by RT-PCR with incremental cycle numbers together with some non-over-expressed (14 and 18) and wild-type lines. Product accumulation of over-expressed lines was visible after 30 cycles, whereas products of non-over-expressed or wild-type lines were not visible after 40 cycles. Actin (ACT) accumulation was used in RT-PCR as the control.

Conclusions and further development

The present work is a first step towards demonstrating the role of four putative transcription regulators in cork. For these selected genes we obtained transgenic potato plants on which to perform further detailed studies of their molecular and physiological function in the periderm. With regards to NAM, we verified cNAM-RNAi silencing and the over-expression of six and five lines respectively. However, we are still working on the confirmation of silencing for the RIK, DEM2, REV and sNAM-RNAi lines.

To discover the physiological role of candidate genes we will perform water permeability, chemical and ultrastructural analysis of the periderm, comparing transgenic and wild-type lines. Furthermore, transcriptome analysis using microarrays will allow target genes affected by up- and down-regulation to be found. The full-length coding sequences and the promoter regions will serve to better characterize protein function and gene regulation. Overall, this will permit a better understanding of the regulatory networks leading to cork formation and differentiation, which to date is practically unknown.

Material and methods

Plant material and growth conditions

Potato plants *(Solanum tuberosum cv Desirée)* were propagated *in vitro* in cabinets and tubers were produced *in vivo* in a walk-in chamber. For *in vitro* propagation, stem cuttings were cultured in MS media (Duchefa) in growth cabinets under a light/dark photoperiod cycle of 16 hours/8 hours at 22ºC. *In vitro* plants were transferred to soil and grown for about two months in a walk-in chamber until tuber production.

RNA isolation and cDNA synthesis

Total RNA was isolated using guanidine hydrochloride (Logemann et al., 1987) from potato root, shoot, leaf, tuber skin and tuber parenchyma tissue. 20 µg of RNA were digested with 4 units μL^{-1} of Turbo DNase (Ambion) in a volume of 40 µL to remove any remaining traces of genomic DNA. After digestion, a further purification step which removes DNase was performed with the RNeasy MinElute CleanUp kit (Qiagen). RNA quality, quantity and purity were checked by formamide-formaldehyde denaturing agarose gel electrophoresis and by the Nanodrop spectrophotometer. First strand cDNA was

synthesized from 2 μg of digested RNA using the SuperScript III reverse transcriptase (Invitrogen) and an oligo$(dT)_{18}$ primer.

Cloning of the full-length coding sequences from potato

Putative orthologous sequences were obtained by BLASTN algorithm (Altschul et al., 1997) using the TAIR, GenBank and The Gene Index Project databases. Alignments were carried out using the Multalin algorithm (Corpet, 1998). Primers were designed using Primer3 software (Rozen and Skaletsky, 2000). PCR was performed using tuber skin cDNA as a template, following the conditions described in the RT-PCR with incremental cycle numbers section. Annealing temperatures were 56ºC for NAM and DEM2, 53ºC for REV, and 58ºC for RIK. PCR products were cloned into pCR4-TOPO (Invitrogen) and sequenced using the BigDye Terminator 3.1 kit (Applied Biosystems).

NAM transcription factor

We designed the forward primer complementary to the potato Tentative Consensus (TC143904) (obtained from The Gene Index Project, http://compbio.dfci.harvard.edu/tgi/) most similar to the cork oak EST (EE743827) (Soler et al. 2007). The reverse primer was designed and degenerated based on the sequences of *Arabidopsis* (GI:18401877, At3g18840) and petunia (*Petunia x hybrida*; GI:21389167) most homologs to the cork oak EST. The forward primer contains a restriction site for BamHI at the 5'end, whereas the reverse primer contains a SalI restriction site. These restriction sites will be used to clone the coding sequence into the over-expression vector. Thus, the primers used for cloning the full-length coding region were 5'-GGATCCGAAACAAGATTGTCATATAATGGAG-3' and 5'-GTCGACTCACCAWATATCGRWGTCCA-3'.

RIK protein

We designed the primers based on the two potato TCs (TC127409 and TC155463) most similar to the tomato sequence (GI:47104870) which, in turn, is the most similar sequence to cork oak EST (EE743879) (Soler et al. 2007). The alignment with tomato showed that the two potato TCs corresponded to the same gene, but did not overlap. The primers used for the cloning were: 5'-TTATTCCGCTCCGTTGAATC-3' and 5'-ACAGCCAATTCCAGGTCAAT-3'.

DEM2 protein

In this case the 5'end coding region was found by 5' Rapid Amplifications of the cDNA Ends (5'RACE; Invitrogen) following manufacturer's protocol and the primers 5'-TCCAGCAAATCTTGGCTATCCCTCACTG-3' and 5'-TCATGGGACTCTTTCCACTACCCTTCCA-3' designed from a potato EST identified in our lab from a potato SSH library (data not shown). We designed the forward primer using the sequence obtained after the 5'RACE. The reverse primer was based on the potato TC136704, the most homologous sequence to the tomato gene (GI:37223343). This tomato gene was the most similar to the potato sequence previously obtained by 5'RACE. Thus, the primers used for the cloning of cDNA were 5'-CATGGGAGCTTCTCACAGTC-3' and 5'-CACTAGATAAATCTTCTTGCACACA-3'.

REVOLUTA HD-ZIPIII protein

We performed an alignment among the *Arabidopsis* sequence GI:145359496 (At5g60690) and the sequences with the highest similarity from potato (BI179320), tomato (GI:47104992) and poplar (*Populus balsamifera subsp. Trichocarpa*; GI:60327620). We designed the forward primer based on the sequence of the potato but, since this sequence does not extend to the stop codon, the reverse primer was designed and degenerated based on the sequences of the tomato, poplar and *Arabidopsis*. Hence, the primers used for the cloning of the full-length coding sequence were 5'-AGGGAAAATGGCTATGGTG-3' and 5'-TYACACRAAWGACCAGTTKAYAA-3'.

Cloning of the regulatory region upstream from the start codon

Regulatory regions were obtained using a GenomeWalker Universal kit (Clontech) and the Advantage polymerase (Clontech) in accordance with the manufacturer's protocol. Genomic DNA was extracted from potato leaves using a protocol based on Xu et al. (2005). Reverse primers used were the following: for the NAM, 5'-AGGGAGGTCCCAAGGCTCAGACTTATTG-3' and 5'-GATGGAACCTGAACCCTGGAGGAAGATT-3'; for the AS1-interacting KH protein, 5'-TGAAACTCTAGGGCAATTATCCTCCGTCA-3' and 5'-TAGTTCGTGCAACTGCAATGCTTCTTTG-3'; for the DEM2, 5'-TCCAGCAAATCTTGGCTATCCCTCACTG-3' and 5'-TCATGGGACTCTTTCCACTACCCTTCCA-3'; and for the REVOLUTA, 5'-TCTGCATAAACCCTCTCCAAAGCCTCAA-3' and 5'-AGCCACCATAGCCATTTTCCCTCAAATC-3'.

Identification of protein domains and cis-regulatory elements

The protein domains were searched for using InterProScan (Zdobnov and Apweiler, 2001; http://www.ebi.ac.uk/InterProScan/). Only best results are shown: e-values of $1.2e^{-92}$ for domain PF02365 in NAM, $5.6e^{-29}$ for domain PTHR15744 in RIK, $5.6e^{-15}$ for domain PF08553 in DEM2, and $6.2e^{-17}$ for domain PF00046, $5.7e^{-51}$ for domain PF01852 and $6.2e^{-86}$ for domain PF08670 in REV. Promoter searches for cis-elements were done *in silico* in potato and *Arabidopsis* using the PlantCARE Web site (Lescot et al., 2002; http://intra.psb.ugent.be:8080/PlantCARE). *Arabidopsis* promoters were obtained from the AGRIS Ohio State University Web site (Davuluri et al., 2003; http://Arabidopsis.med.ohio-state.edu). We used promoter regions of the same length between potato and *Arabidopsis* in order to obtain comparable results (1200 bp for NAM and REV, 768 bp for DEM2 and 431 bp for RIK), and we only selected the cis-regulatory elements conserved between both plants.

Silencing plasmid construction

Specific fragments for gene silencing in potato plants were PCR amplified from the previously cloned full-length sequence using specific primers. For the sNAM-RNAi, the silencing was carried out by means of a 253 bp fragment using the primers 5'-CACCAAACCTTCTAAGGAGGAATGG-3' and 5'-AGCAGCAGCCCAAGTTTAT-3'. For the cNAM-RNAi, it was carried out by means of a 229 bp

fragment using the primers 5'-CACCCAATGGGAGAAAAAGAATGG-3' and 5'-CTGTATTCATGCATAACCCAAT-3'. For the RIK, it was by means of a 246 bp fragment using the primers 5'-CACCCAGATGAAGGAGAGGGAGAA-3' and 5'-TGGCTGTGGGTATGTTATTG-3'. For the DEM2, it was by means of a 275 bp fragment using the primers 5'-CACCTGAGGAATCAGATGATGCAA-3' and 5'-CGGACCACTTATCCTTCTCA-3'. For the REV, it was by means of a 226 bp fragment using the primers 5'-CACCCTCAGCAGCATAGGGAGAGT-3' and 5'-CGACACCTTCTGTTCTGAAAC-3'.

The annealing temperature was 56ºC for all genes, following the conditions described below in the section entitled: *RT-PCR with incremental cycle numbers*. PCR products were cloned in the pENTR TOPO vector (Invitrogen), which contains the recombinant regions attL1 and attL2 flanking the cloned sequence. Insert bearing attL sequences at the borders was PCR-amplified using the primers M13 forward (5'- GTAAAACGACGGCCAG-3') and M13 reverse (5'- CAGGAAACAGCTATGAC-3') at an annealing temperature of 50ºC, and cleaned using QIAquick PCR Purification (Qiagen). The PCR product from pENTR was transferred to the binary destination vector pBIN19RNAi (provided by Prof. Salomé Prat) using LR clonase II (Invitrogen). The PCR-amplified insert and vector were incubated for 5 minutes at 65°C before the addition of the clonase to improve cloning efficiency. Cloning was accomplished overnight at room temperature. Recombination replaced the *ccdB* genes with an amplified fragment yielding a hairpin construct able to trigger gene specific mRNA degradation. Restriction enzyme digestion was used to verify the recombinant construct.

The binary destination vector (pBIN19RNAi) was obtained by subcloning the Gateway RNAi cassette from pH7GWIWG2(II) (Karimi et al., 2002; http://www.psb.rug.ac.be/gateway/) into the pBIN19 vector. For this purpose, the RNAi cassette was excised by a partial XbaI and HindIII digestion and inserted into the XbaI/HindIII sites of the pBIN19 plasmid. This cassette includes a chloramphenicol resistance marker (CmR) and two *ccdB* genes flanked inversely by recombinant *attR1* and *attR2* sequences separated by an intron.

Ectopic over-expression plasmid construction

The NAM coding region and destination vector pBINAR (Höfgen and Willmitzer, 1990) were digested using BamHI and SalI restriction enzymes. We cleaned the digested vector with QIAquick PCR Purification (Qiagen). The digested NAM sequence was loaded into an agarose gel stained with ethidium bromide and excised with QIAquick Gel Extraction (Qiagen). Ligation was accomplished overnight at 16ºC by using T4 DNA ligase (Invitrogen). Thus, the full-length coding sequence was cloned in sense direction under the transcriptional control of the 35S CaMV promoter. Restriction enzyme digestion was used to verify the construct.

Plant transformation

Potato leaves were infected with *Agrobacterium tumefaciens* strain GV2260 transformed with the RNAi recombinant plasmid according to Höfgen and Willmitzer (1988). Potato plants *cv Desirée* were transformed as previously described by Banerjee et al. (2006). Kanamycin-resistant plants were regenerated and grown until tuber development. cNAM-RNAi tuber skin and NAM over-expressing leaf were used for RNA analysis. Transcript accumulation levels were first analyzed by Northern blot and then selected lines were reconfirmed using RT-PCR.

Northern analysis

Samples (20 µg per lane) were separated in a 1.5% formaldehyde-agarose gel (w/v) and transferred onto a nylon membrane (Hybond-N, GE Healthcare). Hybridization was carried out according to Amasino (1986). Filters were hybridized at 42°C and washed in 3 x SSC and 0.5% SDS at 65ºC. The NAM specific primers 5'-CACCAAACCTTCTAAGGAGGAATGG-3' and 5'-GTCGACTCACCAWATATCGRWGTCCA-3'were used to obtain a probe that was [α-^{32}P]-labelled (GE Healthcare) by the random-primed method (Roche).

RT-PCR with incremental cycle numbers

cDNA was 2.5-fold diluted and 5 µL of cDNA was used as a template in a 100 µL reaction. PCR conditions were 2.5mM $MgCl_2$, 0.2mM each dNTP, 0.5 µM each forward and reverse oligonucleotides and 0.05 units μL^{-1} Eurotaq polymerase (Euroclone). Thermal cycling parameters were as follows: 1 cycle at 95ºC for 2 minutes and 35-40 cycles at 95ºC for 20 seconds, 56ºC for 30 seconds and 72ºC for 30 seconds, using a T-Gradient thermocycler (Biometra). Aliquots of 15 µL were taken every five cycles from cycle 20 to 35 for confirmation of over-expression, and every three cycles from cycle 25 to 40 for confirmation of down-regulation. 5 µL of each aliquot was analyzed by agarose gel electrophoresis stained with ethidium bromide as described by Lê et al. (2005). Control amplifications were performed with specific primers to actin (5'-CCTTGTATGCTAGTGGTCG-3' and 5'-GCTCATAGTCAAGAGCCAC-3'). Primers used for NAM transcription factor amplification were 5'-GAAACAAGATTGTCATATAATGGAG-3' and 5'-ACTGATGAAAGATTAGAGCTTACC-3'.

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GENERAL DISCUSSION

Overall, the findings presented in this thesis consist of: i) a very comprehensive list of cork and suberin candidate genes, comprising structural and regulatory proteins, obtained by a transcriptomic approach in the bark of cork oak (*Q. suber*); ii) a time-course expression pattern showing the correlation between environmental factors and the seasonal behaviour of a selection of candidate genes in cork oak bark; iii) the obtaining of transformed potato (*S. tuberosum*) lines to demonstrate the molecular function of four candidate regulatory genes in the cork tissue (NAM, RIK, DEM2 and REV) by means of a reverse genetic approach.

The transcriptomic study of cork is hindered by its own nature. Cork is a barrier tissue produced by phellogen (cork cambium) and can only be mechanically separated from the inner living tissues when the phellogen is active. Furthermore, cork is made of cells which die at maturity and contain high proportions of phenols and tannins. These features make RNA extraction and downstream processes very difficult, and complicate molecular analysis. Despite these difficulties, we were able to detach differentiating cork tissue from the bark of cork oak and perform a successful protocol for extracting RNA. This allowed us to undertake a transcriptomic approach to cork tissue using cork oak, which is the paradigm of suberized tissues. By a SSH, in which somatic embryo tissue was used to subtract common genes, we obtained a list of ESTs from 135 distinct genes induced in cork, encoding structural enzymes and regulatory proteins. Furthermore, a targeted approach by RT-PCR allowed us to complement this list with seven other regulatory proteins related to meristem maintenance and organ patterning. The high cork specificity (checked by microarray and RT-PCR comparing cork and wood) generally shown by the candidate genes is worth noting. This specificity can be attributed to the suitability of cork oak bark as a model tissue for underscoring suberin and cork genes, as it consists of almost pure cork cells that synthesize large amounts of suberin. Moreover, there is a strong agreement between the candidate genes and the putative enzymes necessary for suberin biosynthesis, according to its previously hypothesized metabolic pathway (Kolattukudy, 2001; Bernards, 2002; Franke and Schreiber, 2007).

The analysis of the seasonal behaviour of 12 relevant cork candidate genes, using real time RT-PCR, provided significant information about the role of these genes and the environmental factors that influence their expression in cork oak bark. Although cork is a difficult tissue for molecular work, real time RT-PCR allowed the detection of small gene variations during the growing season that could be correlated with climatic variables. This is a worthwhile contribution considering the paucity of literature available regarding molecular events involved in cork formation and phenology.

Regulatory mechanisms controlling cork formation are practically unknown. Because of the lack of knowledge about this subject, we directed our efforts towards demonstrating the role of regulatory candidate genes in the cork tissue. Based on their transcript profile and their putative molecular function as found in the literature and databases, we selected four genes, respectively encoding NAM, RIK, DEM2 and REV proteins, for performing reverse genetics in the potato. Potato plants were transformed to silence

and/or over-express these genes. At the time of writing this thesis, potato transformed lines are being grown in a walk-in chamber with the aim of producing material for the analysis of transgenic phenotypes.

1. Molecular genetics of cork formation and suberin biosynthesis

1.1. Cork subtractive library

The cork oak SSH library contains 43.5% of the genes related to secondary metabolism. Since suberin is the main component of cork, metabolic pathways involved in its biosynthesis (the acyl-lipid pathway for the aliphatic domain and the phenylpropanoid pathway for the aromatic domain) are the most represented. Metabolic pathways relating to other chemical components of cork, like the isoprenoid pathway for terpenes and the flavonoid pathway for tannins, are also present. Interestingly, regulatory proteins and stress responsive genes were also abundant (19.3% and 9.6% respectively). Regulatory proteins include transcriptional regulators, signal transductors and regulated proteolysis-related genes, identified in cork for the first time. The stress response category comprises genes related to detoxifying enzymes and cell wall strengthening. Interestingly, the miscellaneous category includes genes compatible with the main pathways leading to cork biosynthesis, but whose substrates have not been characterized. It amounted to 9.6%, although they will probably be considered as part of secondary metabolism when their substrates become better characterized.

1.2. Comparison between cork and wood

The microarray comparison between cork and wood confirmed our genes as strong candidates for cork formation and regulation. On the whole, the majority of genes were up-regulated in cork. Both wood and cork synthesize similar aromatic compounds (lignin for wood and aromatic suberin for cork) and some enzymatic activities are expected to be comparable. However, only cork synthesizes aliphatic compounds needed by the aliphatic suberin. Therefore, genes within the acyl lipid category, the most relevant to aliphatic suberin, were more up-regulated in cork than genes in the phenylpropanoid category, the most relevant to the biosynthesis of aromatic compounds. However, two genes in the phenylpropanoids category, both homologs to N-hydroxycinnamoyl/benzoyltransferase, showed a high level of cork up-regulation, pointing to a specific and crucial role in the periderm. In addition, the high up-regulation showed by some genes within the miscellaneous category, which comprises genes coding for enzymes whose substrates have still not been characterized like cytochrome P450s, transporters and acyltransferases, also suggests a specific role in cork.

1.3. Candidate genes for the biosynthesis of suberin

Many of the genes we have reported are potentially important for suberin biosynthesis. Most of them are in accord with the biosynthetic pathways suggested in the literature (Kolattukudy, 2001; Bernards, 2002; Franke and Schreiber, 2007; Graça and Santos, 2007) and on this basis we can construct a diagram of suberin biosynthesis with our candidate genes (Figure1).

The suberin aliphatic pathway starts with the *de novo* biosynthesis of fatty acids, a step that takes place in the chloroplast involving the enzymes dihydrolipoamide S-acetyltransferase and biotin carboxyl carrier protein. The export of fatty acids from the chloroplast is achieved by means of PATE/FAT (palmitoyl-acyl carrier protein thioesterase) and LACS (long-chain acyl CoA synthase) (Bonaventure et al., 2003; Schnurr et al., 2004). The elongation of the carbon chain of long-chain fatty acids to very long-chain fatty acids is catalyzed by fatty acid elongase complexes (FAE). Two genes, KCS (β-ketoacyl-CoA synthase) and KCR (β-ketoacyl-CoA reductase), encode key enzymes of these complexes (Millar and Kunst, 1997; Millar et al., 1999; Todd et al., 1999; Yephremov et al., 1999; Fiebig et al., 2000; Pruitt et al., 2000; Dietrich et al., 2005). Recently, a study has demonstrated that potato CYP86A33 and its *Arabidopsis* ortholog CYP86A1 are involved in the hydroxylation of fatty acids at the terminal (ω) position (Benveniste et al., 1998; Li et al., 2007; Serra et al., submitted). The epoxidation typical of cork oak suberin may be produced by lipoxygenases (LOXs) (Blee and Schuber, 1993; Lequeu et al., 2003). The synthesis of aromatic suberin is based on phenylpropanoid metabolism. It starts with the deamination and hydroxylation of phenylalanine by the enzymes PAL (phenylalanine ammonia-lyase) and C4H (cinnamate 4-hydroxylase) respectively. Other enzymes such as COMT (caffeic acid O-methyltransferase), F5H (ferulate-5-hydroxylase), CCOMT (caffeoyl CoA 3-O-methyltransferase) and 4CL (4-coumarate:CoA ligase) are thought to be important for the synthesis and activation of the hydroxycinnamic acids typical of suberin (Bernards and Lewis, 1998).

It is not known if suberin monomers are transported to the apoplast via Golgi-derived vesicles or via ATP-binding cassette (ABC) transporters (Franke and Schreiber, 2007). However, the finding of two cork up-regulated ABC transporters in the library supports the hypothesis that at least some suberin precursors may be transported by these transporters to the cell wall. Although our library does not contain any class III peroxidase involved in the assembly of aromatic monomers (Kolattukudy, 2001; Bernards, 2002), it does contain three genes coding for laccases. Laccases are extracellular oxidases capable of coupling phenylpropanoids with a proposed role in lignin and suberin biosynthesis (Kiefer-Meyer et al., 1996; LaFayette et al., 1999; Ranocha et al., 2002; Ehlting et al., 2005; Liang et al., 2006).

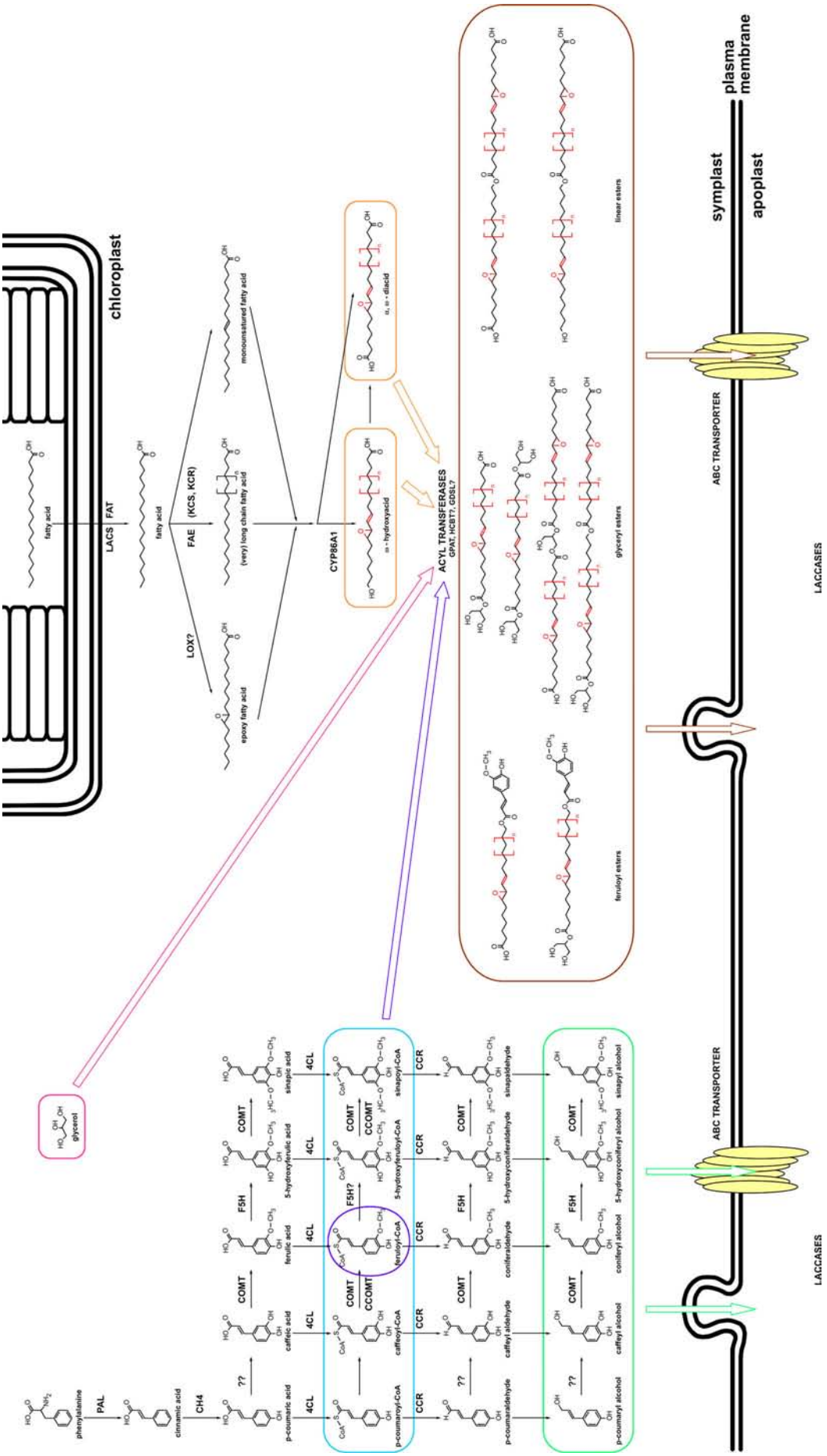
chloroplast
LACS
FAT
fatty acid
fatty acid
FAE
(KCS, KCR)
(very) long chain fatty acid
monounsaturated fatty acid
LOX?
epoxy fatty acid
CYP86A1
ω - hydroxyacid
α, ω - diacid
ACYL TRANSFERASES
GPAT, HCBT?, CDSL?
linear esters
glyceryl esters
feruloyl esters
plasma membrane
symplast
apoplast
ABC TRANSPORTER
ABC TRANSPORTER
LACCASES
LACCASES
glycerol
phenylalanine
PAL
cinnamic acid
CH4
??
p-coumaric acid
4CL
p-coumaroyl-CoA
CCR
p-coumaraldehyde
??
p-coumaryl alcohol
caffeic acid
caffeoyl-CoA
caffeyl aldehyde
caffeyl alcohol
ferulic acid
feruloyl-CoA
coniferaldehyde
coniferyl alcohol
5-hydroxyferulic acid
5-hydroxyferuloyl-CoA
5-hydroxyconiferaldehyde
5-hydroxyconiferyl alcohol
sinapic acid
sinapoyl-CoA
sinapaldehyde
sinapyl alcohol
COMT
CCOMT
F5H
F5H?

Figure 1. Simplified model of suberin biosynthesis, based on Bernards (2002), Boerjan et al. (2003), Franke and Schreiber (2007) and Graça and Santos (2007). Suberin is a biopolymer consisting of an aliphatic domain cross-linked with an aromatic domain. The biosynthetic pathway of the aromatic monomers derives from the phenylpropanoid metabolism, whereas the biosynthetic pathway for the aliphatic monomers involves modification and elongation of fatty acids. Note that only the enzymes found in our SSH library are shown in the diagram, although more enzymes are necessary to complete the biosynthesis. The phenylpropanoid metabolism, with the enzymes PAL, CH4, 4CL, COMT, CCOMT, F5H and CCR, produces two classes of products: hydroxycinnamic acids (in blue) and monolignols (in green). Monolignols are thought to be transported to the apoplast either by an ABC transporter or via Golgi-derived vesicles, and are probably responsible of the linking to the primary cell wall. Hydroxycinnamic acids activated with Coenzyme A, mainly ferulic (in purple), may be esterified with modified fatty acids. Long-chain fatty acids are exported from the chloroplast by a process involving FAT and LACS, and they can be elongated by FAE, epoxidated by LOX or desaturated (possible modifications are marked in red). Finally, these fatty acids are hydroxylated at the ω-position by CYP86A1 or processed by other enzymes giving rise to ω-hydroxyacids and α,ω-diacids (in orange). Glycerol (in pink) is esterified with ω-hydroxyacids and α,ω-diacids. Acyltransferases are the enzymes involved in all these esterifications. To date, only the role of GPAT has been confirmed to esterify fatty acids with glycerol, but HCBT or GDSL may be involved in other esterifications of suberin monomers, involving ferulates and hydroxylated fatty acids. These esterifications are thought to occur in the symplast, although they could also occur in the apoplast. Then, suberin building blocks (in brown) formed after esterification are thought to be transported to the apoplast via either an ABC transporter or Golgi-derived vesicles. Cross-linking of aromatic components could be performed in the apoplast by extracellular laccases.

The assembly of the suberin glycerol polyester is thought to occur by esterification of the carboxyl groups of α,ω-diacids and ω-hydroxyacids with the alcohol groups of glycerol and ω- and mid-chain hydroxy fatty acids (Kolattukudy, 2001). We found two cork up-regulated genes coding for glycerol-3-phosphate acyltransferases, enzymes which have been demonstrated to play an important role in the synthesis of aliphatic polyesters and suberin by the esterification of fatty acids to glycerol (Suh et al., 2005; Beisson et al., 2007). Furthermore, our library contains a high number of GDSL lipase/hydrolases that are highly up-regulated in cork. Taking into account that some GDSL lipase/esterases have been suggested to act on cutin metabolism (Kannangara et al., 2007; Reina et al., 2007), a putative role in suberin assembly cannot be disregarded. On the other hand, the linkages between aliphatic and aromatic suberin monomers are thought to be due to esterification between ferulates and fatty acids (Adamovics et al., 1977; Bernards and Lewis, 1992; Lotfy et al., 1994; Graça and Santos 2007). Our library contains one gene encoding an acyltransferase classed in miscellaneous and two HCBTs, all members of the BAHD superfamily and highly up-regulated in cork. These genes could be responsible for the linkages between the two suberin domains, since some members of the BAHD acyltransferase superfamily catalyze the esterification of hydroxycinnamates with fatty acids (Lotfy et al., 1995). However, more studies are needed in order to confirm this hypothesis.

2. Molecular genetics of cork regulation

2.1. Environmental factors affecting the cork tissue

At the beginning of the growing season, when phellogen is highly active, thin-walled active cells are produced, but towards the end of the season cell divisions become rare and thick-walled cells are formed (Waisel, 1995). This suggests that some differences occur during the annual formation of the cork ring, which may be a consequence of environmental factors. To study this process, a seasonal transcript profile pattern of 12 genes was analyzed in the bark of cork oak using real time RT-PCR. These genes include enzymes of the main metabolic pathways (CYP86A1, GPAT, FAT, HCBT, F5H, bAS and ANR) together with transcription factors (NAM and WRKY) and stress responsive proteins (HSP17.4, ANN and APX). The level of expression in cork at different time points during the growing season was correlated with environmental factors, and statistical analysis allowed the grouping of genes and climatic variables. By providing helpful information about the function of candidate genes, this study contributes to a better knowledge of cork ring formation and phenology.

Although expression differences were not very pronounced, CYP86A1, HCBT, GPAT, WRKY and NAM genes tend to have their maximum expression levels in June, the month when periderm formation is at full activity and cork harvesting starts in north-eastern Spain. Expression correlations of candidate genes HCBT, WRKY and NAM with CYP86A1 and GPAT, which have a demonstrated role in relation to suberin (Beisson et al., 2007; Li et al., 2007; Serra et al., submitted), support the role of the first three genes in cork formation and suberin biosynthesis. On the other hand, F5H and FAT expression is high in spring and low in summer. F5H is a well-known control point of lignin monomer composition, as plants with F5H mutation accumulate guaiacyl (G) lignin, whereas its over-expression leads to a syringyl (S) lignin accumulation (Meyer et al., 1998). FAT regulates the amount of saturated fatty acids exported from plastids, acting as a major control point of saturated fatty acid fluxes (Bonaventure et al., 2003). Therefore, it can be hypothesized that their expression differences may induce a change in suberin monomer composition. In contrast, the HSP17.4 and ANN expression level is low in spring and high in summer. Both genes are related to stress responses (Puigderrajols et al., 2002; Gorecka et al., 2007) and their expression pattern correlates with a protective role against the heat and drought conditions of Mediterranean summers. This behaviour makes these genes good candidates as markers of tree stress, especially in the case of HSP17.4, which has much more pronounced expression differences.

2.2. Candidate genes for the regulation of cork formation

Cork and suberin regulation is virtually limited to what is contained in the work of Soliday et al. (1978), which showed that abscisic acid (ABA) induces suberization in potato wound periderm. However, in our

approach using cork oak, we found several regulators relating to plant hormones which could be involved in cork and suberin regulation, such as a phytosulfokine receptor (PSKR) and the ethylene forming enzyme aminocyclopropane-carboxylate (ACC) oxidase. Furthermore, some genes in our library may be in some way regulated by ABA, such as the annexin (Lee et al., 2004).

Cambial cell maintenance requires genes like CLAVATA and KNOX (Groover et al., 2006). Furthermore, the radial patterning of the vascular system is thought to be controlled by the HD-ZIP/KANADI genetic system and by the gene ASYMMETRIC LEAVES1 (AS1) (Hawker and Bowman, 2004; Schrader et al., 2004; Carlsbecker and Helariutta, 2005; Zhao et al., 2005). Using SSH, we found the AS1-interacting KH protein (RIK), predicted to be necessary for the correct role of AS1, which is a KNOX genes repressor (Phelps-Durr et al. 2005). In contrast, using a targeted screening strategy, we found ESTs from KNOX, KANADI and HD-ZIPIII transcription factors in cork, suggesting that similar mechanisms could be conserved between the maintenance of cambium and phellogen cells and between the radial patterning of the vascular system and the periderm.

It is well known that some MYB genes, specifically R2R3-MYB, are lignin regulators (Tamagnone et al., 1998; Patzlaff et al., 2003; Karpinska et al., 2004; Goicoechea et al., 2005; Fornalé et al., 2006). Something similar is known about some NAC transcription factors, which have been related to cell wall thickening in xylem (Kubo et al., 2005; Mitsuda et al., 2005; Ko et al., 2007; Mitsuda et al., 2007; Zhong et al., 2007). Our library contains MYB (including an R2R3-MYB) and NAM (a subgroup of the NAC multigene family) genes, some of them highly up-regulated, indicating their putative role in the differentiation of cork cells. In addition, the library also contains a WRKY transcription factor highly induced in cork, a gene family which may modulate gene expression by binding to the W-boxes (cis-regulatory elements) of some genes, including cytochromes P450 monooxygenases (Mahalingam et al., 2003; Narusaka et al., 2004).

2.3. Functional studies of regulatory candidate genes for cork in potato

Our list of cork regulatory genes is valuable for hypothesizing molecular mechanisms acting on cork and suberin regulation, but, in the absence of functional genetic studies, the role of these genes in cork remains unproven. We therefore selected four genes (NAM, RIK, DEM2 and REV) from among our candidates for reverse genetics approach in potato. Successful silencing and over-expression has been confirmed for NAM, and verification of silencing for the remaining lines is already planned. Although functional characterization of these genes has not been completed, the present work provides material basic for detailed characterization of the physiological and molecular function of these putative regulators in cork. Experiments using these transgenic lines will probably be very helpful for a deeper understanding of cork regulatory networks.

3. Future perspectives

In this thesis we achieved a better understanding of cork formation and regulation by the identification of candidate genes and the characterization of some of them. According to its annotations, these genes code for structural enzymes, stress responsive proteins and regulatory factors. A more detailed study of gene tissue expression would allow us a better characterization of the candidate genes. However, cork is a very difficult tissue for obtaining good histological sections for the observation under the optic microscopy, so we discarded techniques like *in situ* hybridization or immunolocalization. Instead, RT-PCR, Northern blot and microarrays proved to be valuable tools to establish tissue expression pattern. To complete the characterization of some genes, we analyzed their temporal expression in cork tissue along the growing season. This study gave us some clues about the role of these candidate genes in the formation of cork and, in general, it helped us understanding the environmental factors influencing the formation of this tissue. The temporal expression pattern of more genes will increase our knowledge about the formation and regulation of cork at molecular level. It would be also worthy to perform global proteomic or metabolomic approaches to the formation of cork, since this will greatly complete our research at genomic level. However, taking into account the scarce experience of our laboratory in doing these hard and laborious techniques, we will not embark in such a huge project in the near future. However, maybe in a further future, with the help of a European consortium, we could be able to face these techniques.

With this work we obtained a comprehensive list of candidate genes for cork formation and we started characterizing their expression pattern using cork oak, which is the paradigm for cork formation. On the other hand, we based the discussion of the putative role of these genes on putative orthologs from others species, especially *Arabidopsis*. Since cork oak has not been fully sequenced, we could not be completely sure about the orthology relationship we found and, although we were, this would not always imply that the genes play identical functions. Therefore, to probe the role of our candidate genes in the formation of cork we performed functional genetics using potato, which can be easily transformed and grown in the laboratory. Potato is an interesting model for the study of cork formation because enough amount of tuber periderm can be isolated and is a crop of agronomic interest. However the lack of seeds and the polyploidy hinder genetic studies in this plant. In this work we focused on four regulatory genes. Thus, to complete the phenotypic analysis of silenced and over-expressed potato lines for these genes will be one of the priorities for the near future. We will compare the periderm of transgenic and wild-type plants at chemical, ultrastructural and physiological level and, if differences are detected, which would imply an obvious phenotype, we will perform more experiments to better characterize the function of the genes in the cork formation and regulation. Some of these experiments may include the analysis of the global expression pattern of the transgenic lines using microarrays to identify putative targets of these regulatory genes, the purification of functional proteins to *in vitro* characterize their activity and the obtaining of antibodies to identify cis-regulating elements by means of chromatin immunoprecipitation and footprinting. However, not only regulatory genes are planned to be studied by functional genomics,

since some structural genes candidates for the biosynthesis and deposition of suberin are now under study in our laboratory, concretely the potato putative orthologs of *Arabidopsis* CYP86A1, CER6 and HCBT among others. The achievement in doing all these experiments will surely lead the understanding of cork formation to a much greater level than it is at present.

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CONCLUSIONS

1. The bark of cork oak is a good model for the identification of candidate genes for cork formation:

1.1. One hundred and thirty-five genes induced in cork were isolated and identified by the subtraction of common cDNAs with somatic embryo. The predicted molecular function of many genes was compatible with the chemical composition of cork and suberin. Regulatory and stress-responsive genes were also found.

1.2. Transcriptomic analysis revealed that most of these genes were up-regulated in cork versus wood, which reinforced their expected role in cork formation.

1.3. Seven regulatory genes (KNOX, KANADI, HD-ZIPIIIs and BR receptor) with a putative function in phellogen maintenance and organ patterning, which could not be identified by means of the subtraction with somatic embryo, were isolated using a targeted approach.

2. A set of genes was grouped according to their transcript profile during the growing season of cork oak bark.

2.1. HCBT, NAM, CYP86A1, WRKY, GPAT, and to a lesser extent bAS, showed their highest expression in June, the period when cork growth rate is maximal in the sampling area.

2.2. FAT and F5H showed maximum expression when the temperature was at its lowest and relative humidity at its highest, whereas HSP17.4 and ANN showed the opposite behaviour.

3. Genetic modified potato plants for the cork candidate regulatory genes NAM, RIK, REV and DEM2 were obtained. Transgenic plants will improve knowledge about the role of these genes in the periderm.

3.1. Successful up- and down-regulation of NAM was confirmed.