FUNCTIONAL GENETICS OF SUBERIN: THE ROLE OF CYP86A33 AND STKCS6 IN POTATO TUBER PERIDERM

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Memòria redactada per Olga Serra i Figueras, inscrita al Programa de Doctorat en Ciències: Química i Física de les Molècules i els Materials, Biotecnologia i Ciències de la Salut de la Universitat de Girona, per adquirir el grau de Doctor Europeu per la Universitat de Girona

Aquest treball s'ha realitzat al Laboratori del Suro del Departament de Biologia sota la direcció de **Mercè Figueras Vall·llosera i Marisa Molinas de Ferrer.**

Les directores de tesi   Autora

Mercè Figueras   Marisa Molinas   Olga Serra
i Vall·llosera   de Ferrer   i Figueras

AGRAÏMENTS

La tesi que tens a les teves mans mostra la culminació del treball científic que vaig iniciar fa més de quatre anys al Laboratori del Suro. No obstant, per tal de fer realitat aquesta tesi ha calgut l'esforç d'alguns, la paciència d'uns altres i el recolzament de molts.

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humilitat i el toc de *freakisme* m'han transformat els moments de crisi a rialles. A vosaltres, els qui teniu l'esperit d'Espais Comuns també us he d'agrair aquesta tesi: Marçal, Pere, Olaya, Gela, Mire, Alexandra, Jess, Ariadna, Anna (Gaxi), Núria, Marc L, Lluís S., Gisela, Marc Y., Marta, Montse, Sònia, Vicky, Àlex, Eva, Sílvia, Marga, Noe, Gerard, Roger, Laia M., Arantxa, Ari, Dolors, Magalí, Clara, Mariona, Anna V. i probablement algun que em deixi... Igualment, també vull donar les gràcies a tota la gent que em van fer les estades a Madrid i a Bonn més agradables i gratificants. Entre ells a Bonn: la Caro, en Kosala, l'Alexander, la Pannaga, en René, la Martina, en Patrik, l'Andrea i la Tina, i a Madrid: la Cristina, en Carlos, en Miguel, en Luka, en Juanma, en Jean-Micheal, l'Abe, l'Anna i molts més. Als meus amics també els hi he d'agrair que escoltessin i intentessin entendre, encara que de vegades sorpresos, les incoherències de fer un doctorat. A més, voldria agrair a tota la gent que s'ha bolcat donant-me ànims i mostrant-me el seu suport en aquesta dissortada, esgotadora i penosa recta final de tesi.

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CONTENTS

SUMMARY

The periderm, which protects the mature or secondary organs of plants, consists of the phellem or cork in its outer side. The phellem is a multilayered tissue of dead cells with suberized walls that affords protection against dehydration and pathogens. These suberized walls of phellem contain suberin and suberin associated waxes, both fractions responsible of the hydrophobic nature of periderm. Suberin is a fatty acid and glycerol-based polyester that contains phenolic compounds. The aliphatic compounds present in suberin are mainly long (C18) and very long (>C18) chain fatty acids (LCFA; VLCFA) with functionalized carboxylic or alcohol groups in their ω-terminal carbon. The suberin associated waxes are mainly derivatives of VLCFA. Thus, the ω-hydroxylation and elongation of fatty acids must be significant steps for the biosynthesis of suberin and wax compounds.

To better understand the biosynthesis and function of suberin in periderm, in our laboratory we obtained a list of candidate genes for suberin biosynthesis by selecting those genes highly up-regulated in phellem. In this thesis, the functional characterization of two of these genes, the putative ω-hydroxylase CYP86A33 and the putative ketoacyl-CoA synthase StKCS6, is performed by their RNA interference (RNAi)-mediated silencing in potato. In both cases, the silencing effects were analyzed at chemical, ultrastructural and physiological level.

CYP86A33 is a putative ω-hydroxylase, candidate for the functionalization of the ω-terminal carbon of suberin aliphatic compounds. The CYP86A33 deficiency leads to a great reduction of the main suberin monomers, the C18:1 ω-hydroxyacid and α,ω-diacid, together with a smaller reduction of the rest of suberin compounds. All these reductions have an effect on the suberin total amount that decreases by 60%. In contrast to suberin, the wax content increases in more than 2-fold. Besides, in *CYP86A33*-RNAi periderms the lamellar ultrastucture of suberin is deeply distorted and the permeability to water increases. All these results demonstrate the significance of the ω-oxidized fatty acids for the deposition and assembly of suberin compounds as well as for the periderm water barrier function.

StKCS6 is a putative ketoacyl-CoA synthase, candidate for the elongation of VLCFA and derivatives of suberin and waxes of potato periderm. StKCS6 deficiency leads to a decrease of compounds with chain-length C28 and higher and an increase of those of chain-length C26 and lower. Even though this shift towards shorter chain lengths of C_{26} and lower in *StKCS6*-silenced periderms, relevant changes in the relative amount of different types of compounds are not observed. Moreover, in these transgenic periderms structural defects, either in the suberin cell wall or in the cellular organization of this tissue, are not perceived. However, the StKCS6 deficiency impairs the water barrier function of periderm, suggesting that the fatty acid chain-length can contribute to the sealing properties of periderm.

The results obtained in this thesis are discussed as a whole to highlight their contribution in the biosynthesis of suberin and wax compounds, in the suberin ultrastructure and in the properties of the periderm hydrophobic barrier.

RESUM
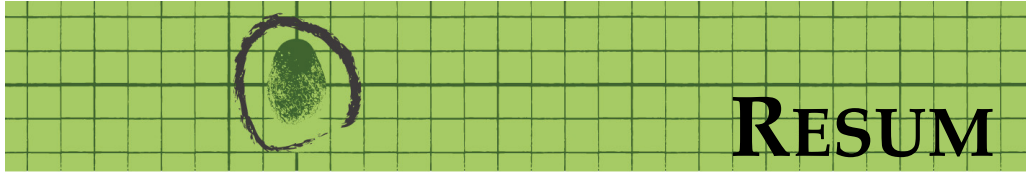
La peridermis, la qual protegeix els òrgans madurs o secundaris de les plantes, consta del fel·lema o suro a la seva part externa. El fel·lema és un teixit pluriestratificat de cèl·lules mortes amb parets cel·lulars suberificades que protegeix contra la deshidratació i els patògens. La suberina i les ceres associades que impregnen les parets suberificades del fel·lema són responsables de la naturalesa hidrofòbica del periderm. La suberina és un polièster d'àcids grassos i glicerol que conté compostos fenòlics. Els compostos alifàtics que formen la suberina són principalment àcids grassos de cadena llarga (C18) o molt llarga (> C18) que contenen grups carboxílics i alcohòlics funcionals a l'extrem ω-terminal. Les ceres associades a la suberina són bàsicament derivats d'àcids grassos de cadena molt llarga. Així, la ω-hidroxilació i l'elongació d'àcids grassos són dos processos claus per a la biosíntesi dels components de la suberina i de les ceres.

Per tal de poder entendre millor la ruta biosintètica de la suberina i la seva funció en la peridermis, en el nostre laboratori varem obtenir gens candidats per la biosíntesi de la suberina mitjançant la selecció d'aquells gens que estaven sobreexpresats en el fel·lema. En aquesta tesi es presenta la caracterització de dos d'aquests gens, la hipotètica ω-hidroxilasa d'àcids grassos CYP86A33 i la hipotètica ketoacyl-CoA sintasa StKCS6, mitjançant el seu silenciament per RNA d'interferència (RNAi) en patata. En ambdós casos es van analitzar els efectes del silenciament a tres nivells: químic, estructural i fisiològic.

CYP86A33 pel caràcter d'ω-hidroxilasa d'àcids grassos és candidat per la funcionalització del carboni terminal ω dels compostos alifàtics de la suberina. La deficiència de CYP86A33 comporta una gran reducció de la quantitat dels monòmers principals de la suberina, l'àcid gras ω-hidroxilat i l'α,ω-diàcid C18:1, juntament amb una reducció més petita de la resta de compostos de la suberina. Totes aquestes reduccions provoquen que la quantitat de suberina dipositada en les parets cel·lulars es vegi reduïda en un 60%. Pel que fa a les ceres, contràriament a la suberina, la seva quantitat es veu incrementada en més de dues vegades. En la peridermis de les plantes silenciades en *CYP86A33* s'observa una greu alteració de la

disposició lamel·lar de la suberina que va acompanyada d'un increment en la permeabilitat a l'aigua. Tots els resultats en conjunt demostren la rellevància dels àcids grassos ω-oxidats en la deposició i l'assemblatge de la suberina, així com també en la funció barrera de la peridermis.

StKCS6 pel seu caràcter d'elongasa d'àcids grassos és candidata per síntesi dels àcids grassos o derivats de cadena molt llarga de la suberina i les ceres de la peridermis. La deficiència en StKCS6 comporta que els compostos de la peridermis de cadena igual o més llarga de 28 carbonis es redueixin i que els de cadena igual o més curta de 26 s'incrementin. A pesar d'aquest escurçament de cadenes, no s'aprecien canvis rellevants en la quantitat relativa dels diferents tipus de compostos. A més, tampoc s'observen canvis ultraestructurals de la paret de suberina ni en l'organització cel·lular de la peridermis. No obstant, la deficiència de StKCS6 danya la funció barrera de la peridermis, el que suggereix que la llargada de la cadena dels àcids grassos pot contribuir a les propietats impermeabilitzants de la peridermis.

Els resultats obtinguts en aquesta tesi es discuteixen en conjunt per posar de relleu la seva contribució en la biosíntesi dels compostos de suberina i ceres, en la ultraestructura de la suberina i en les propietats de la barrera hidrofòbica de la peridermis.

RESUMEN
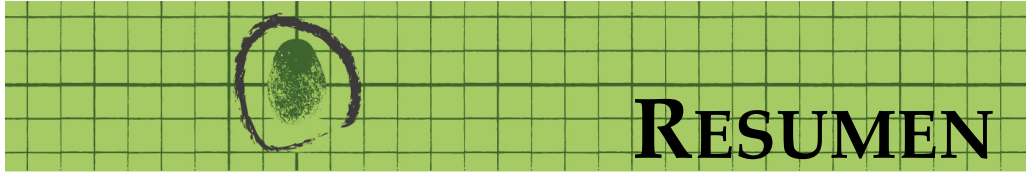
La peridermis, la cuál protege los órganos maduros (secundarios) de las plantas, contiene en su parte externa felema o corcho. El felema es un tejido pluriestratificado de células muertas con paredes celulares suberificadas que protege de la deshidratación y los patógenos. La suberina y las ceras asociadas que impregnan las paredes suberificadas del felema son responsables de la naturaleza hidrofóbica de la peridermis. La suberina es un poliéster de ácidos grasos y glicerol que contiene compuestos fenólicos. Los compuestos alifáticos que forman la suberina son principalmente ácidos grasos de cadena larga (C18) y muy larga (>C18) que contienen grupos carboxílicos o alcohólicos funcionales en su carbono ω-terminal. Las ceras asociadas a la suberina son básicamente derivados de ácidos grasos de cadena muy larga. Así, la ω-hidroxilación y la elongación de ácidos grasos deberían ser procesos claves para la biosíntesis de los componentes de la suberina y de las ceras.

Para poder entender mejor la ruta biosintética de la suberina y su función en la peridermis, en nuestro laboratorio obtuvimos una colección de genes candidatos para la biosíntesis de la suberina mediante la selección de aquellos genes sobreexpresados en el felema. En esta tesis se presenta la caracterización funcional de dos de éstos genes, la hipotética ω-hidroxilasa *CYP86A33* y la hipotética ketoacyl-CoA sintasa *StKCS6*, mediante su silenciamiento por RNA de interferencia (RNAi) en patata. En ambos casos se analizaron los efectos del silenciamiento a tres niveles: químico, estructural y fisiológico.

CYP86A33 por su carácter de ω-hidroxilasa de ácidos grasos es candidato para la funcionalización de los grupos ω de los compuestos alifáticos de la suberina. La deficiencia de CYP86A33 comporta una gran reducción de la cantidad de los monómeros principales de la suberina, el ácido graso ω-hidroxilado y el α,ω-diácido C18:1, acompañada de una reducción más pequeña en el resto de componentes de la suberina. Todas esas reducciones conllevan que la cantidad de suberina depositada en las paredes celulares se ve reducida en un 60%. En referencia a las ceras, contrariamente a la suberina, su cantidad se ve incrementada en más de dos veces. Además, en la peridermis de las plantas silenciadas se observa una gran alteración

de la disposición lamelar de la suberina acompañada de un incremento en la permeabilidad al agua. Todos los resultados en conjunto demuestran la relevancia de los ácidos grasos ω-oxidados en la deposición y ensamblaje de la suberina, así como también en la función barrera de la peridermis.

StKCS6 por su carácter de elongasa de ácidos grasos es candidato para la síntesis de los ácidos grasos o derivados de cadena muy larga de la suberina y las ceras de la peridermis. La deficiencia en StKCS6 conlleva que los compuestos de la peridermis de cadena igual o más larga de 28 carbonos se reduzcan y que los de cadena igual o más corta de 26 carbonos se incrementen. A pesar de este acortamiento de cadenas, no se aprecian cambios relevantes en la cantidad relativa de los diferentes tipos de compuestos. Además, tampoco se observan cambios ultraestructurales en la pared de suberina ni en la organización celular de la peridermis. No obstante, la deficiencia de StKCS6 daña la función barrera de la peridermis lo que sugiere que la longitud de la cadena de los ácidos grasos puede contribuir a las propiedades impermeabilizantes de la peridermis.

Los resultados obtenidos en esta tesis se discuten en conjunto para poner de manifiesto su contribución en la biosíntesis de los compuestos de suberina y ceras, en la ultraestructura de la suberina y en las propiedades de la barrera hidrofóbica de la peridermis.

INTRODUCTION

1. Periderm, a plant lipophilic barrier

One of the features that allowed plants to colonize the land was the development of lipophilic barriers, such as the cuticle and the periderm, to avoid water loss. The cuticle is a continuous non-cellular hydrophobic thin membrane (0.1-10 μm thick) deposited in the outermost cell walls of the epidermis of above-ground organs (Riederer and Schreiber, 2001). The periderm is found in subterraneous root organs (exodermis and tuber skin) and also develops to replace the cutinized epidermis when it is damaged by wounding or by secondary growth (like tree trunks in woody species) (Lendzian, 2006).

The periderm is produced by a secondary meristematic layer called phellogen or cork cambium. Phellogen normally arises outside the vascular cambium in the subepidermal tissue of stems or in the pericycle of roots. It generates the phellem or cork centrifugally and the cork parenchyma or phelloderm (not always present) centripetally. All together, phellogen, phellem and phelloderm make up the periderm (Lendzian, 2006) (Figure 1).
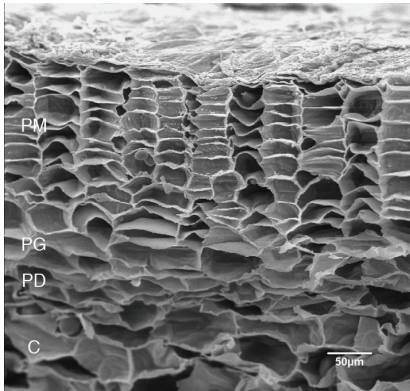
Figure 1. **Periderm structure**. Scanning electron microscope micrograph showing potato tuber periderm made up of phellem (PM), phellogen (PG) and phelloderm (PD), which are regularly arranged in contrast with the parenchymatic cortical cells (C).

The barrier properties of periderm are provided by the phellem multilayered tissue and specifically by their apoplastic lipid compounds: suberin and suberin associated waxes. As phellem cells develop, suberin and suberin associated waxes are deposited in their cell walls, become isolated from the water and nutrient supply and subsequently die, thereby forming the protective barrier (Sabba and Lulai, 2002). Suberin is also deposited in the endodermis of roots (Casparian bands), in the bundle sheath cells of grass leaves and in the hilum area (funiculus) of seeds. Although a true periderm is not developed in these tissues, the hydrophobic nature of suberin confers water barrier properties as well.

1.1. Periderm aliphatic compounds: wax and suberin

Most of chemical suberin studies have been focused in the phellem or cork of both cork oak (*Quercus suber*) bark and potato (*Solanum tuberosum*) tuber periderm, probably due to the large amount of suberin they contain. Suberin is an unknown biopolymer in many aspects. Although the monomeric chemical composition of suberin seems to be more clearly understood with the recent elucidation of glycerol as a key compound (Moire *et al.,* 1999), very little is known about the enzymes involved in their biosynthesis. Besides, other features like monomer transport to the apoplast, monomer esterification, macromolecular organization and regulation of the whole suberization process remain to be elucidated. Due to the lack of information about suberin and taking into account that cutin shares similar features with suberin (as both are plant polyesters of a hydrophobic nature), cutin has often been helpful to better understand suberin. For this reason, multiple comparisons between the two plant polyesters will be made in this work.

1.1.1. Chemical composition of suberin

Suberin consists of an aliphatic polyester esterified to some phenolics and cross-linked with an aromatic lignin-like domain bounded to cell wall carbohydrates (Bernards, 2002). The chemical composition of suberin was described after the breaking of ester linkages or transesterification (Kolattukudy and Agrawal, 1974; Franke *et al.*, 2005; Graça and Santos, 2007). Generally, this depolymerization releases glycerol, hydroxycinnamic acids and a mixture of aliphatic compounds ranging from C16 to C32 with hydroxy and carboxylic acid functionalities (Graça and Santos, 2007).

The aliphatic compounds of suberin are long and very long chain fatty acids (LCFA, VLCFAs) and derivatives such as α,ω-diacids, ω-hydroxyacids, primary alcohols and mid-chain-oxidized fatty acids. Although the same profile of α,ω-diacids, ω-hydroxyacids and glycerol seems to be prevalent in suberized tissues, suberin composition varies between plant species and tissues where it occurrs (Graça and Santos, 2007). Compared with potato suberin, the suberin from the cork oak bark is more abundant in saturated and mid-chain oxidized fatty acids and derivatives (Graça and Santos, 2007) and the suberin from *Arabidopsis* is shorter since it rarely contains VLCFAs and derivatives longer than C26 (Franke *et al.*, 2005). In contrast to the chemical composition of suberin, cutin polyester rarely contains VLCFA and derivatives. Besides, the

typical α,ω-diacids of suberin are not usual cutin compounds, although they are found in *Arabidopsis* cutin (Franke *et al.*, 2005).

The aromatic compounds obtained after depolymerization are a mixture of hydroxycinnamic acids, especially ferulic acid, and derivatives. The aromatic fraction of suberin has been compared to lignin by some authors (Kolattukudy 1980, Bernards, 2002). Although both suberin and lignin contain monolignols, other hydroxycinnamic acids and derivatives such as feruloyltyramine have only been described in suberin (Bernards *et al.*, 1995).

1.1.2. Chemical composition of waxes

Waxes are present in the cuticle, in suberized tissue and in the pollen seed coat. Unlike the insoluble polyesters cutin and suberin, waxes are referred to as the "soluble lipid fraction" or "extractives" for their solubility in organic solvents like chloroform. Although waxes are characterized to be represented by VLCFA and derivatives, their chemical composition is highly variable depending on the organ and plant species analyzed. Waxes also include triterpenoids and minor secondary metabolites, such as sterols and flavonoids in the cuticles of *Arabidopsis* (Kunst and Samuels, 2003) and tomato fruit (Vogg *et al.*, 2004) as well as in the periderm of cork oak bark (Castola *et al.*, 2005). It is noteworthy that the VLCFA and derivatives composition of suberin associated waxes greatly differs from that of cutin associated waxes. Whereas in suberin associated waxes the main compounds are free fatty acids, primary alcohols, alkanes and alkyl esters of ferulic acid (ferulic acid esters), in cutin associated waxes the major compounds include alkanes and ketones. Cutin associated waxes also contain alkyl esters but they are obtained by the reaction of very long chain alcohols with fatty acids (Lai *et al.,* 2007), not with ferulic acid. Recently, monoacylglycerols have been reported as minor compounds in suberin associated waxes in roots, but not in cutinized tissues (Li *et al.*, 2007b).

1.1.3. Biosynthesis of wax and suberin aliphatic compounds

The wax and suberin aliphatic monomers are basically fatty acids and their derivatives. The ubiquitous C16 and C18 (also C18:1) fatty acids synthesized by the plastid enzyme machinery act as precursors for the rest of the compounds. After being exported from the plastids, C16 and C18 fatty acids are supposed to be elongated and modified by endoplasmic reticulum (ER)

enzymes to produce all the wax and suberin aliphatic compounds. It is important to know that these fatty acid precursors are also used to synthesize the cutin polyester, the membrane glycerolipids and the storage lipids (triacylglycerols). It has recently been shown that a glycerol-phosphate acyltransferase 5 (GPAT5) is a key enzyme for suberin deposition in the cell walls (Beisson *et al.*, 2007; Li *et al.*, 2007a). However, the subcellular localization and the molecular mechanism of GPAT5 remain unknown.

Fatty acid elongation

Fatty acid elongation is required to produce the VLCFA and derivatives compounds of suberin and suberin associated waxes. The C16 and C18 fatty acids (LCFA) synthesized in the plastids bear an acyl carrier protein (ACP). Prior to the elongation of these precursors to VLCFA, three steps are required (Schnurr *et al.*, 2004): the release of the ACP by an acyl-ACP thioesterase (like FATB; Bonaventure *et al.*, 2003), the activation to a CoA-thioester by a long-chain acyl-CoA synthetase (LACS) and the transfer to the ER. The elongation of LCFA to VLCFA wax and suberin precursors is carried out by an ER membrane-bound fatty acid elongase (FAE) complex (Figure 2). The FAE sequentially adds two carbon moieties to the growing acyl chain through a four-step reaction series catalyzed by four distinct enzymes: condensation of malonyl-CoA with the long-chain acyl-CoA, reduction to β-hydroxyacyl-CoA, dehydratation to enoyl-elongated acyl-CoA and a second reduction of the enoyl-CoA (Samuels *et al.*, 2008). The condensing reaction is carried out by the 3-ketoacyl-CoA synthase (KCS), which is the rate-limiting step (Cassagne *et al.*, 1994; Suneja *et al.*, 1991) and the most sensitive to substrate chain length (Todd *et al.*, 1999). It is thought that multiple individual elongase systems are required to elongate a fatty acid from C18 to C32 (Samuels *et al.*, 2008). Several studies have been carried out regarding plant KCS involved in the biosynthesis of cutin associated waxes such as KCS6/CER6/CUT1 (Millar *et al.*, 1999; Jenks *et al.*, 1995), KCS1 (Todd *et al.*, 1999) and FDH (Lolle *et al.*, 1997) from *Arabidopsis*; LsCER6 from tomato (*Lycopersicum esculentum*) (Vogg *et al.*, 2004; Leide *et al.*, 2007) and GhCER6 from cotton (*Gossypium hirsutum*) (Qin *et al.*, 2007). Moreover, a wide-study expressing known and putative *Arabidopsis* KCSs in yeast has shown the variable saturated and unsaturated fatty acid product range of each KCS (Trenkamp *et al.*, 2004), which could explain the relative high number of KCSs in *Arabidopsis* (21). Despite identifying all these KCSs for the biosynthesis of cutin associated waxes, until now only KCS1 (Todd *et al.*, 1999) and very recently KCS2 (Franke *et*

al. 2008) have been involved in elongating suberin monomers in *Arabidopsis*. In this thesis (Chapter 2), we report on the phenotypic effects that StKCS6 deficiency causes to the aliphatic compounds of suberin and their associated waxes in potato tuber periderm.

Once VLCFAs are produced, they will be further modified to obtain the fatty acid derivatives of suberin and/or suberin associated waxes. It is expected that alcohols and alkyl esters are produced via an acyl-reduction pathway, whereas alkanes are produced via a decarbonylation pathway (Kunst and Samuels, 2003). In contrast with suberin associated waxes, in the wax fraction of cuticle further modifications in alkanes are needed to yield the ketones and secondary alcohols. Suberin ω-hydroxyacids and the corresponding α,ω-diacids are produced via a ω-oxidation pathway.
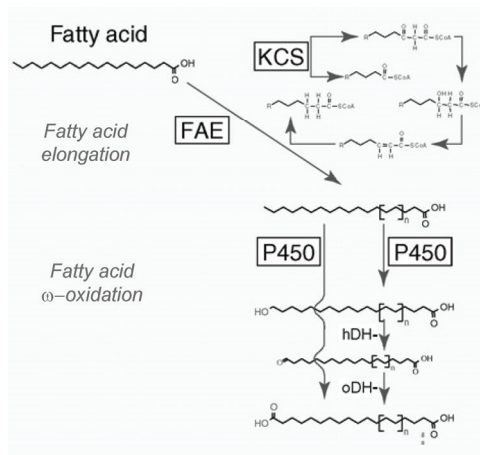
Figure 2. Fatty acid elongation and ω-oxidation steps to yield suberin compounds. The fatty acid is elongated by the FAE complex in which the KCS is the enzyme that condenses the malonyl-CoA with the growing fatty acid. Cytochromes P450 are the enzymes that catalyze the oxidation of the ω-terminal group of the fatty acids.
This is a part of the figure extracted from the proposed model for the suberin biosynthetic pathway (Franke and Schreiber, 2007).

ω-Oxidation of fatty acids

ω-Hydroxyacids and α,ω-diacids, together with glycerol, are the major monomers released after suberin polyester transesterification. Their synthesis was demonstrated years ago in potato tuber disks, in which radiolabeled oleic acid (C18:1) was incorporated into ω-hydroxyoleic acid and the corresponding diacid (Dean and Kolattukudy, 1977). The oxidation of the fatty acid in the ω-position (terminal position) is catalyzed by NADPH-dependent cytochromes P450 monooxygenases (Figure 2), especially from the CYP86, CYP94 and CYP92 subfamilies (Benveniste *et al.*, 1998; Kandel *et al.*, 2006; Le Bouquin *et al.*, 2001). Although the conversion of ω-hydroxyacid to the corresponding α,ω-diacid normally involves a two-reaction

step catalyzed by ω-hydroxyacid and ω-oxoacid dehydrogenases (Kolattukudy, 2001), the tobacco CYP94A5 and the *Arabidopsis* CYP94C1 have been shown to directly catalyze the oxidation of fatty acids to the corresponding α,ω-diacid (Kandel *et al.*, 2007; LeBouquin *et al.*, 2001). In this thesis, the effects of *CYP86A33* downregulation on the suberin and periderm function are presented in Chapter 1.

1.1.4. Macromolecular assembly of suberin compounds

Suberin is deposited in the secondary wall of suberized cells. Kolattukudy (1980), based on the chemical depolymerization of suberin and the resemblances between suberin and cutin and lignin, proposed a model where the aliphatic compounds were structured in a matrix like cutin and linked to the phenolic domain, which was polymerized by oxidative coupling like lignin. This phenolic domain would covalently link the aliphatic suberin structure to the carbohydrates of the cell wall. However, this model did not elucidate the lamellation (alternated opaque and translucent lamellae) of suberin observed by a transmission electron microscope (TEM) (Figure 3a). Studies using the cotton green-lint mutant (Lg), whose fibres are suberized with a large proportion of waxes, helped to understand the suberin macromolecular structure. On one hand, treatment with a VLCFA synthesis inhibitor showed shorter electron translucent lamellae suggesting that the aliphatic compounds are "stacked" perpendicular to the lamellae plane (Schmutz *et al.*, 1996). On the other, the treatment with an aromatic compound synthesis inhibitor was associated with the disappearance of electron opaque lamellae, which were replaced by empty spaces, demonstrating that the polyaromatics are relevant for the formation of these lamellae (Schmutz *et al.*, 1993). Moreover, finding glycerol in suberin (Moire *et al.,*1999; Schmutz *et al.*, 1993) and the studies based on partial suberin depolymerization (Graça and Santos, 2006a; Graça and Santos, 2006b; Graça and Pereira, 2000) have also shed light on the poorly understood macromolecular structure of suberin. Bernards (2002) proposed a model in which aliphatic monomers, corresponding to the translucent lamellae, were regularly oriented perpendicular to their lamellae plane and esterified with aromatic compounds and glycerol located in the opaque lamellae (Figure 3b). Glycerol, as a key compound, would provide the alcohol groups for ester linkages, permitting the growth of the polyester in a three dimensional network. This regular opaque and translucent lamellae
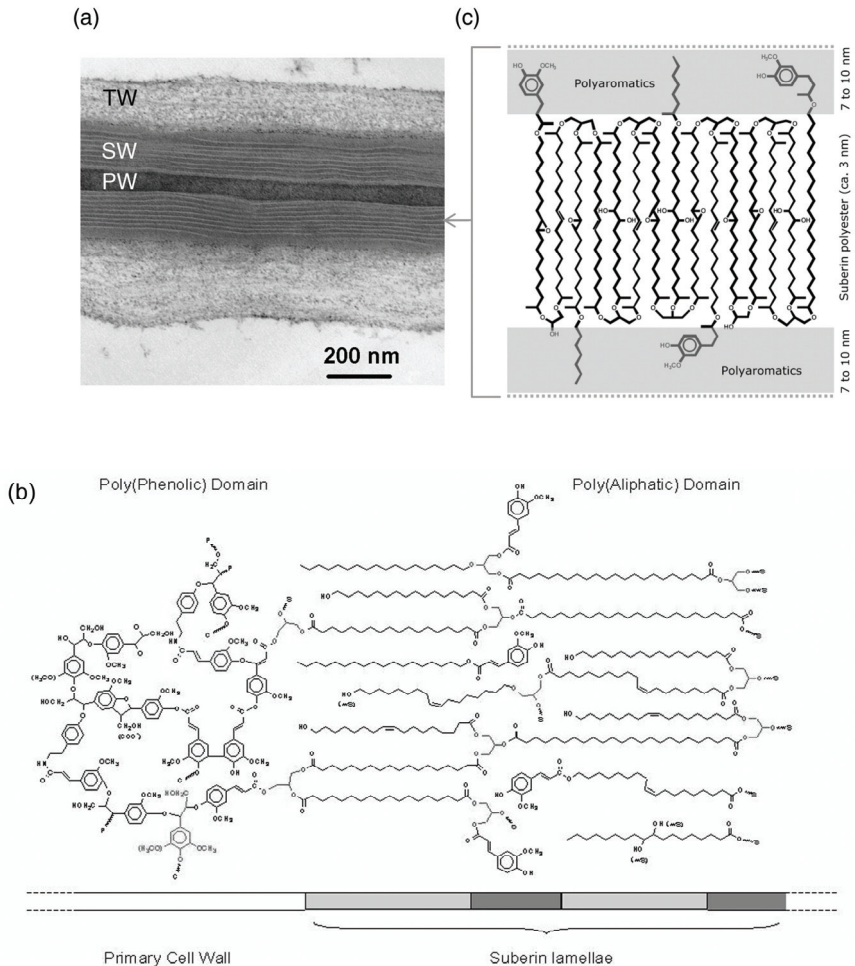
Figure 3. Suberin macromolecular structure under TEM and proposed models for suberin macromolecular assembly. (a) Suberized cell wall from two cells of potato tuber phellem separated by the middle lamella (not seen) of the polysaccharide primary wall (PW). The polysaccharide tertiary wall (TW) is also observed. The suberized secondary wall (SW) shows the typical opaque and translucent lamella alternating structure. (b) Tentative model proposed by Bernards (2002) for the assembly of suberin compounds in a macromolecular structure. The polyphenolic domain is shown restricted to the primary cell wall and covalently attached to carbohydrate units. The polyaliphatic domain is represented by a glycerol-based polyester. In the model, the predominantly aliphatic zones would yield the translucent bands observed in TEM micrographs, while the phenolic rich zones would yield the opaque bands (C: carbohydrate; P: phenolic; S: suberin). (c) Model proposed by Graça and Santos (2007) to explain the suberin lamellae structure seen under TEM. They proposed that glycerol and α,ω-diacids compounds constituted the framework of the suberin assembly.

structure was named the polyaliphatic domain despite the presence of some aromatic monomers. The polyphenolic domain was formed exclusively by aromatic compounds which were suggested to link the polyaliphatic domain to the cell wall carbohydrates. Graça and Santos (2007), taking into account all their results from suberin partial depolymerizations, suggested a model in which the basis of the suberin macromolecular structure was constituted by α,ω-diacids anchored at both sides to glycerol (Figure 3c). Ferulic acid would assure the linkage between the polyaromatics (opaque lamellae) and aliphatics (translucent lamellae). The several lamellae of aliphatic polyester would be connected by ω-hydroxyacids, crossing through the polyaromatic lamellae. In the translucent lamellae, intra and intermolecular bounds were postulated between the mid-chain-oxidized monomers. However, despite the efforts to understand the molecular structure of suberin, conclusive evidence linking the suberin chemical composition and ultrastructure is lacking.

1.2. Water barrier function of potato tuber periderm

The cork tissue of periderm contains aerenchymatous cork areas called lenticels, which are paths for the "regulated" water vapour and the oxygen and carbon dioxide exchange between inside and outside. In fact, lenticels are formed in the periderm by the divisions of cells apparently continuous with the phellogen, located below the stomata of the original epidermis replaced by the periderm (Tyner *et al.*, 1997). In contrast, the unregulated water loss goes through the cork layer of the periderm, thus determining the state of the periderm water barrier function (Lendzian *et al.*, 2007).

1.2.1. Water permeability measures

The use of well-defined enzymatically isolated periderms is a good strategy to measure the transfer of molecules via the periderm controlling the presence/absence of lenticels (Schreiber *et al.*, 2005; Vogt *et al.*, 1983) in a way that is similar to the one widely used for cuticles (Riederer and Schreiber, 2001). The treatment of periderms with fungal cellulases and pectinases leads to the isolation of the heavily suberized cells that are forming the phellem. The suberin deposited in the cells protects the polysaccharide cell wall from the hydrolytic enzymes without changing the structure of the phellem (Vogt *et al.*, 1983). Therefore, in a strict sense, the term "isolated periderm" or "periderm membranes" should be replaced by "isolated phellem" as phellogen and phelloderm become digested. However, the term periderm

is still being used as it has been done in the past by different authors (Schreiber *et al.*, 2005; Stark *et al.*, 1994; Vogt *et al.*, 1983).

The water permeability of the potato tuber periderm is determined by gravimetric methods using stainless steel or brass transpiration chambers of a specific diameter. The isolated periderms are mounted on these water-filled chambers with the physiological inner side of the periderm facing the inner side of the chamber (Figure 4). The permeability values are calculated based on the loss of the chamber weight caused by the water evaporated across the periderm.
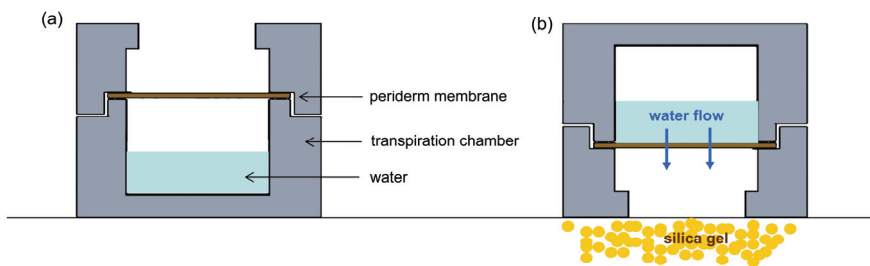
Figure 4. **Diagram of the longitudinal section of the typical transpiration chamber used to measure the water permeability across isolated cuticles or periderms by gravimetric methods.** (a) Periderm mounted on the transpiration chamber filled by water. (b) Once mounted, the transpiration chambers were turned upside down to ensure the direct contact between the water and the periderm, kept in closed boxes containing dry silica gel and stored at a constant temperature. The water is forced to pass through the periderm by evaporation to the silica gel.

Using modified transpiration chambers and tritiated water, it was possible to describe two pathways for water transport across the periderm (Figure 5) (Vogt *et al.*, 1983). Pathway 1 is characterized to have fast water diffusion and low permeability values and could be represented by middle lamellae and a primary wall orientated in a radial direction parallel to the direction of water flow. Pathway 2 is characterized to have slow water diffusion and high permeability and could be represented by the cell walls formed by middle lamellae, the primary wall, the suberized wall and the tertiary wall together with the wax fraction, orientated perpendicular to the water flow (Vogt *et al.*, 1983). Using the gravimetric method, which takes measurements at day intervals, it is not possible to distinguish the water diffusion through

pathways 1 and 2, and therefore the measured permeability represents the total permeability of both pathways (Schreiber *et al.*, 2005).
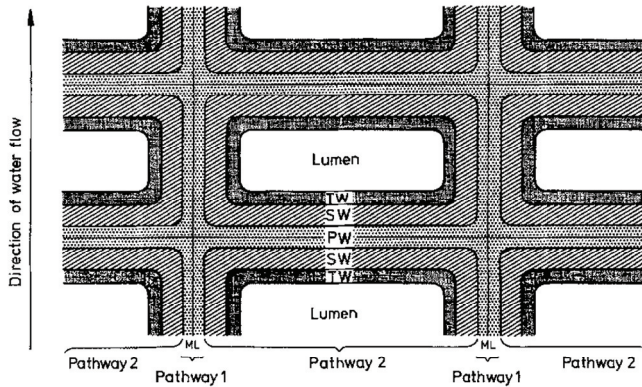
Figure 5. Schematic drawing of a cross-section of a periderm membrane indicating the two different pathways of water movement. The drawing is not to scale. Extracted from Vogt *et al.*, (1983).

1.2.2. Role of suberin and waxes in the water barrier function

Periderm membranes consist of two components: i) the extractable or organic solvent-soluble fraction known as wax and ii) the non-extractable or insoluble material formed by suberin and polysaccharides (Lendzian, 2006). Until now, the wax and suberin contribution to maintaining the water barrier properties of potato tuber periderm has seemed ambiguous and very biased depending on the potato variety studied (Lendzian, 2006). Likewise, the role of the chain-length profile and the substance class distribution of the aliphatic periderm compounds, as well as their physical assembly, have not yet been determined. Waxes have been proposed as space fillers, supporting the three-dimensional network of suberin based on the shrinkage produced in periderms when this wax fraction is removed. However, more evidence is needed because in potato periderm, where the wax fraction is very small, the shrinkage is still very high (Lendzian, 2006). Waxes have also been identified as key compounds in maintaining the periderm water barrier function. After wax extraction, the water permeability of tuber periderm membranes increases by a factor of 100 (Schreiber *et al.*, 2005; Vogt *et al.*, 1983), although this increased permeability was not observed in all the potato varieties (Lendzian, 2006).

Besides, the importance of wax in potato tuber periderm has been demonstrated using trichloroacetate as an inhibitor of fatty acid chain elongation. After treatment, the periderm experienced a great decrease of wax compounds and an inhibition of the development of diffusion resistance of the tissue to water vapour (Soliday *et al.*, 1979). Regarding the insoluble material formed by suberin and polysaccharides, as far as we know, no one has provided any direct evidence correlating these compounds to the periderm water barrier function.

In potato, the immature/mature periderm is defined based on its susceptibility to the excoriation of the skin (skinning) (Lulai and Orr, 1993). During the storage period, the periderms become resistant to excoriation, and reach the mature stage when the transpiration rate is minimum and constant (Schreiber *et al.*, 2005). At this stage of maturity, measures of periderm water permeability were around 10^{-10} m s^{-1} (Schreiber *et al.*, 2005; Vogt *et al.*, 1983). This decreased water permeability at maturity is attributed to the huge amount of suberin and waxes deposited in the cell wall during this storage period, especially during the first days of storage (Schreiber *et al.*, 2005). However the deposition of suberin and waxes cannot by itself explain the 100-times higher permeability seen in wound periderms, which amounts to 50-60% of the total suberin and wax load of native periderm (Schreiber *et al.*, 2005). Other factors, such as the physical assembly of all periderm components, might therefore contribute to the periderm water barrier properties.

2. Molecular tool: RNA interference-mediated silencing

2.1. The mechanism of gene silencing

Gene silencing was initially perceived as an unpredictable and inconvenient side effect of introducing transgenes into plants (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). Since the discovery of the gene silencing phenomenon in 1998 (Fire and Mello 1998), silencing pathways have been characterized. At present it is widely accepted that many eukaryotes benefit from a highly abundant class of single-stranded small RNAs (20-30 bp) that trigger gene silencing through RNA-RNA and possibly also RNA-DNA interactions, hence called silencing RNAs (sRNAs). These sRNAs participate in a wide range of processes such as transcriptional silencing of heterochromatin, post-transcriptional regulation of mRNA and silencing of invading viral genomes or transgenes (Brodersen and Voinnet, 2006; Lippman and

Martienssen, 2004; Vaucheret, 2006). sRNAs are derived from the RNA cleavage (Dicer family) of longer, partially or fully double-stranded RNA (dsRNA) molecules, produced by inter or intra-molecular interaction. There are two types of sRNA: i) micro RNAs (miRNAs) produced by longer single-stranded imperfect fold-back mRNAs which mediate post-transcriptional control of endogenous transcripts and ii) short-interfering RNAs (siRNAs) produced by longer double-stranded perfectly complementary RNAs. The siRNAs come from viral replication or through the action of RNA-dependent RNA polymerase on single-stranded plant RNA. They mediate the post-transcriptional regulation of endogenous/exogenous (including viral and transgene) transcripts and transcriptional silencing of transposable elements (Ossowski *et al.*, 2008).

2.2. RNA interference mediated silencing for functional genetics.

From a practical perspective, complementary long RNA able to produce double-stranded RNA was reported to efficiently silence an endogenous loci and a transgene in tobacco (Waterhouse *et al.*, 1998). Since then, gene silencing has been used as a specific and powerful technique to knock down the expression of target genes and subsequently evaluate their physiological role in plants. Now we know that the classical use of long antisense RNAs, resulting in the downregulation of the complementary mRNA is mediated by sRNAs as well as the unexpected silencing in which a sense transgene overexpression results in the downregulation of the transgene and the homologous endogenous gene (sense PTGS) (Ossowski *et al.*, 2008). A more recently developed and most efficient silencing method in plants is the use of the inverted repeat (ir) post-transcriptional gene silencing (PTGS), also called the RNA interference (RNAi) or hairpin RNAi (hpRNAi) approach (Watson *et al.*, 2005). The hpRNAi, controlled by a promoter and a terminator, consists of two transgene sequences oriented inversely and separated by a spacer region, normally a short intron. *In planta*, the transcription of this hpRNAi leads to a dsRNA which is processed by Dicer into heterogeneous siRNAs. One strand of the siRNAs associates itself with Argonaute (AGO), the catalytic subunit of RNA-induced silencing complexes (RISCs). AGO binds by complementarity both small and longer endogenous mRNA and cuts the target transcript in the corresponding position 10/11 of the sRNA. Due to the heterogeneity of the siRNAs, the AGO-dependent cleavage is found in many sites of the target transcript. The resulting fragments of the cleavage are degraded or used as primers for the RNA-dependent RNA polymerase to synthesize new dsRNAs which will be

processed as the hpRNAi, hence the amplification of the process leading to strong RNAi-mediated silencing (Ossowski *et al.*, 2008). One of the advantages of the RNAi-mediated silencing is that the hpRNAi construct is genetically dominant and therefore the silencing effects can be screened in the same plant or first progeny without needing to produce homozygous lines (Helliwell and Waterhouse, 2003). Thus, RNAi-mediated silencing has been identified as the appropriate strategy for functional genetic approaches in non-model polyploid plants with restricted capacity for sexual reproduction such as the potato.

OBJECTIVES

The research in our laboratory is focused on understanding the molecular processes that govern suberized tissues. Despite the importance of suberized tissues for land plants, an easily detected phenotype for suberin deficiency has not been reported, indicating that forward genetic approaches are inappropriate to identify genes involved in the suberization process. A more suitable strategy would be to identify genes putatively involved in suberized tissues and, afterwards, confirm their real role using a reverse genetic approach. Specifically, we followed a two-step strategy: i) the isolation of candidate genes for suberin biosynthesis through suppression subtractive hybridization (SSH) libraries (presented as background studies in this section) and ii) the development of a reverse genetic approach in potato to downregulate these target genes and subsequently analyze the effects in the chemical composition, structure and water barrier function of the tuber periderm (presented in the Chapters 1 and 2).

1. Background studies: identification of candidate genes of suberin biosynthesis

The periderms of cork oak (*Quercus suber*) bark and potato (*Solanum tuberosum*) tuber skin are paradigms of suberized tissues due to the large amount of suberin that their cork or phellem cells synthesize. These periderms were used to underscore genes involved in the suberin biosynthesis and periderm formation by constructing two SSH libraries (Diatchenko *et al.*, 1996). The SSH technique was used to obtain expressed sequence tags (ESTs) in the periderm (tester) but not in the control tissue (reference or driver) and to enrich ESTs of rare transcripts. The first SSH library was constructed using the cork bark of field-grown cork oak and the proliferative mass of *in vitro*-grown cork oak embryo as tester and driver tissues respectively. To distinguish the candidates of periderm among those involved in the suberin aliphatic or aromatic fraction, the library was printed on a microarray and cohybridized with cork and wood (lignified tissue) cDNA (Soler *et al.*, 2007). At the same time, a second SSH library containing ESTs preferentially expressed in potato tuber skin (or periderm) was constructed, using as driver tissue the parenchymatous tissue of potato tubers.

2. Functional genetics of StKCS6 and CYP86A33 by RNA interference-mediated silencing in potato tubers

Potato tuber periderm, a model to study suberin biosynthesis and function

Arabidopsis is the model plant for molecular studies in dicotyledonous because it is a relatively short life-cycle plant with fully-sequenced genome and large molecular resources available. It was recently identified as a valuable model for suberin functional studies due to the development of methods to detect and analyze suberin in roots (Franke *et al.*, 2005). To date, however, the lack of a well-developed direct methodology to measure the efficiency of the suberin barrier in *Arabidopsis* for water transport is not available, probably due to the smallness of *Arabidopsis* roots (Schreiber *et al.*, 2005) and the complex devices needed. Moreover, the study of periderm features cannot be approached in *Arabidopsis* because it is difficult to find stem periderm areas free of cuticle and root periderm areas free of endodermis. In contrast, the methodology to study the water barrier transport of potato tuber periderm has been fully developed (Schreiber *et al.*, 2005; Vogt *et al.*, 1983). The potato, although it does not have the same molecular tools as *Arabidopsis*, is an outstanding model for functional genetic studies of suberin biosynthesis and function, since sufficient amounts of tuber periderm can be easily obtained for chemical, ultrastructural and physiological studies. Finally, it is also significant that potato is an important crop plant whose tubers are of great economic interest.

The potato, like many crop plants, is polyploid (The NSF Potato Genome Project, http://www.potatogenome.org/nsf5/potato_biology/). The use of mutagenesis is therefore not suitable because polyploidy hinders the obtaining of homozygous progeny. On the contrary, RNA interference (RNAi)-mediated silencing technology has allowed stable knockdown of target genes in traditionally non-model plants such as the potato. These plants are of more agronomic interest and more appropriate to study certain processes, such as periderm suberification.

CYP86A33 and StKCS6 genes, candidates for suberin biosynthesis

The hydrophobic nature of suberin is attributed to the aliphatic domain. The main monomers released after suberin depolymerization are long chain and very long chain fatty acids (LCFA; VLCFAs), some of them functionalized in the ω or terminal carbon. Thus, pivotal reactions to

yield these main suberin compounds must be the fatty acid ω-hydroxylation catalyzed by cytochrome P450 monooxygenases and the elongation of LCFA catalyzed by the ER membrane-bound fatty acid elongase complex. This elongation pathway is also crucial for the biosynthesis of suberin associated waxes, as these waxes are mainly composed of VLCFAs and derivatives. Taking into account the importance of ω-oxidation and elongation steps in suberin biosynthesis, the specific goal of the present work is to use functional genetic approaches to study genes suspected of being involved in these enzymatic reactions in potato. With this purpose in mind, two genes encoding a putative cork oak fatty acid ω-hydroxylase member of the CYP86A subfamily (*CYP86A32* from now on) and a putative potato ketoacyl-CoA synthase (*StKCS6* from now on) were selected from the cork oak and potato SSH libraries respectively. *CYP86A32* was strongly up-regulated in cork versus wood (Soler *et al.*, 2007) and its expression pattern during the cork growing season was consistent with a function for suberin biosynthesis (Soler *et al.*, 2008). The transcript profiles in potato tissues of the putative potato orthologous gene of *CYP86A32* (*CYP86A33* from now on) and the *StKCS6* gene were consistent with suberin biosynthesis and these genes were consequently characterized by a reverse genetic approach.

To analyse the *CYP86A33* and *StKCS6* gene function in suberin and periderm, RNAi-mediated silencing technology was used. The two selected genes were respectively downregulated in potato tuber periderm and the resulting phenotype was analyzed in three independent silenced lines. A diagram showing the different steps of the proposed strategy is shown below:
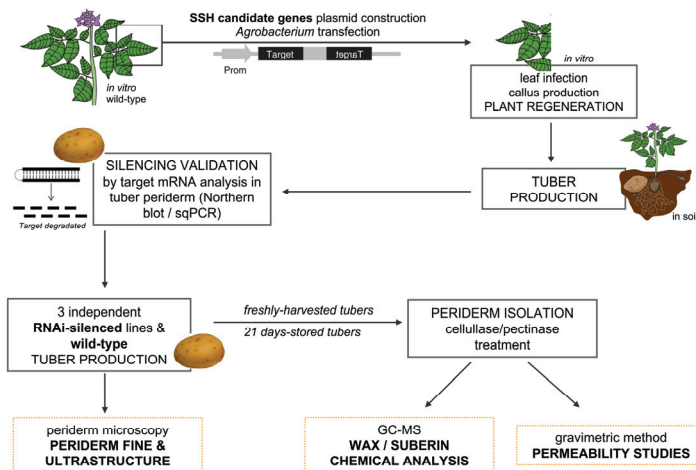
Specific objectives involving CYP86A33 and StKCS6

The main objective of this thesis is to better understand the function of ω-oxidation pathway in periderm through *CYP86A33* gene deficiency, and VLCFA elongation pathway through *StKCS6* gene deficiency, with emphasis on their role in periderm aliphatic compound biosynthesis, ultrastructure and water barrier function.

The specific objectives are:

1. Study the role of *CYP86A33* in suberin (Chapter 1).

    1.1. Analyze the expression pattern of *CYP86A33*.

    1.2. Isolate the *CYP86A33* full-length coding sequence.

    1.3. Demonstrate by chemical analysis that CYP86A33 is an enzyme involved in suberin biosynthesis.

    1.4. Analyze the function of CYP86A33 in suberin macromolecular structure by transmission electron microscopy (TEM).

    1.5. Study the effects of *CYP86A33* downregulation on periderm water barrier by gravimetric methods.

2. Study the role of *StKCS6* in potato periderm (Chapter 2).

    2.1. Analyze the expression pattern of *StKCS6*.

    2.2. Isolate the StKCS6 full-length coding sequence.

    2.3. Demonstrate that StKCS6 is involved in the biosynthesis of VLCFA and derivatives of suberin and wax by chemical analyses.

    2.4. Analyze the function of *StKCS6* in the suberin macromolecular structure by TEM.

    2.5. Study the effects of *StKCS6* downregulation on the periderm water barrier by gravimetric methods.

3. Discuss the significance of our finding in relation to the global knowledge of suberin biosynthesis and periderm function.

CHAPTER 1

CYP86A33 targeted gene silencing in potato tuber alters suberin composition, distorts suberin lamellae and impairs the periderm's water barrier function

CYP86A33 **targeted gene silencing in potato tuber alters suberin composition, distorts suberin lamellae and impairs the periderm's water barrier function.**

ABSTRACT

Suberin is a cell wall lipid-polyester found in the cork cells of the periderm offering protection against dehydration and pathogens. Its biosynthesis and assembly, as well as its contribution to the sealing properties of the periderm, are still poorly understood. Here, we report on the isolation of the coding sequence *CYP86A33* and the molecular and physiological function of this gene in potato (*Solanum tuberosum*) tuber periderm. *CYP86A33* was downregulated in potato plants by RNA interference (RNAi)-mediated silencing. Periderm from *CYP86A33* silenced plants revealed a significant decrease in its suberin load (~60%) and greatly reduced levels of C18:1 ω-hydroxyacid (~70%) and α,ω-diacid (~90%) in comparison with wild-type. The typical regular ultrastructure of suberin, consisting of dark and light lamellae, disappeared and the thickness of the suberin layer was clearly reduced. In addition, the water permeability of the periderm isolated from *CYP86A33* silenced lines was 3.5-times higher than that of the wild-type. Thus, our data provide convincing evidence for the involvement of CYP86A33 in establishing suberin structure and function.
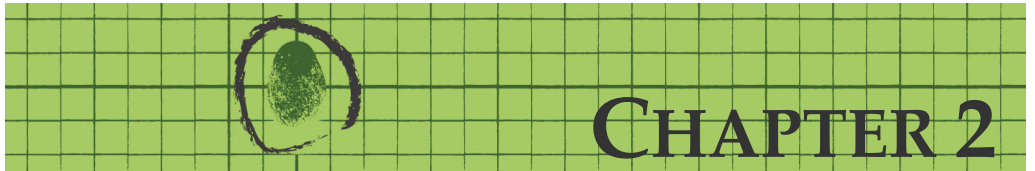
CHAPTER 2

Silencing of *StKCS6* in potato periderm leads to reduced chain lengths of suberin and wax compounds and increased peridermal transpiration

Silencing of *StKCS6* in potato periderm leads to reduced chain lengths of suberin and wax compounds and increased peridermal transpiration

ABSTRACT

Very long chain aliphatic compounds occur in the suberin polymer and associated wax. Up to now only few genes involved in suberin biosynthesis have been identified. Here we report on the isolation of a potato (*Solanum tuberosum*) *3-ketoacyl-CoA synthase* (*KCS*) gene and the study of its molecular and physiological relevance by means of a reverse genetic approach. This gene, called *StKCS6*, was stably silenced by RNA interference (RNAi) in potato. Analysis of the chemical composition of silenced potato tuber periderms indicated that *StKCS6* down-regulation has a significant and fairly specific effect on the chain length distribution of very long-chain fatty acids (VLCFAs) and derivatives, occurring in the suberin polymer and peridermal wax. All compounds with chain lengths of C_{28} and higher were significantly reduced in silenced periderms, whereas compounds with chain lengths of C_{26} and lower accumulated. Thus, *StKCS6* is preferentially involved in the formation of suberin and wax lipidic monomers with chain lengths of C_{28} and higher. As a result, peridermal transpiration of the silenced lines was about 1.5-times higher than that of wild-type. Our results convincingly show that *StKCS6* is involved in both suberin and wax biosynthesis and that a reduction of the monomeric carbon chain-lengths leads to increased rates of peridermal transpiration.

DISCUSSION

The main function of periderm suberin and their associated waxes is to protect plant secondary (mature) organs against dehydration (Bernards, 2002). However the suberin/wax chemical and physical properties that confer this physiological role to periderm remain unclear. Moreover, no direct relationship has been established between the composition, ultrastructure and sealing properties of suberin. The biosynthetic pathways proposed for suberin and wax compounds have been hypothesized on the basis of suberin and wax chemical composition (Bernards, 2002; Franke *et al.*, 2005; Kolattukudy, 2001). However, most steps of these pathways remain unconfirmed, probably due to technical difficulties inherent to the nature of suberized tissues.

To shed some light on suberin biosynthetic pathways, in this thesis we report on the study of two genes involved in the two major steps of suberin monomer biosynthesis: fatty acid ω-oxidation (*CYP86A33*) and elongation of long chain fatty acids (LCFA) (*StKCS6*). Our approach aims to characterize these genes by RNA interference (RNAi)-mediated silencing in potato tuber periderm. *CYP86A33* and *StKCS6* were selected based on their identification in two suppression subtractive hybridization (SSH) periderm libraries. The cork oak *CYP86A32*, the putative ortholog of potato *CYP86A33* and *Arabidopsis CYP86A1*, was isolated from the cork oak bark SSH library (Soler *et al.*, 2007) and the potato *StKCS6* from the potato tuber SSH library. In this thesis, we demonstrate that CYP86A33 is responsible for the biosynthesis of the ω-oxidized suberin major compounds and StKCS6 for the biosynthesis of the longest very long chain fatty acids (VLCFAs) and derivatives in potato tuber periderm. Moreover, it has been demonstrated that CYP86A33 is fundamental in organizing the suberin lamellar ultrastructure and both CYP86A33 and StKCS6 are critical to maintaining the water barrier function afforded by suberin polyester and their associated waxes. Therefore, using potato periderm as a model has made it possible to directly relate changes in suberin and wax chemical composition with the suberin ultrastructure and the water barrier function of periderm.

Very recently and during the course of research done in this thesis, other authors have approached the molecular genetics of suberin biosynthesis with a strategy based on the analysis of *Arabidopsis* mutants for suberin candidate genes. This alternative approach has revealed that four *Arabidopsis* genes encode suberin related-enzymes: *CYP86A1* (At5g58860), encoding a fatty acid ω-hydroxylase (Höfer *et al.*, 2008; Li *et al.*, 2007a); *GPAT5* (At3g11325), encoding

a glycerol-phosphate acyltransferase (Beisson *et al.*, 2007); and *KCS1* (At1g01120) (Todd *et al.*, 1999) and *KCS2* (At1g04220) (Franke *et al.*, 2008) encoding ketoacyl-CoA synthases.

All together, our results in potato and the results obtained in *Arabidopsis* represent a major advance in the knowledge of the biosynthesis and assembly of suberin. Moreover, our results are also related with the physiological role of periderm. The following sections are focussed on how our work and that of the other authors have contributed to a better understanding of the chemical and physiological properties of suberin.

1. Suberin biosynthesis

The phenotypic effects in CYP86A33 and StKCS6 deficient periderms are consistent with the role expected for these enzymes in the ω-oxidation and elongation pathways of suberin aliphatic precursors previously suggested (Bernards, 2002; Franke *et al.*, 2005; Kolattukudy, 1981). Figure 1 summarizes the biosynthesis of the suberin and suberin associated waxes showing the reactions that the potato and *Arabidopsis* suberin related-enzymes are probably catalyzing.
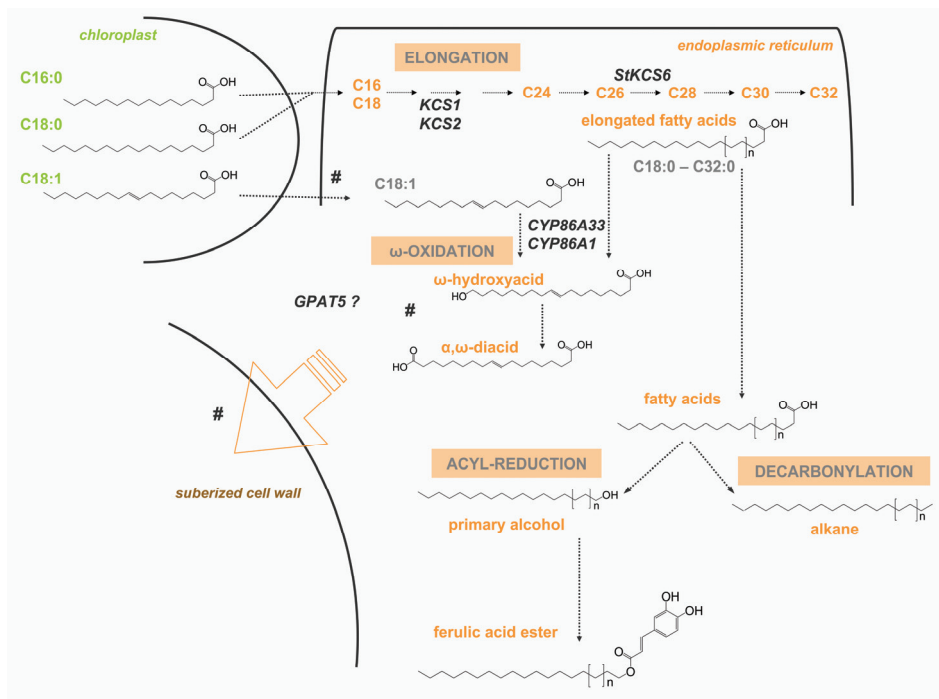
1.1. VLCFAs elongation pathway

The downregulation of *StKCS6* reports results in periderm consistent with those seen for cuticle waxes in the mutants of its putative orthologs in *Arabidopsis* (Millar *et al*., 1999; Jenks *et al.*, 1996) and tomato (Leide *et al.*, 2007; Vogg *et al.*, 2004). In contrast to AtKCS6/CER6/CUT1, which was designated as wax-specific KCS (Samuels *et al.*, 2008), our results demonstrate that StKCS6 is involved in the synthesis of very long chain linear compounds of suberin and suberin associated waxes. The deficiency in StKCS6 affects the elongation of C26 VLCFAs and derivatives to longer homologous compounds, which correspond to 15% and 65% of suberin and wax monomers of wild-type tuber periderm respectively. Despite the reduction in the longest VLCFAs and derivatives, the total load of each type of compound fraction was not greatly affected due to the simultaneous accumulation of homologous series of shorter chained compounds. This increase in shorter chain wax compounds, putatively synthesized by the acyl-reduction pathway (primary alcohols and ferulic acid esters), suggests that the enzymes of this pathway are not rate-limiting; hence shorter fatty acids can be transformed to primary alcohols and subsequently to ferulic acid esters by the acyl-reduction pathway (Figure 1). Moreover, the fact that StKCS6 deficiency causes the same effect in the chain-length profile in both suberin and wax fractions indicates that suberin and wax compounds could share the elongation pathway. However, it is not known whether wax biosynthesis is independent of that of suberin, or that waxes might be suberin precursors that have not been polymerized or might be derived by some suberin post-

Figure 1. General biosynthetic scheme for the aliphatic suberin and suberin associated wax compounds. The biosynthetic pathways leading to the fatty acids and derivatives which will be incorporated into the suberin and suberin associated waxes are based on previous publications and on cutin wax biosynthesis. The layout shows the direction of the main pathways, but only the enzymes known to be involved in suberin are depicted: StKCS6, KCS1, KCS2, CYP86A33, CYP86A1 and GPAT5. Fatty acid precursors are exported from the plastids to the endoplasmic reticulum to be modified. The elongation process controlled by KCSs yields the VLCFA precursors. It has been suggested that KCS1 and KCS2 act on fatty acids below C26 and StKCS6 on C26 VLCFAs. These elongated fatty acids, as well as the C18:0 and C18:1 fatty acids, can be ω-oxidized by NADPH-dependent cytochrome P450 monooxygenases, such as CYP86A33 and CYP86A1, to yield the ω-hydroxyacid and subsequently the α,ω-diacid compounds. Likewise, the acyl-reduction and the decarbonylation pathway use the same VLCFA precursors to produce primary alcohols, ferulic acid esters and alkanes. All these compounds can be incorporated as suberin and/or suberin associated waxes. The putative reactions that GPAT5 might catalyze are highlighted (#) (for more details see the text).

deposition hydrolytic event (Li *et al.*, 2007b). Taking into account that in *CYP86A33* silenced periderms the reduction in suberin content is accompanied by a wax increase, the latter hypothesis seems unlikely. In *Arabidopsis*, the elongases KCS1 (Todd *et al.*, 1999) and KCS2 (Franke *et al.*, 2008) have been involved to the elongation of suberin compounds below C26.

1.2. ω-Oxidation pathway

The *CYP86A33* downregulation reports a deficit of C18:1 ω-hydroxyacids and α,ω-diacids, the two major suberin compounds (~50% of wild-type suberin), suggesting that CYP86A33 is a suberin fatty acid ω-hydroxylase. So far, only one other cytochrome P450 from the same 86A subfamily, CYP86A1, has been demonstrated to be a suberin effector (Höfer *et al.*, 2008; Li *et al.*, 2007a). Based on the fact that CYP86A1, the *Arabidopsis* putative orthologous gene of CYP86A33, is not able to produce α,ω-diacids (Benveniste *et al.*, 1998), it is quite probable that CYP86A33 cannot yield α,ω-diacids either. However, without demonstrating the *in vitro* biochemical activity of CYP86A33, the ability to completely oxidize the ω-terminal group cannot be entirely ruled out.

Although it seems that the elongation pathway occurs before the ω-oxidation pathway (as shown in Figure 1), there are some intriguing results to show that the ω-oxidized monomers could be elongated after being oxidized (Höfer *et al.*, 2008; Pollard *et al.*, 2008). This is supported because *cyp86a1* plants are defective in C18:0 and C18:1 ω-oxidized monomers (Höfer *et al.*, 2008; Li *et al.*, 2007a) whereas *in vitro* results have shown that the C18:0 fatty acid was not a substrate for CYP86A1 and that the greatest activity was obtained towards the C16:0 fatty acid instead of the C18:1 fatty acid substrates (Benveniste *et al.*, 1998).

1.3. Glycerol entrance in the suberin biosynthesis

The finding that GPAT5 is acting on the formation of suberin fatty acid precursors (Beisson *et al.*, 2005; Li *et al.*, 2007a) was significant for suberin biosynthesis. GPATs catalyzes the transfer of an acyl group to the sn-1 position of glycerol 3-phosphate to form the 2-lysophosphatidic acid (Murata and Tasaka, 1997). Hence three different possibilities emerge for GPAT5 activity and glycerol incorporation into suberin (# in Figure 1): i) formation of fatty acid-glycerol units to be further oxidized; ii) formation of oxidized fatty acid-glycerol units to be polymerized; or iii) addition of acyl chains to a growing glycerol-containing lipid polyester

network (Beisson *et al.*, 2007; Pollard *et al.*, 2008). Although none of these hypotheses can be excluded, the ectopic overexpression of GPAT5 in *Arabidopsis* resulting in the accumulation of monoacylglycerols as waxes supports fatty acids as substrates for GPAT5 (Li *et al.*, 2007b). These results suggest that the CYP86A ω-hydroxylases could act on acylglycerols. In contrast, for fatty acid elongation, it has been suggested that KCSs act on fatty acids (Samuels *et al.*, 2008), but not on acylglycerols. This is supported because StKCS6 also synthesizes wax compounds whose biosynthesis seems to be independent of glycerol in contrast with the glycerol-based suberin polyester. However, taking into account the complexity of the suberin polyester assembly and the unknown connectivity between monomers, more experiments are required to confirm these hypotheses and clarify suberin biosynthesis.

2. Suberin molecular organization and ultrastructure

The models that try to explain the macromolecular structure of suberin are based on lipid biochemistry, suberin structural studies and suberin chemical analysis. Our results contribute to a better explanation of these proposed models. Suberin ultrastructure appears under transmission electron microscope (TEM) as a thickening of the secondary cell wall, consisting of alternating opaque and translucent lamellae. At the structural level, the compound distributions that make up these regular lamellae are not known, although the monomer connectivity has been speculated based on partial depolymerizations and hypothetical assumptions (Graça and Santos, 2007; Pollard *et al.*, 2008). These hypotheses identify the linkage of α,ω-diacids and glycerol monomers as the framework of the suberin macromolecular structure. The disorganization of the suberin lamellae seen in *CYP86A33* silenced periderms, deficient in C18:1 α,ω-diacids and glycerol, provides valuable evidence supporting this hypothesis. However, whether or not these crucial monomers are associated to form an extensive cross-linking with glycerol esterified at all three hydroxyl groups remains unclear (Pollard *et al.*, 2008).

Moreover, in *CYP86A33* silenced periderms, the great decrease of C18:1 major compounds inherently implies changes in the suberin monomeric composition in relation to the wild-type (i) increasing the average chain length of the acyl compounds (ii) but without showing dominance of any chain length. These chemical defects associated with the suberin lamellae destabilization also supports the Schmutz *et al.* results showing the translucent lamella is

directly related to the average length of the acyl chains (Schmutz *et al.*, 1996). The fact that in *StKCS6* downregulated periderms a decrease in the average chain length does not lead to suberin lamellae destabilization does not contradict these conclusions by Schmutz *et al.*, as in *StKCS6* deficient periderms the C18:1 α,ω-diacid monomer amount remains unchanged compared to the wild-type. By contrast, chemical treatments with inhibitors of multiple fatty acid elongases in the wound periderm of potato tubers (Soliday *et al.*, 1979) and in fibres of the green-lint mutant of cotton (Schmutz *et al.*, 1996) produced an important alteration of the suberin lamellation but also induced a much greater reduction in VLCFAs and derivatives and in the major α,ω-diacid compounds. Taken together, these results suggest that the major suberin α,ω-diacid monomer, C18:1 in potato, cork oak and *Arabidopsis* (Franke *et al.*, 2005; Graça and Santos, 2007) and C22 in green cotton fibres (Yatsu *et al.*, 1983), could be key, together with glycerol, to establishing the lamellar suberin structure as was suggested before (Schmutz *et al.*, 1996).

In conclusion, CYP86A33 and, consequently, ω-oxidation can be considered crucial for the correct arrangement of the suberin lamellae or, in other words, for creating the framework in which other suberin monomers are sustained. Taking into account the valuable results obtained for *CYP86A33* silenced periderm, further insights into its altered suberin macromolecular structure could be gained, for example, by partial suberin depolymerization.

3. Water diffusion path in periderm

Potato periderm forms an effective barrier to protect the tuber from unregulated water loss. Despite the agronomical importance of this periderm function, which guarantees the tuber integrity during storage and until consumption, the periderm characteristics accountable for it are not fully understood. The significance of the suberin and wax load to avoid water loss across the tuber periderm is evidenced through tuber maturation (or storage). The periderms from freshly-harvested tubers, which contain respectively 68% and 47% of the suberin and wax load of periderms from 21-day-stored tubers, have up to 1.9-fold higher permeability than that of 21-day-stored tubers (Table 1). Taking into account that periderms from freshly-harvested and 21-day-stored tubers are almost identical at the qualitative level, an indirect relationship between suberin and wax amount and permeability could be presumed to be in agreement with the results reported before (Schreiber *et al.*, 2005). However, we cannot rule

out that other factors during the storage affecting the polysaccharide cell wall or the physical assembly of the suberin and wax compounds could also contribute to improving the periderm water barrier function.

Table 1. Comparison between the main characteristic features of periderm membranes isolated from potato tubers (wt means wild-type; all the data is compared to 21-day-stored wild-type tubers).

Sample	Suberin	Suberin associated waxes	Water permeability	Suberin ultrastructure
Wild-type 21-day-stored tubers	- ~ 460 ug cm^{-2}	- ~ 20 ug cm^{-2}	- ~ 10^{-10}	- regular lamellation
Wild-type freshly-harvested tubers	- 68% of 21-day-stored load	- 47% of 21-day-stored load	- 1.9-times 21-day-stored permeability	- regular lamellation
CYP86A33-RNAi tubers (21-day-stored periderm)	- 40% of wt load - ↓ ω-oxidized monomers - lose of main suberin monomers - ↑ carbon median chain-length	- 2.4-times wt load	- 3.5-times wt permeability	- lamellar disorganization - clumps - thinner lamella
StKCS6-RNAi tubers (21-day-stored periderm)	- = wt load - ↓ VLCFA and derivatives ≥ C28 - ~ 2 carbon chain-length reduction	- 63% wt load - ~ 2 carbon chain-length reduction	- 1.5-times wt permeability	- regular lamellation

The results reported in this thesis (see Table 1) demonstrate for the first time the role of two enzymes, CYP86A33 and StKCS6, in the resistance of periderm to water loss. CYP86A33

deficiency results in impairment of the water barrier function, increasing permeability 3.5-times that of the wild-type. Although this defective physiological function could be attributed to the reduction of the suberin load to 40% of that of the wild-type, the disorganized lamellar ultrastructure made up of suberin compounds can also be a factor in this increased permeability. Taking into account the great chemical and ultrastructural shortcomings of *CYP86A33*-RNAi periderms, higher permeability rates were expected for them. Therefore, we can speculate that the more than 2.5-times increased wax content in the *CYP86A33*-RNAi periderms could be taking part in slowing down this expected permeability. This is not surprising considering that the removal of waxes gives rise to an increase of permeability by 100-fold when the periderms are devoid of them (Schreiber *et al.*, 2005; Vogt *et al.*, 1983).

StKCS6 deficiency impairs the periderm barrier to water although much more weakly than in the CYP86A33 deficient periderms. The *StKCS6*-RNAi transgenic periderm blocks the production of longer VLCFAs and derivatives and accumulates the shorter homologous compounds without yielding a major distortion of the type of aliphatic compounds in periderm. Therefore, the moderate but significant increase in the permeability of *StKCS6*-RNAi silenced periderms seems to be a consequence of the reduction of longer VLCFAs and derivatives. Evidence that longer compounds could be more efficient at avoiding water loss has not been previously reported.

CONCLUSIONS

1)   The fact that CYP86A33 and StKCS6 are enzymes of suberin biosynthesis indicates that suppression subtractive hybridization (SSH) technique is a good methodology to identify candidate genes of a particular process. Moreover the identification of other demonstrated suberin efectors such as GPAT5 in the cork oak periderm SSH library points these listed candidate genes as highly-probable suberin-related.

2)   The full length coding sequence of cork oak *CYP86A32* and potato *CYP86A33* show the highest homology with the *Arabidopsis* fatty acid ω-hydroxylase *CYP86A1*, suggesting a fatty acid ω-hydroxylase function for both enzymes.

3)   The expression pattern of *CYP86A33* gene is restricted to potato suberized tissues supporting the expected role of CYP86A33 in suberin biosynthesis.

4)   The suberin chemical analysis of *CYP86A33*-silenced periderm confirms the involvement of this gene in the ω-oxidation step of suberin biosynthesis to yield the suberin main compounds, C18:1 ω-hydroxyacids and α,ω-diacids.

5)   The reduction in C18:1 ω-hydroxyacids and α,ω-diacids results in a decrease on the rest of suberin monomers (including glycerol and ferulic acid), hence ω-functionalized compounds are key for the suberin deposition in the cell wall.

6)   The distortion of the suberin lamellar ultrastructure under TEM of *CYP86A33*-silenced periderms supports the hypothesis that the main α,ω-diacid suberin compound (C18:1 in potato suberin) linked to glycerol at both ends is the base for suberin's three-dimensional structure.

7)   In CYP86A33-silenced periderms, the reduction of suberin to 40% of that of wild-type together with the distortion of suberin ultrastructure leads to an impairment of periderm water barrier function.

8)   The full-length coding sequence of potato *StKCS6* shows the highest homology with other KCS6-like genes of other plant species suggesting a ketoacyl-CoA synthase function for this enzyme.

9)   The high accumulation of *StKCS6* transcript in periderm supports a function for StKCS6 in the biosynthesis of periderm aliphatic compounds. *StKCS6* gene expression in cutinized tissues reveals that this enzyme is probably involved in potato cuticular wax biosynthesis.

10) The chemical analysis of *StKCS6*-silenced periderm confirms the involvement of this gene in the elongation of very long chain fatty acid (VLCFA) to yield suberin monomers and suberin associated wax compounds with chain-length longer than C26.

11) The fact that StKCS6 downregulation leads to the same defects in the chain-length lipid profile of suberin and suberin associated waxes supports that suberin and wax compounds share the same elongating pathway. The fact that in *CYP86A33* downregulated periderm the wax content increases simultaneously with a suberin reduction reinforces that the biosynthetic pathways of both suberin and suberin associated waxes use the same precursors.

12) In *StKCS6*-silenced periderms, the reduction of VLCFA and derivatives longer than C26 and the minor reduction in wax load lead to an impairment of periderm water barrier function.

13) CYP86A33 and StKCS6 are fundamental for all the maturation process occurring after tuber harvesting, as the effects of their downregulation are seen in freshly-harvested and 21-day-stored tubers.

14) Results presented here demonstrate that potato periderm represents an excellent model for future studies to analyze the relationship between transport properties of suberized barriers and their qualitative and quantitative chemical composition.

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