Plant cuticle development: insights from molecular cloning of *CER13* and protein-protein interaction studies

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SUMMARY

Cuticle, the outermost layer covering all primary aerial surfaces of vascular land plants, is essential for the protection and normal development of the plant and has as main components cutin and wax. Although numerous genes are involved in the biosynthesis and regulation of cuticular components, only few have a known function.

To contribute to the understanding of regulatory mechanisms involved in cuticle development, two approaches have been taken in this project. These involved on the one hand mapping and characterizing a locus involved in wax regulation and metabolism and on the other hand, identifying putative interactors for three proteins involved in cuticular pathways. The first approach concerns the mapping and characterization of *eceriferum13* (*cer13*) mutant *Arabidopsis thaliana.* Obtained in L*er*-0 background by fast neutron treatment, the *cer13* mutant has less epicuticular wax and its semi-dwarf phenotype, as well as its responsiveness to photoperiod indicate a possible implication in regulatory mechanisms. After fine mapping to a region of 46 candidate genes, *RESURRECTION1* (*RST1*, AT3G27670) was selected as candidate for *CER13*, due to the fact that its mutation causes altered epicuticular wax phenotype. Sequence analysis of *RST1*, encoding a putative transmembrane protein of unknown function, revealed a 950 bp deletion resulting in a frame shift and a premature STOP codon in *CER13* cDNA. The allelism test performed by crossing *cer13* to *rst1-3* was positive, indicating a single locus. To investigate the mechanism responsible for the reduced wax levels in *cer13*, we performed a whole-genome ATH1 microarray analysis. Previously, the biochemical pattern of wax composition was found to be highly similar between *cer13*, *cer3* and *cer7*. Interestingly, *CER3* is down-regulated in both *cer13* and *cer7* transcriptomes, indicating that the three genes could be involved in the same pathway. We also compared *cer13*-misregulated genes to a MASTA (MicroArray overlap Search Tool and Analysis) database of more than 600 microarray datasets. This analysis suggested that *CER13* provides a link between wax biosynthesis and salicylic acid-mediated signaling, as well as similar expression patterns to cold and drought stress.

The second approach undertaken in this study consists in using three cuticular genes as baits in a Y2H cDNA library screening. All three genes that were selected for this screening are involved in the cuticle development in an unusual manner. The putative interactors found for the subtilisin-like serine protease ALE1, which is required for the proper formation of epidermis and cuticle around the embryo (Tanaka et al., 2001), are subunits a and b of the CSN5, a component of the COP9 signalosome. The putative acyl transferase *CER2* is particular in that it localizes to the nucleus. A porin and two immunophilins were identified as putative interactors. The *PALMITOYL PROTEIN THIOESTERASE* (*PPT*) is a gene up-regulated in three independent cuticular mutants and an ATP-dependent helicase as well as an ADP-ribosilation factor were isolated as putative interactors.

These results highlight the complexity of the regulatory mechanisms behind the formation and the function of the cuticle and contribute to the understanding of plant defenses.

ZUSAMMENFASSUNG

Die Kutikula, die äußerste, alle primären oberirdischen Organe von Gefäßpflanzen bedeckende Schicht, ist unerläßlich zum Schutz und für die normale Entwicklung der Pflanze und besteht hauptsächlich aus Cutin und Wachsen.

Wenngleich zahlreiche Gene in Biosynthese und Regulation von cuticulären Bestandteilen involviert sind, ist doch nur für wenige eine genaue Funktion bekannt.

Um zu einem besseren Verständnis regulatorischer Mechanismen, die an der Kutikula-Entwicklung beteiligt sind, beizutragen, wurden im Rahmen dieses Projektes 2 Strategien verfolgt. Dazu gehörte auf der einen Seite die Kartierung und Charakterisierung eines an Wachs-Metabolismus und –Regulation beteiligten Gen-Locus und auf der anderen Seite die Identifizierung putativer Interaktoren dreier Proteine, die in kutikuläre Entwicklungswege involviert sind.

Im Rahmen der ersten Strategie wurde die Arabidopsis thaliana *eceriferum13* (*cer13*) Mutante kartiert und charakterisiert. Die *cer13* Mutante - durch Bombardierung mit schnellen Neutronen im L*er*-0 Hintergrund erhalten - besitzt weniger epicuticuläres Wachs und sowohl ihr halb-zwergenwüchsiger Phänotyp als auch ihre photoperiodische Sensitivität, geben einen Hinweis auf eine mögliche Beteiligung an regulatorischen Mechanismen.

Nachdem durch Fein-Kartierung eine Eingrenzung auf eine Region mit 46 putativen Kandidaten-Genen erreicht werden konnte, wurde RESURRECTION1 (RST1, AT3G27670) als Kandidat for CER13 ausgewählt, da eine Mutation dieses Gens einen veränderten epicuticulären Wachs-Phänotyp verursacht.

Sequenz-Analyse von RST1, das für ein putatives Transmembran-Protein unbekannter Funktion codiert, offenbarte das Vorhandensein eine 950 bp umfassenden Deletion in der CER13 cDNA, die eine Verschiebung des Leserahmens und ein vorzeitiges STOP-Codon zur Folge hat.

Durch Kreuzung von *cer13* mit *rst1-3* konnte der allelische Charakter der Mutationen bestätigt werden, was für die Verantworlichkeit eines einzelnen Locus spricht.

Um die Mechanismen zu untersuchen, die für den reduzierten Wachsgehalt in *cer13* verantwortlich sind, wurde eine ATH1 Microarray Analyse durchgeführt. Zuvor war

die große Ähnlichkeit des biochemischen Musters der Wachs-Zusammensetzung in *cer13*, *cer3* und *cer7* beschrieben worden.
Interessanterweise ist CER3 sowohl in *cer13* also auch im *cer7* Transkriptome herunterreguliert, was darauf hindeutet, dass die drei Gene in einen gemeinsamen Entwicklungsweg involviert sind.
Ein Vergleich der in *cer13* missregulierten Gene mit einer MASTA (MicroArray overlap Search Tool and Analysis) Datenbank von über 600 Microarray-Experimenten legte nahe, dass CER13 eine Verbindung zwischen Wachs-Biosynthese und Salicylsäure-vermittelten Signalwegen herstellt, und zeigte Ähnlichkeit des Transkriptmuster der Mutante mit Kälte- und Trockenstreß-Bedingungen.
Die zweite hier verfolgte Strategie bestand im Durchmustern einer Yeast-2-Hybrid-Bibliothek, zur Identifizierung der Interaktoren dreier für die Kutikula-Entwicklung relevanter Proteine.
Putative Interaktoren für die Subtilisin-ähnliche Serine-Protease ALE1, die für korrekte Ausbildung von Epidermis und Kutikula des Embryos (Tanaka et al., 2001), benötigt wird, sind die Untereiheiten a und b von CSN5, einer Komponente des COP9 Signalosoms.
Die putative Acyl-Transferase CER2 ist besonders in ihrer nukleären Lokalisierung.
Ein Porin und 2 Immunophiline wurden als putative Interaktoren identifiziert.
Für PALMITOYL PROTEIN THIOESTERASE (PPT), ein in drei unabhängigen Kutikula-Mutanten hochreguliertes Gen, wurden eine ATP-abhängige Helicase und ein ADP-Ribosilierungsfaktor als putative Interaktoren identifiziert.
Diese Ergebnisse betonen die Komplexität regulatorische Mechanismen, die der Bildung und Funktion der Kutikula zugrundeliegen und tragen zum Verständnis der pflanzlichen Abwehr bei.

INTRODUCTION

STRUCTURE OF THE PLANT CUTICLE

A distinctive characteristic of all epidermal cell types is the presence of cuticle covering their outer surface as a continuous lipophilic layer, which forms a barrier over the aerial organs of land plants during their primary stages of development (Holloway, 1982; Kunst et al., 2005). Although the features of the plant cuticle vary considerably between species, organs and developmental stages, its main components are cutin, and wax (Jeffree, 1986; Riederer and Schreiber, 2001; Jeffree, 2006). Cutin is a polyester mainly comprising hydroxy and hydroxy-epoxy C16 and C18 fatty acids, as well as glycerol (Kolattukudy, 2001). Waxes are both intracuticular (embedded in the cuticular matrix) as well as epicuticular (deposited on the outer surface of the cell wall), as depicted in the schematic representation below.

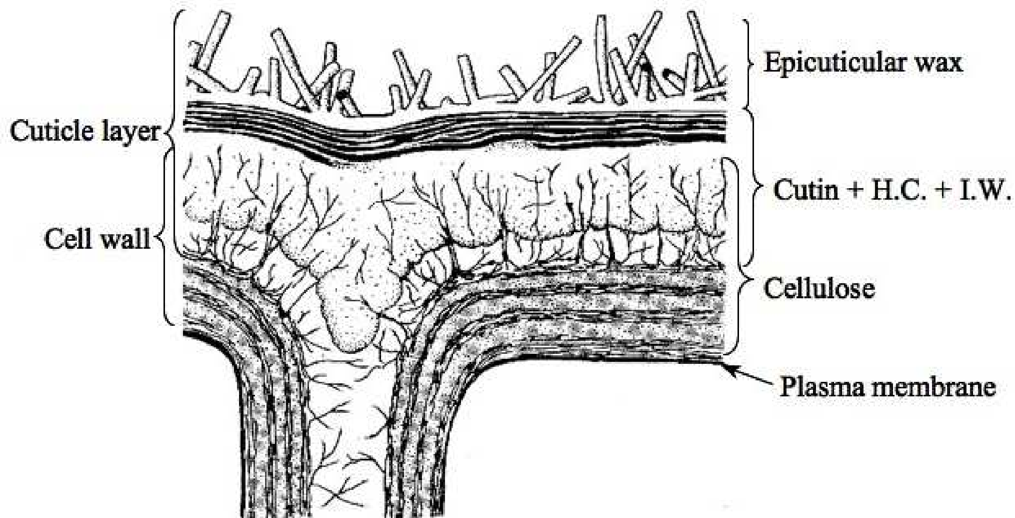


Figure 1. Schematic representation of the plant cuticle structure (adapted from Jeffree, 1986). H.C., hydrolysable compounds; I.W., intracuticular wax.

Waxes consist of a heterogeneous mixture of very long-chain fatty acids (VLCFAs) and their monomeric derivatives, wax esters, as well as secondary metabolites such as triterpenoids, phenylpropanoids, and flavonoids (Shepherd et al., 1995; Kunst et

al., 2005). As they are physically very closely associated, it is difficult to distinguish between the relative contribution of the cutin matrix and that of cuticular waxes to the physical properties and the biological roles of the cuticle. However, in biochemical experiments, the cutin and cuticular waxes are usually analyzed separately, due to the soluble properties of the waxes versus the cutin polymer, which remains insoluble.

THE ROLES OF PLANT CUTICLE

The chemical structure described above gives the cuticle a set of highly protective features and these were studied initially as to limiting nonstomatal water loss and gaseous exchanges, controlling the absorption of lipophilic compounds, and providing mechanical strength and viscoelastic properties (Baker et al., 1982; Riederer and Schreiber, 2001).

The plant cuticle is considered to be the first physical barrier to protect the plant against biotic and non-biotoc stress factors. As far as the biotic stress factors are concerned, the cuticle can be penetrated by fungal pathogens either through the natural openings in the epidermis (such as stomata) or by active cutinase-secretion. In addition to its barrier function, the plant cuticle was proposed to be a signaling source for potential pathogens (Lin and Kolattukudy, 1978), by means of its breakdown products. Thus, fungi would sense the presence of cutin monomers on the plant surface and would induce the formation of cutinase, required for invasion (Kolattukudy, 1985). Cutin monomers have been shown to induce the production of cutinase in *Fusarium solani f.sp. pisi* (Lin and Kolattukudy, 1978; Woloshuk and Kolattukudy, 1986) as well as the formation of appresoria in fungi such as *Erysiphe graminis f.sp. hordei* (Francis et al., 1996), the rice blast fungus *Magnaporthe grisea* (Gilbert et al., 1996), *Puccinia graminis f.sp. tritici* and the anthracnose fungus *Colletotrichum gloeosporioides*. In their turn, plans also sense molecules released during pathogen interactions. For example, products resulting from a breakdown of the plant cell wall were reported to act as elicitors of defense mechanisms (Boller, 1995). *Botritis cinerea* is a ubiquitous fungal pathogen that causes significant damage to many crop plants and perturbations of the cuticular layer rendered *Arabidopsis* plants fully immune to the fungus (Chassot et al., 2007).

Abiotic factors such as light and water deficit have been shown to stimulate wax biosynthesis in vascular plants (Thomas and Barber, 1974; Bengtson et al., 1978; von Wettstein-Knowles et al., 1979). Furthermore, light was shown to be essential for CER6 transcription, and osmotic stress as well as the presence of abscisic acid enhanced CER6 transcript accumulation (Hooker et al., 2002).

Other than protecting the plant from biotic and non-biotic environmental stress factors (Schweizer et al., 1996), the cuticle was also reported to have a developmental role. Transgenic *Arabidopsis* plants expressing a cutinase from *Fusarium solani* f sp *pisi* display an altered ultrastructure of the cuticle, enhanced permeability to solutes and strong postgenital organ fusion. Furthermore, pollen could germinate on fully differentiated leaves of cutinase-expressing plants (Sieber et al., 2000). From this experiment, as well as from the numerous mutants characterized up to date it is clear that an intact, functional cuticular layer is needed for normal epidermal differentiation and organ formation. It was suggested that cuticle permeability also influences cell-to-cell communication by enhancing or attenuating the passage of signal molecules (Pruitt et al., 2000). For example, such signals could be required for organ adhesion, when they would be moving across the cuticle, or for mediating signaling between trichomes and stomata, when moving within the developing epidermis (Lolle et al., 1997; Krolikowski et al., 2003).

CUTICULAR AND WAX-RELATED MUTANTS

In *Arabidopsis*, as well as in other species, several mutants have been identified to be defective in wax and/or cutin formation, thus facilitating the identification of enzymes associated with the cutin and wax pathways.

Mutants in the wax biosynthesis pathway

Some of the enzymes catalyzing various steps in the wax pathway have been characterized or their function has been proposed based on the phenotype of the corresponding mutants. The identification of *eceriferum* (*cer*) mutant lines (Koornneef et al., 1989) has led to the isolation and characterization of various genes associated with cuticular wax metabolism in *Arabidopsis*. *CER1* has been proposed to encode an aldehyde decarbonylase (Aarts et al., 1995). Several genes playing a role in the fatty acid elongation pathway that generates very long chain fatty acid (VLCFA) wax precursors have also been characterized. They include the *FATTY ACID ELONGATION1* (*FAE1*) homologs (James et al., 1995), *FIDDLEHEAD* (*FDH)* (Yephremov et al., 1999; Pruitt et al., 2000), *3-KETOACYL-CoA SYNTHASE1* (*KCS1*) (Todd et al., 1999), *CUT1/CER6*, and *CER60* (Millar et al., 1999; Fiebig et al., 2000). *CER6* was suggested to be the key condensing enzyme for wax biosynthesis in *Arabidopsis,* due to its expression throughout all stages of the stem and leaf development, as well as in the inflorescence (Hooker et al., 2002). *CER2* encodes a putative CoA-dependent acyltransferase, apparently located in the nucleus (Xia et al., 1996; Kunst and Samuels, 2003). Although the precise function of the gene is still unknown, *cer2* stems have more fatty acids with C24 but less with C26, C28 and C30, less C29 secondary alcohols and more C26 and C28 primary alcohols (Rashotte et al., 2001). Furthermore, its nuclear localization is intriguing for an acyl transferase. Many of the *cer* mutants remain still to be characterized and the isolation of their corresponding genes might bring valuable information into the mechanisms of wax metabolism.

Mutants involved in cutin metabolism

Several reports have also provided insights into the biosynthesis of cutin monomers in plants and this subject has been extensively reviewed (Yephremov and Schreiber, 2005; Nawrath, 2006; Pollard et al., 2008; Samuels et al., 2008). For example, the identification of the *WAX2* gene (Chen et al., 2003) showed that the protein it encodes has 32% similarity to *CER1* and contains certain regions with homology to sterol desaturases and short-chain dehydrogenases/reductases. It was suggested therefore that *WAX2* plays a metabolic role in both wax and cutin synthesis, thus indicating a link between wax and cutin metabolism. *ADHESION OF CALYX EDGES/HOTHEAD* (*ACE/HTH*) is proposed to be an oxidase catalyzing the formation of dioic acids from ω-hydroxy acyl-CoAs (Krolikowski et al., 2003; Kurdyukov et al., 2006). The *Arabidopsis LACERATA* (*LCR*) gene (Wellesen et al., 2001) encodes a cytochrome P450; enzyme activity assays using the recombinant *LCR* protein showed that it could efficiently catalyze the formation of ω-hydroxy fatty acids (ranging from C12 to C18:1). Expression of the *LCR* gene is predominant in inflorescence and siliques, as well as in roots and young seedling tissue and it is the first cytochrome P450 fatty acid ω-hydroxylase for which a mutant has been isolated.

Cuticle metabolism and the regulation of cuticular pathways

Results of microarray analysis conducted in our group (Voisin, 2008) on three independent cuticular mutants revealed that a palmitoyl-protein thioesterase (*PPT,* AT5G47330) is almost ten fold up-regulated in three mutants, as compared to wild type. In humans, *PPT* is a lysosomal long-chain fatty acyl hydrolase that removes fatty acyl groups from modified cysteine residues in proteins, and the defective enzyme causes infantile neuronal ceroid lipofuscinosis, a recessive hereditary neuro-degenerative disorder (Vesa et al., 2002).

The mutation in a subtilisin-like serine protease led to impaired cuticle formation and consequently adhesion between the endosperm and embryo, as well as fusion of cotyledons and leaves. The mutant is called *abnormal leaf shape1* (*ale1*) and the corresponding *ALE1* gene is preferentially expressed during seed development, showing a weak transcript expression in young embryo and a strong one within the endosperm cells closely surrounding the developing embryo (Tanaka et al., 2001).

Although many members of this family of proteases were reported in plants (Siezen, 1997; Schaller, 2004), little is known about their precise role.

When overexpressed in *Arabidopsis*, the SHINE clade of AP2 domain-containing transcription factors have been reported to activate wax biosynthesis, to alter cuticle properties, and to confer tolerance to drought stress (Aharoni et al., 2004).

Mutants characterized so far have led to the identification of genes involved in the metabolic pathways leading to the cuticle formation, though only few of these genes have a precise function assigned. Furthermore, the high variety as to the nature of the proteins implicated in cuticle development, whether they have a known or unknown function, is an indicator of a regulatory network behind cuticular metabolism. However, the nature of such a network is yet elusive.

AIM OF THE THESIS

To contribute to the understanding of regulatory mechanisms involved in cuticle development, two approaches have been taken in this project. The first approach concerns the mapping and characterization of a wax mutant of *Arabidopsis thaliana* and the second approach aims at finding interactors for proteins known to be involved in cuticle development.

The collection of *eceriferum* mutants is a valuable tool in understanding the metabolic as well as the regulatory pathways involved in cuticle development. One of the *CER* genes that had not been cloned is *CER13*. The *cer13* mutant shows a glossy phenotype on stem and siliques and together with *cer10* and *cer3*, it was also reported to exhibit organ fusions (Jenks et al., 2002), an indicator of substantial disturbance in the cutin metabolism (Lolle et al., 1992; Lolle et al., 1998). As our group is interested in a possible link between wax and cutin biosynthesis, the *cer13* mutant was previously selected for further characterization and positional cloning (Faust, 2006). The aim of this project was to clone the gene and to further characterize the *cer13* mutant.

Numerous genes are involved in cuticle development and/or its regulation (Pollard et al., 2008; Samuels et al., 2008). The high diversity as to the nature of the proteins involved in these processes indicates that a broad regulatory network, still far from being elucidated, governs the regulation of cuticle development and that such a network is present at all key developmental stages of the plant. To contribute to the understanding of the mechanisms behind the regulation of cuticle development and in the attempt to have an integrative approach, three genes were chosen for further investigation, by screening a cDNA library using the Y2H sistem. All three genes are involved in the cuticle development in an intriguing manner. *ALE1* encodes a subtilisin-like serine protease and it is expressed in the endosperm surrounding the developing embryo. It was proposed that the ALE1 protein causes a signal from the endosperm that is required for the proper formation of the epidermis and cuticle around the embryo (Tanaka et al., 2001). Finding the molecular nature of such a signal would give more insight as to the regulation of cuticle development at the very beginning of a plant's life. The acyl transferase *CER2* is the second gene

chosen for further investigation, due to the fact that it is localized in the nucleus, which is surprising for a protein with such a function. *PPT* is a third gene, which might provide valuable insight into the mechanisms that a plant commonly uses to compensate for a deficient cuticle formation, due to its up-regulation in three independent cuticular mutants. To investigate the molecular function of *ALE1*, *CER2* and *PPT*, the three genes were used as baits in three yeast two-hybrid cDNA library screenings.

RESULTS

CHARACTERIZATION OF *CER13* AND MOLECULAR IDENTIFICATION OF THE GENE

The *cer13* mutant has a pleiotropic phenotype

Obtained in Landsberg *erecta* (L*er*-0) background by fast-neutron treatment, the *cer13* mutant has a glossy appearance on stems and siliques (Koornneef et al., 1989). Together with *cer10* and *cer3*, *cer13* was also reported to exhibit organ fusions (Jenks et al., 2002), an indicator of substantial disturbance in cutin biosynthesis, transport or assembly (Lolle et al., 1992; Lolle et al., 1998; Sieber et al., 2000; Nawrath, 2006). Although the organ fusion phenotype was not observed throughout this project, it is known that the strength of the allele, as well as variations in growth conditions, especially humidity, can prevent the adhesion of organs (Lolle et al., 1997). The *cer13* mutant has a semi-dwarf phenotype compared to the wild-type, indicating a possible role in signaling mechanisms involved in the regulation of cuticle development. Since our group is interested in regulatory aspects of cuticle development, the *cer13* mutant was selected for further characterization and positional cloning. The chemical composition of leaf and stem cuticular wax in *cer13* was analysed by Rashote et al. (2001) and was reported to be decreased, as compared to the wild type. Chemical composition analysis of cutin, as well as seed coat polymeric lipids were previously performed in our group by Dr. Andrea Faust and the seed coat phenotype was observed by scanning electron microscopy. While several cutin components were reported to be reduced in the *cer13* mutant as compared to L*er*-0, the seed coat analysis did not indicate any difference in the lipid polyester composition and the size of *cer13* seeds was reported to be smaller, with a modified seed surface (Faust, 2006).

During the map based cloning performed in this project, it was notable that plants from the F2 generation that were homozygous for the *cer13* locus were easier to

screen when grown only in long day conditions. Therefore, to further characterize *cer13*, the mutant was simultaneously grown with the wild-type (WT) in short day (SD, 8 hours light) and long day (LD, 16 hours light) conditions, with the purpose of observing whether there is any change in the wax crystal morphology. At fructification stage, *cer13* plants are shorter than wild-type plants, when grown in both SD and LD conditions (figure 2, graph 1). Due to the presence of wax crystals, WT L*er*-0 shoots and siliques have a light, opaque appearance, as compared to those of *cer13*, which have a more intense green color and a glossy surface.

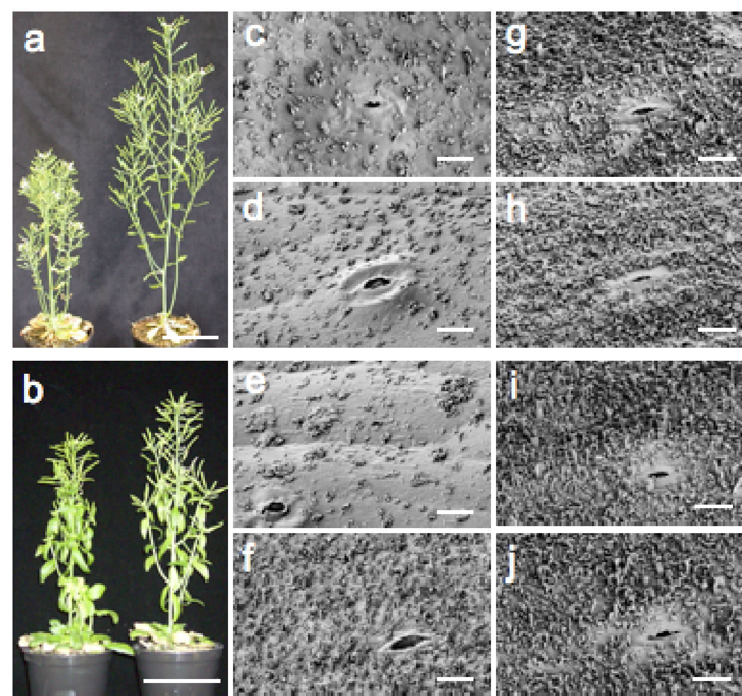
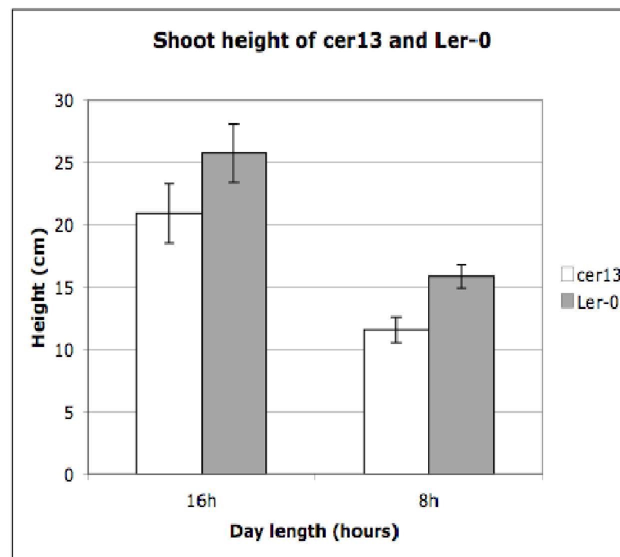


Figure 2. General morphology, epicuticular wax phenotype and shoot height of *cer13* as compared to L*er*-0, in long day (18h) and short day (8h) conditions. a, b – general morphology of *cer13* (left) and L*er*-0 (right); a, plants grown in SD for two weeks and subsequently transferred to LD (adapted, from Faust, 2006); c, d, g, h – LD; b, e, f, i, j – SD; c, d, e, f – *cer13*; g, h, i, j – L*er*-0; c, g, e, i – epicuticular wax on shoots; d, h, f, j – epicuticular wax on siliques; Scale bars: a, b: 5 cm; c – j: 10 μm.

Electron micrographs show less wax crystals, predominantly formed as plates on the stems and siliques of *cer13*, as compared to the L*er*-0 wild type (WT), which exhibits homogeneously spread and very numerous wax crystals formed as blocks, as well as plates. Block crystals on stems and siliques of *cer13* are very few and they cluster, instead of being homogeneously distributed on the organ surface (figure 2, c, d, e). When grown in short day conditions, as compared to long day conditions

(figure 2, f), there are more wax crystals on shoots but not on siliques of *cer13*, although they are still less numerous than on the shoots of L*er*-0 WT.

Although the height of *cer13* mutant plants can vary, the extent of the variation is yet unclear. Plants grown initially in SD for two weeks and subsequently transferred to LD exhibit a higher difference in height as compared to the wild type (figure 2, a). However, plants grown simultaneously in the late winter season did not show a higher variation in LD conditions as compared to SD conditions (Graph 1).



Graph 1. Shoot height of *cer13* versus L*er*-0, in 16h and respectively 8h light. The measurements were made on 30 plants for each case. Error bars represent standard deviation.

Such phenotypes indicate that the CER13 protein might have a regulatory function in wax metabolism.

The *CER13* gene maps to a region of 46 genes on chromosome III

The *cer13* mutant, obtained by fast neutrons in Landsberg *erecta* (L*er*-0) background, was previously mapped on chromosome III, at 47,3cM on the Lister and Dean RI map, relatively to the SSLP marker AthGAPab (Rashotte et al., 2004). For the map based cloning approach, an F2 generation of *cer13* crossed to Colombia (Col-0) was obtained as a mapping population. Rough mapping lead to a genetic region of 3,1 Mbp that was thus established between two single nucleotide polymorphism (SNP) markers located on BAC clones F20C19 (left border) and

respectively F21A17 (right border) (Faust, 2006). The fine mapping carried out in this project revealed an area of 140 kb in chromosome III, containing 46 genes, on BAC clones MGF10 and K16N12 (figure 3).

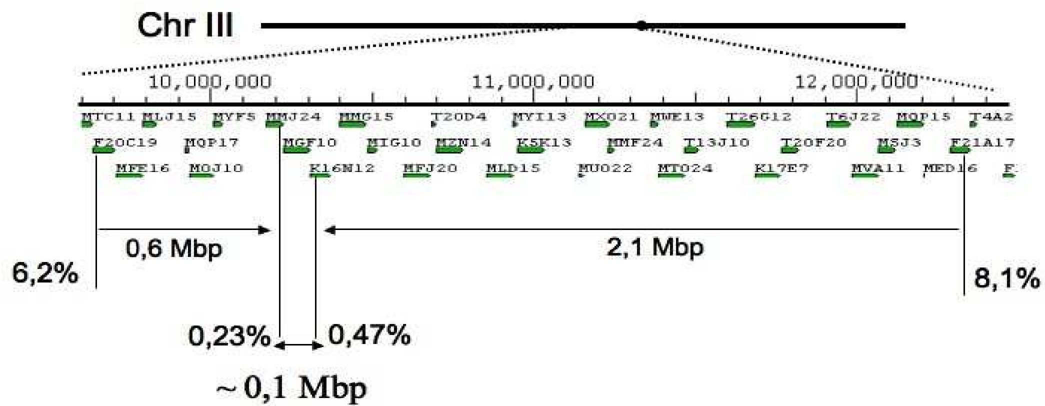


Figure 3. Fine mapping of *cer13*. Chr III, chromosome III; Green block arrows, BACs; Mbp, mega base-pairs; recombination is given in percentage, at the respective location.

A candidate gene approach led to the selection of At3G27670 as *CER13* candidate, due to the fact that its mutation was previously described to cause decreased epicuticular wax load as well as altered morphology and viability of seeds. The selected candidate was previously characterized as *RESURRECTION1 (RST1*) (figure 4). The gene is unique in the *Arabidopsis* genome, has an annotated size of 8160 nucleotides (nt) in the genomic DNA and encodes a predicted protein of 1841 amino acids (aa) (Chen et al., 2005).

Due to its large size, we decided to further amplify the selected candidate gene in two fragments, as described in methods. The PCR amplification of the first fragment resulted in a product of the expected size when using both WT and *cer13* genomic DNA as template. However, in the case of the second fragment, the size was the expected 4918 nt when using L*er*-0 WT DNA as template, whereas the fragment amplified from *cer13* genomic DNA template was shorter (figure 4, c).

Sequencing of the first fragment revealed identity between the L*er*-0 and *cer13* genomic DNA, whereas the second PCR fragment revealed a deletion of 950 nucleotides in the region between nucleotides 7770 – 8720 of the gene, corresponding to part of the 18th exon, the entire 18th intron and 19th exon, as well as part of the 19th intron (Figure 4, b).

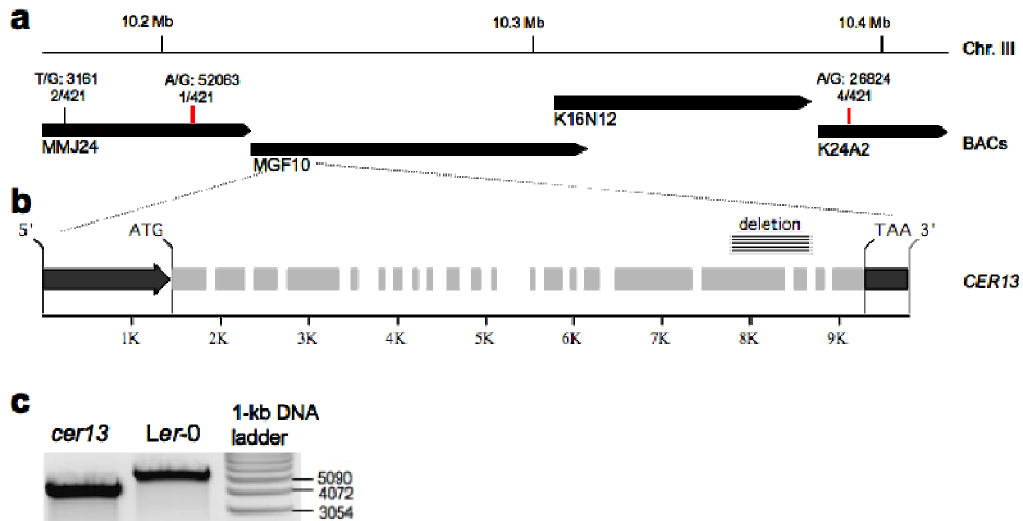


Figure 4. Identification of *CER13*. a, Genetic map of chromosome three region with distances in Mb above. BAC clones are marked with black, block arrows. The fraction of recombinant plants detected in the mapping population is indicated above the back clone, as well as the SNP polymorphism Col-0/L*er*-0, with its position on the BAC. b, Schematic representation of *CER13* gene. Black arrow: 5'UTR; grey boxes: exons; spaces: introns; black box: 3'UTR; deletion is marked with lined box; size of the gene is marked in kb (K). c, PCR amplification of candidate gene using *cer13* and as compared to L*er*-0 WT DNA. 1kb DNA ladder is used as marker.

The *cer13* locus is allelic to *rst1*

To have a biological confirmation of the identity of *CER13*, an allelism test was perfomed by crossing *cer13* to *rst1*, using *cer13* as female parent, fertilized with *rst1-3* pollen. Considering that *cer13* is a mutant obtained in L*er*-0 background, and since the *erecta* phenotype is recessive, an F1 generation of a successful cross is expected to have the general morphology of Col-0 ecotype for the *ERECTA* phenotype and to lack wax if the two mutants are allelic. Indeed, the F1 generation of *cer13* x *rst1-3* had the the *ERECTA* phenotype, and the shoots and siliques had the glossy appearance typical to the *cer13* and the *rst1-3* mutants. Figure 5, c shows less wax crystals on stems of an F1 generation plant, proving that indeed *cer13* is allelic to *rst1*.

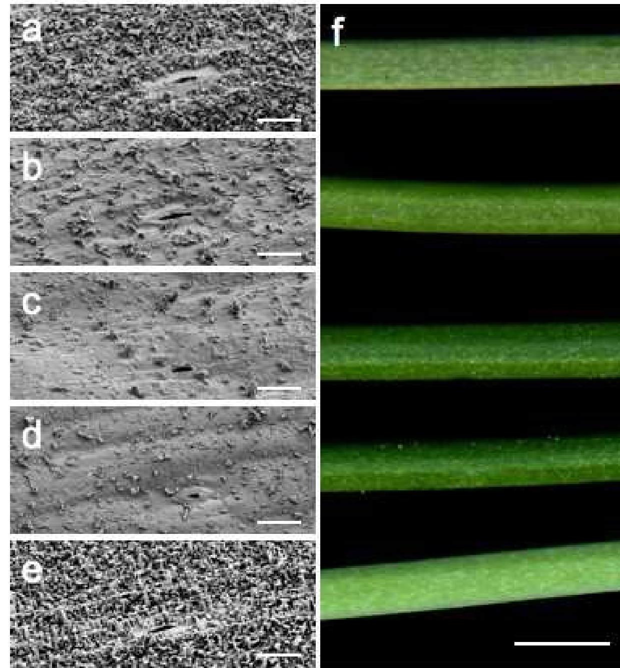


Figure 5. Epicuticular wax phenotype on stems of lines used for the allelism test. a – e, stem fragments analysed by SEM; f, shoot fragments observed under the binocular; a, WT Ler-0; b, *cer13*; c, F1: *cer13* x *rst1-3*; d, *rst1-3*; e, WT Col-0; f , from up to down: WT L*er*-0, *cer13*, F1: *cer13* x *rst1-3*, *rst1-3* and WT Col-0.

The deletion in *cer13* results in a truncated version of the protein

The predicted amino acid sequence encoded by the allele carrying the 950 bp deletion diverges from the RST1 sequence after the amino acid 1420 and terminates prematurely at amino acid 1425 (figure 6). Thus, in the mutant, the 950 bp deletion causes a frame shift and a premature STOP codon, leading to a truncated version of *CER13*. The resulting protein is 421 amino acids shorter than the wild-type protein.

```
                   10        20        30        40        50        60        70        80        90       100       110       120
CER13_MT   MASYATLLEKTRVPQPSIQRFAVISVFSKLRSAPEQFGSEAEAGREAISFCLTSESITVVDQSVHELCRLVSDSVLDLSRGLLELQSALEGCDSKLVSLFVKGLGFLIRIGYERNNGNWK
CER13_WT   MASYATLLEKTRVPQPSIQRFAVISVFSKLRSAPEQFGSEAEAGREAISFCLTSESITVVDQSVHELCRLVSDSVLDLSRGLLELQSALEGCDSKLVSLFVKGLGFLIRIGYERNNGNWK
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                  130       140       150       160       170       180       190       200       210       220       230       240
CER13_MT   FNSTENHPFVRIQSSRVETQTELLHQVSLFVMHNRRLGMVGVCEFLEPFLNFTILRIPLSDSSSSLFARELISSMASLCCSSRHEALPIFRLLMRCLKYIPGNNLEVIVKILVDAYTVVV
CER13_WT   FNSTENHPFVRIQSSRVETQTELLHQVSLFVMHNRRLGMVGVCEFLEPFLNFTILRIPLSDSSSSLFARELISSMASLCCSSRHEALPIFRLLMRCLKYIPGNNLEVIVKILVDAYTVVV
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                  250       260       270       280       290       300       310       320       330       340       350       360
CER13_MT   RDLVGTGLEVTEVHLLGVQLVDGVLFLCASPHVQTTEQESVIESLKHLLAVQKDLGLAYSHDLSLVVLSLVFMLAKSTVEHEQLCILKFLLFLLKWKTESENLSVKDAAGSSVESLLLFP
CER13_WT   RDLVGTGLEVTEVHLLGVQLVDGVLFLCASPHVQTTEQESVIESLKHLLAVQKDLGLAYSHDLSLVVLSLVFMLAKSTVEHEQLCILKFLLFLLKWKTESENLSVKDAAGSSVESLLLFP
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                  370       380       390       400       410       420       430       440       450       460       470       480
CER13_MT   ITALMSSPSKSIKVAASKVLSIVENFLVTVSNAPKIEVHTSKGDSPLSRVGSVVFRFMQQLWHQNDYTPSTSSFLRVAYTNGSEKQETYLGPVTWNSLLREHAERFWDKKKLSASFCLSQ
CER13_WT   ITALMSSPSKSIKVAASKVLSIVENFLVTVSNAPKIEVHTSKGDSPLSRVGSVVFRFMQQLWHQNDYTPSTSSFLRVAYTNGSEKQETYLGPVTWNSLLREHAERFWDKKKLSASFCLSQ
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                  490       500       510       520       530       540       550       560       570       580       590       600
CER13_MT   EIPILLGAVAGVMVMHPSLGADAIGSLTIIGGIDSKMSVPLLLAVLYFSNLLSRTNVPCQSLLSKLLGLLPSLAAQQVMIPLVVQTITPMLRKDAKGLLYATAIRLLCQTWVVNDRAFSS
CER13_WT   EIPILLGAVAGVMVMHPSLGADAIGSLTIIGGIDSKMSVPLLLAVLYFSNLLSRTNVPCQSLLSKLLGLLPSLAAQQVMIPLVVQTITPMLRKDAKGLLYATAIRLLCQTWVVNDRAFSS
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                  610       620       630       640       650       660       670       680       690       700       710       720
CER13_MT   LQEVLRPQGFIEYISERHICISMAASIHDVCKRHPDRGVDLILSVQACIESQNCPVRALGFQSLSHLCEADVIDFYTAWDVIKKHAQHIKLDPLLAYSVCHLLKWGAMDAEAYPEDAENV
CER13_WT   LQEVLRPQGFIEYISERHICISMAASIHDVCKRHPDRGVDLILSVQACIESQNCPVRALGFQSLSHLCEADVIDFYTAWDVIKKHAQHIKLDPLLAYSVCHLLKWGAMDAEAYPEDAENV
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                  730       740       750       760       770       780       790       800       810       820       830       840
CER13_MT   LNILWEIGSSMQKPHDSQWTKARVSAIVALGQYEVSFMENKFSDFNKNCTYLLFSETNAEILNALEDLSIKIMIHEHSVRRRYVREKKVPGSKIEKLLDVIPQVIFPAGKEIKTGELPGA
CER13_WT   LNILWEIGSSMQKPHDSQWTKARVSAIVALGQYEVSFMENKFSDFNKNCTYLLFSETNAEILNALEDLSIKIMIHEHSVRRRYVREKKVPGSKIEKLLDVIPQVIFPAGKEIKTGELPGA
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                  850       860       870       880       890       900       910       920       930       940       950       960
CER13_MT   ALLCLSYNPRDVKFGSSRSFHDVHFQYEEAFRVVVKSLQLSRNISLALISLQSLKAFMRRWMRANILSIDATTKELSSDKTSKATNNIMKSLVHMAEEALPRCAENIALALGALCAALPA
CER13_WT   ALLCLSYNPRDVKFGSSRSFHDVHFQYEEAFRVVVKSLQLSRNISLALISLQSLKAFMRRWMRANILSIDATTKELSSDKTSKATNNIMKSLVHMAEEALPRCAENIALALGALCAALPA
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                  970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
CER13_MT   ASHNIKASASKFLLSWLLEHEHEHRQWTAGISLGLISSSLHVTDHKQKFQNISGLLEVLCSSKSTLVKGACGVGLGFSCQDLLTRTEASASSDIDSDSYRNQEERLLGRIVRLLSSILHG
CER13_WT   ASHNIKASASKFLLSWLLEHEHEHRQWTAGISLGLISSSLHVTDHKQKFQNISGLLEVLCSSKSTLVKGACGVGLGFSCQDLLTRTEASASSDIDSDSYRNQEERLLGRIVRLLSSILHG
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                 1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
CER13_MT   FLHTPCDILESLSALFPPGEEDNVIGLPQLLDESSDDFDDDTWGIAGLIIGLGMSVGAIYRAGKKDAVVKIKNLIVSWIPYADSLKQTSGSNSKVSVRLFSVGSCLALPIVITFCQKVEL
CER13_WT   FLHTPCDILESLSALFPPGEEDNVIGLPQLLDESSDDFDDDTWGIAGLIIGLGMSVGAIYRAGKKDAVVKIKNLIVSWIPYADSLKQTSGSNSKVSVRLFSVGSCLALPIVITFCQKVEL
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                 1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
CER13_MT   FDAHEVDDIIGCFKDLISELLIVRKSGALRKRLLMASCIGAGDLLGSVLNEGIHPVKIESVKELLELFKKCYSGLYPPVAHFGGMLGVVNVLGAGAGNLVYSHPRPRAPPASSEENEISY
CER13_WT   FDAHEVDDIIGCFKDLISELLIVRKSGALRKRLLMASCIGAGDLLGSVLNEGIHPVKIESVKELLELFKKCYSGLYPPVAHFGGMLGVVNVLGAGAGNLVYSHPRPRAPPASSEENEISY
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                 1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440
CER13_MT   VSGPLLSNAYFTQQLTPVVQEIFLIAQNTKDRQLQHYAAWAISILRTYMRSGESSSLSNENQSDDSDRNSISHNVPEHTMVMKLAQGLTNPSFPLAGSPLISGTC*
CER13_WT   VSGPLLSNAYFTQQLTPVVQEIFLIAQNTKDRQLQHYAAWAISILRTYMRSGESSSLSNENQSDDSDRNSISHNVPEHTMVMKLAQGLTNPSFPLAGSPLNIGTMASALRCLSHAPRLPN
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                 1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560
CER13_MT
CER13_WT   LDWGATIRRLMKQETQTDVTQSGDVPKEITLREECFKFSLAHASEFDELLAFLDELSELSRFKALEESLQSCLLCHLGGLMRIFSGSRMNKLFDDVSCFVVSLSSDQTYSCDQKSSLRVS

                 1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680
CER13_MT
CER13_WT   CWKGLSQCLEETSLESSEYVTKIEKCIELLFAVLPVASQSPRADQMGSVKEWSEAVTCLQKSHRDWLYKFLQVSNLEPGNEKTNFQGDLKKIQAKAKLAKLGSVPFSELGKLKAIILNCE

                 1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
CER13_MT
CER13_WT   ESDIWDVLIEIVAALHHAEGGIKRQWLIDAVEISCVSSHPSTAIIFVGLLSSICCEYMPFLNLDRSTVLSDMSVTVTSLLSDPSYEVVTEPFISFLWTSLERVYSFATESDANARLSSQQ

                 1810      1820      1830      1840
CER13_MT
CER13_WT   IAQSERDKAPMLVKVMHYICVAFRDHLPLEKQLRLASMDMS*
```

Figure 6. ClustalW alignment of CER13 amino-acid sequence in WT and mutant (MT). Identical amino-acids are underlined. The STOP codon is indicated by “*”.

CER13/RST has nine predicted trans-membrane domains

The ARAMEMNON database enables direct comparison of the predictions of seven different TM span computation programs and the predictions of subcellular localization by eight signal peptide recognition programs. The proteins related to the query are displayed and dynamically generated as a protein family structure (Schwacke et al., 2003). This on-line tool (http://aramemnon.botanik.uni-koeln.de.) was used in the present study with the CER13/RST1 rice homolog OS01G07490. The outcome of the analysis indicates that CER13/RST1 could have up to 9 trans-membrane domains when aligned with its rice homolog (figure 7). This indicates that the protein may function as a receptor or transporter in the regulation of wax biosynthesis.

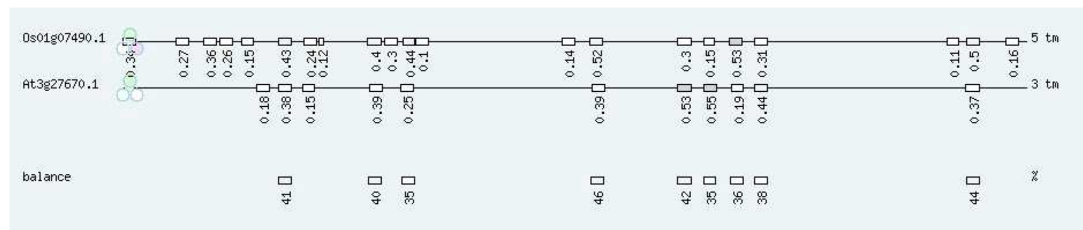

Figure 7. Transmembrane span alignment using the ARAMEMNON database. First line: sequence of the CER13/RST1 rice homolog OS01G07490. Second line: sequence of CER13/RST1. Bottom line: trans-membrane domains predicted for CER13/RST1

There is no fluorescence signal when DsRED is fused to the C-terminus part of the full length CER13

The RST1 protein was reported to have a predicted localization in the mitochondria (Chen et al., 2005). To verify this experimentally, a full-length genomic clone was obtained as described in methods. As in the construct used, the expression of the *CER13* gene is driven by the native promoter, a successful transgene would also indicate the localization of the protein in specific plant tissues. No signal was found for the fluorochrome fused to the C-terminal part of the CER13 protein after checking various organs of several lines, at different developmental stages of *Arabidopsis* transformants by confocal laser scanning microscopy (CLSM). Reasons for this result might include the large size of the protein, as well as the fact that it is predicted to have several trans-membrane domains (Chen et al., 2005). Constructs of

truncated versions of the gene expressed under the native promoter or under the 35S promoter might still elucidate the sub-cellular localization of CER13 fused to a fluorochrome.

CER13 is expressed in ovules and in the epidermis of young organs close to the apical meristem

According to the *Arabidopsis* eFP browser (http://bbc.botany.utoronto.ca/efp) (Winter et al., 2007), *CER13* is ubiquitously expressed; however, previously perfomed RT-PCR indicated higher transcripts in leaves and flowers (Chen et al., 2005). To locate the expression of *CER13* in plant tissues, we prepared two probes for in situ hybridization. The in situ experiment including the hybridization procedure and the immunohistochemical detection was conducted by Dr. Nadia Efremova as described in methods. In this experiment, transverse sections through flowers and longitudinal sections through the shoot apex have shown a positive signal. In the case of transverse sections through flowers (figure 8, a), a signal was detected in the tapetum cells of the anthers as well as in ovules. A signal was also detected in epidermal cells of developing leaves, in the proximity of the apical meristem (figure 8, b).

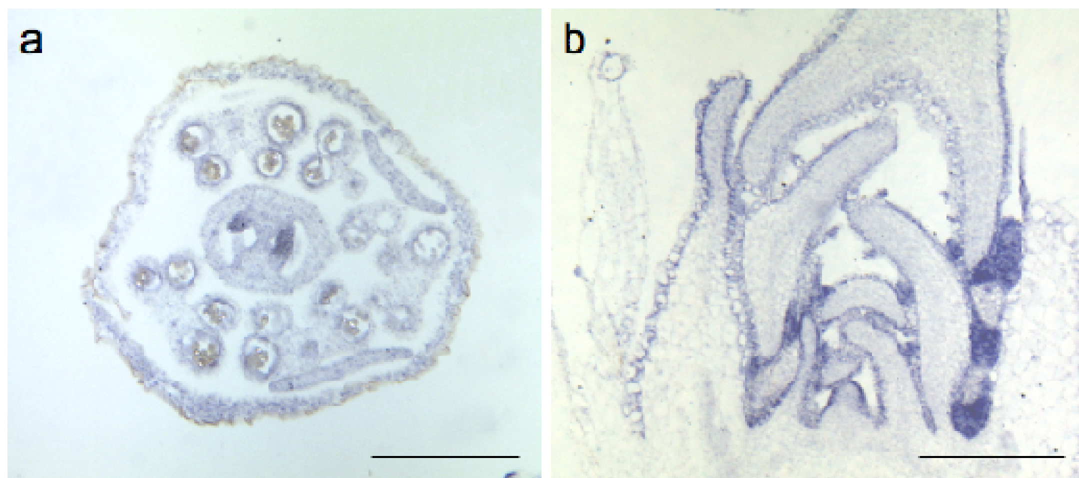

Figure 8. In situ hybridization. a, transverse section through flower; b, longitudinal section through shoot apex. Scale bars: 200µm

Genes involved in various metabolic pathways are miss-regulated in *cer13*

The CER13 protein shows no sequence similarity to any known protein that would suggest a putative function. To gain insight into the metabolic or regulatory pathway involving *CER13*, or into the mechanisms that could regulate its function, a transcriptome analysis was conducted using the *Arabidopsis* ATH1 gene-chip (Affymetrix). The resulting data was statistically analyzed as described in methods. Miss-regulated genes with a pfp value of less than 0.003 and a fold-change of at least 1.9 were further investigated. The total number of genes meeting both criteria was eighty-one, out of which thirteen were down-regulated and sixty-eight were up-regulated. Out of sixty-eight up-regulated genes, eleven encode proteins of unknown function, which were excluded from further investigation. The remaining fifty-seven up-regulated genes as well as all thirteen down-regulated genes were analysed using various tools from the Genevestigator V3 program (Zimmermann et al., 2004) (www.genevestigator.com). A clustering analysis in the "development" profile of the Genevestigator V3 program is shown in figure 9, as a heat map, indicating the predicted expression of each gene in the respective developmental stage.

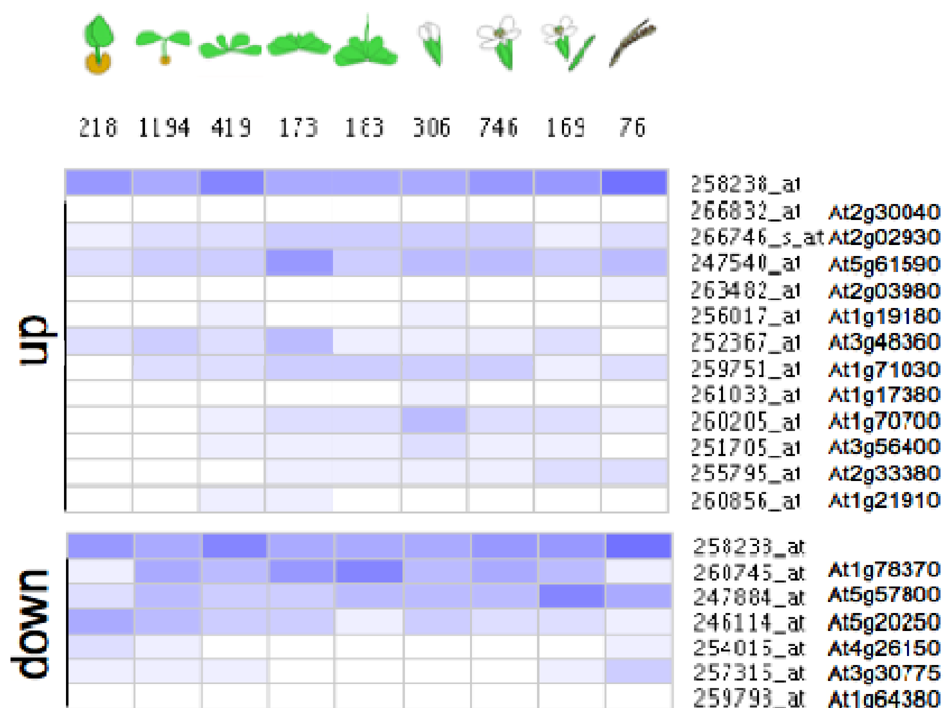

Figure 9. Clustering analysis in heat map format of up-regulated and down-regulated genes in *cer13* transcriptome, using the Genevestigator V3 program. First row images represent the following developmental stages: germinated seed, seedling, young rosette, developed rosette, boulting, young flower, developed flower, flowers and siliques and mature siliques. Numbers below developmental

stage images represent the number of arrays scored. The first row in each heatmap represents the expression of probe 258238_at, corresponding to *CER13*. The other probes have the corresponding AT number in the column to the right and correspond to up- and down-regulated genes as indicated in Table1.

Several of the top up- and down-regulated genes are presented in table 1. The *MAPKKK14* gene (AT2G30040) is up-regulated in several stress response mechanisms like light (Khanna et al., 2006) and cold (Lee et al., 2005), as well as hormone treatment with indole-3-acetic acid (IAA) and brassinolide (BL) (Goda et al., 2004). qRT-PCR was conducted as described in methods for the confirmation of the microarray experiment and it indicated a 3,85 fold up-regulation of *MAPKKK14*. The *JAZ* genes are widely expressed throughout plant development and are implicated in jasmonic acid signaling. The JAZ proteins were reported to function as repressors of jasmonate signaling and to be degraded through the SCF^{COI1}-dependent 26S proteasome pathway (Chini et al., 2007). At least ten of the *JAZ* genes are rapidly induced by jasmonate treatment (Staswick, 2008) and three of them (*JAZ1*, *JAZ5* and *JAZ9*) are up-regulated in the *cer13* transcriptome, compared to wild-type. Several transcription factors are miss-regulated in *cer13* and three of them contain an AP2 domain. Although all three accessions presented in table 1 (At5g61590, At1g21910 and At1g64380) encode members of various subfamilies of the ERF (ethylene response factor), overexpression of AP2 domain-containing transcription factors was reported to activate wax biosynthesis, to alter cuticle properties, and to confer drought tolerance in *Arabidopsis* (Aharoni et al., 2004). However, the transcription factors miss-regulated in *cer13* are involved mainly in pathogen - response mechanisms.

Table 1. Top miss-regulated genes in *cer13* transcriptome, as compared to WT L*er*-0. FC1- fold change according to microarray analysis; FC2 – fold change according to qRT-PCR.

	FC1	Gene	Accession	FC2
up-regulated	18.9	MAPKKK14__MAPKKK14 (Mitogen-activated protein kinase kinase kinase 14); kinase	At2G30040	3.85
	5.3	ATGSTF3_GST16__ATGSTF3 (GLUTATHIONE S-TRANSFERASE 16); glutathione transferase	At2G02930	-
	5.3	AP2 domain-containing transcription factor family protein	At5g61590	-
	3.7	GDSL-motif lipase/hydrolase family protein	At2g03980	-
	3.2	JAZ1_TIFY10A__JAZ1/TIFY10A (JASMONATE-ZIM-DOMAIN PROTEIN 1)	At1g19180	2.64
	3.1	BT2__BT2 (BTB AND TAZ DOMAIN PROTEIN 2); protein binding / transcription factor/ transcription regulator	At3g48360	-
	2.6	ATMYBL2__ATMYBL2 (Arabidopsis myb-like 2); DNA binding / transcription factor	At1g71030	-
	2.5	JAZ5_TIFY11A__JAZ5/TIFY11A (JASMONATE-ZIM-DOMAIN PROTEIN 5)	At1g17380	1.90
	2.3	JAZ9_TIFY7__JAZ9/TIFY7 (JASMONATE-ZIM-DOMAIN PROTEIN 9)	At1g70700	-
	2.2	WRKY70__WRKY70 (WRKY DNA-binding protein 70); transcription factor	At3g56400	-
	2.1	RD20__RD20 (RESPONSIVE TO DESSICATION 20); calcium ion binding	At2g33380	-
	2.1	AP2 domain-containing transcription factor family protein	At1g21910	-
down-regulated	11.5	ATGSTU20__ATGSTU20 (Arabidopsis thaliana Glutathione S-transferase (class tau) 20); glutathione transferase	At1g78370	-
	3.0	CER3_FLP1_WAX2_YRE__CER3/FLP1/WAX2/YRE (ECERIFERUM 3); catalytic	At5g57800	2.74
	2.2	DIN10__DIN10 (DARK INDUCIBLE 10); hydrolase, hydrolyzing O-glycosyl compounds	At5g20250	1.61
	2.2	CGA1__CGA1 (CYTOKININ-RESPONSIVE GATA FACTOR 1); transcription factor	At4g26150	-
	2.0	PRODH_ERD5_ATPDH_ATPOX_AT-POX_PRO1__ERD5 (EARLY RESPONSIVE TO DEHYDRATION 5); proline dehydrogenase	At3g30775	-
	1.9	AP2 domain-containing transcription factor, putative	At1g64380	-

CER3/WAX2/YRE/FLP1* is a wax gene three-fold down-regulated in *cer13

The *ECERIFERUM3* (*CER3/WAX2/YRE/FLP1)* gene was reported to be required for wax biosynthesis and the CER3 protein was proposed to be implicated in cutin production, due to the fact that strong *cer3* alleles display organ fusions. However, leaf cutin analysis of two *cer3* alleles did not reveal significant differences in cutin load or composition, indicating that CER3 has no major role in leaf cutin formation.

(Rowland et al., 2007). The *CER3* gene expression is down-regulated in the *cer7* mutant, the latter corresponding to a putative 3'-5' exoribonuclease and a core subunit of the RNA processing and degrading exosome (Hooker et al., 2007). In the *cer13* transcriptome, *CER3* is the only gene known to be involved in wax biosynthesis and it is three fold down-regulated. This result suggests that the CER13 protein indirectly regulates wax biosynthesis via CER3.

In silico meta-analysis of microarrays reveals overlap between the *cer13* transcriptome and other stress-response pathways.

To find out whether the expression pattern of the *cer13* transcriptome has any similarity to other mutants or treatments, we performed an in silico analysis using MASTA (MicroArray overlap Search Tool and Analysis), as described in methods. This analysis revealed overlaps between the microarray experiment involving the contrast *cer13* compared to wild type and microarray experiments involving several types of stress-related contrasts. There are numerous coupling-phase (up-regulated genes overlapping with up-regulated genes; down-regulated genes overlapping with down-regulated genes) and repulsion-phase overlaps (up-regulated genes overlapping with down-regulated genes) with the up-regulated genes in the *cer13* transcriptome and only few overlaps with the down-regulated genes. The most prominent overlaps occurred with factors such as cold/heat stress, drought stress, wounding and salicylic acid (SA)-related mutants and treatments.

<u>Overlaps with cold and heat stress</u>

The *sfr* (*sensitive to freezing*) mutants of *Arabidopsis* are impaired in freezing tolerance after cold acclimation (McKown et al., 1996; Byeong et al., 2002). Up-regulated genes in the *cer13* transcriptome showed repulsion phase overlaps with genes in cold-treated *sfr* mutants as well as in heat-stressed wild-type plants, but coupling-phase overlaps with genes induced in cold-treated wild-type plants.

<u>Overlaps with drought stress</u>

Up-regulated genes in *cer13* showed coupling-phase overlaps with genes induced in eight drought-stress contrasts. Only with two contrasts there were five overlapping

genes, whereas with the remaining six contrasts the number of overlapping genes was between nine and fifteen.

Overlaps with wounding

Genes up-regulated in the *cer13* transcriptome overlapped with up-regulated genes in three wounding contrasts (coupling-phase overlaps). The numbers of overlapping genes were eight (contrast one hour after wounding versus non-wounded plants), seven (three hours after wounding versus non-wounded) and nine (six hours after wounding versus non-wounded). Up-regulated genes in *cer13* showed repulsion-phase overlaps with wounding contrasts of fifteen (nine overlapping genes) and thirty minutes (thirteen overlapping genes) after wounding compared to non-wounded plants.

Overlaps with pathogen-related pathways

Systemic acquired resistance (SAR) is a plant defense response induced by pathogen attack. The accumulation of salicylic acid (SA) in *Arabidopsis* precedes the onset of SAR. The signaling molecule SA induces nuclear translocation of the transcription cofactor NPR1 (NONEXPRESSER OF PR GENES) to activate many genes required for disease resistance (Kinkema et al., 2000). WRKY transcription factors have also been implicated in regulating the response against pathogen infection. Many WRKY genes are rapidly induced after treatment with elicitors associated with infection (Chen and Chen, 2000; Yoda et al., 2002; Dong et al., 2003). WRKY transcription factors are also known to regulate the expression of NPR1 (Yu et al., 2001).

Benzothiadiazole (BTH) is a synthetic functional analog of SA and it induces SAR (Gorlach et al., 1996). The *npr1* mutants cannot express pathogenesis-related (PR) genes and are unable to develop SAR in response to SA treatment. Thus, they have an enhanced susceptibility to pathogens.

Up-regulated genes in *cer13* showed coupling-phase overlaps with genes induced in four contrasts involving BTH treatment on *wrky18*, in three contrasts with BTH treatment on *npr1* and in two contrasts involving SA. Figure 10 shows a fragment of a MASTA analysis graphical output, containing the coupling-phase overlaps

between the contrast *cer13* versus WT and the contrasts involving *npr1* (C30, C28 and C26).

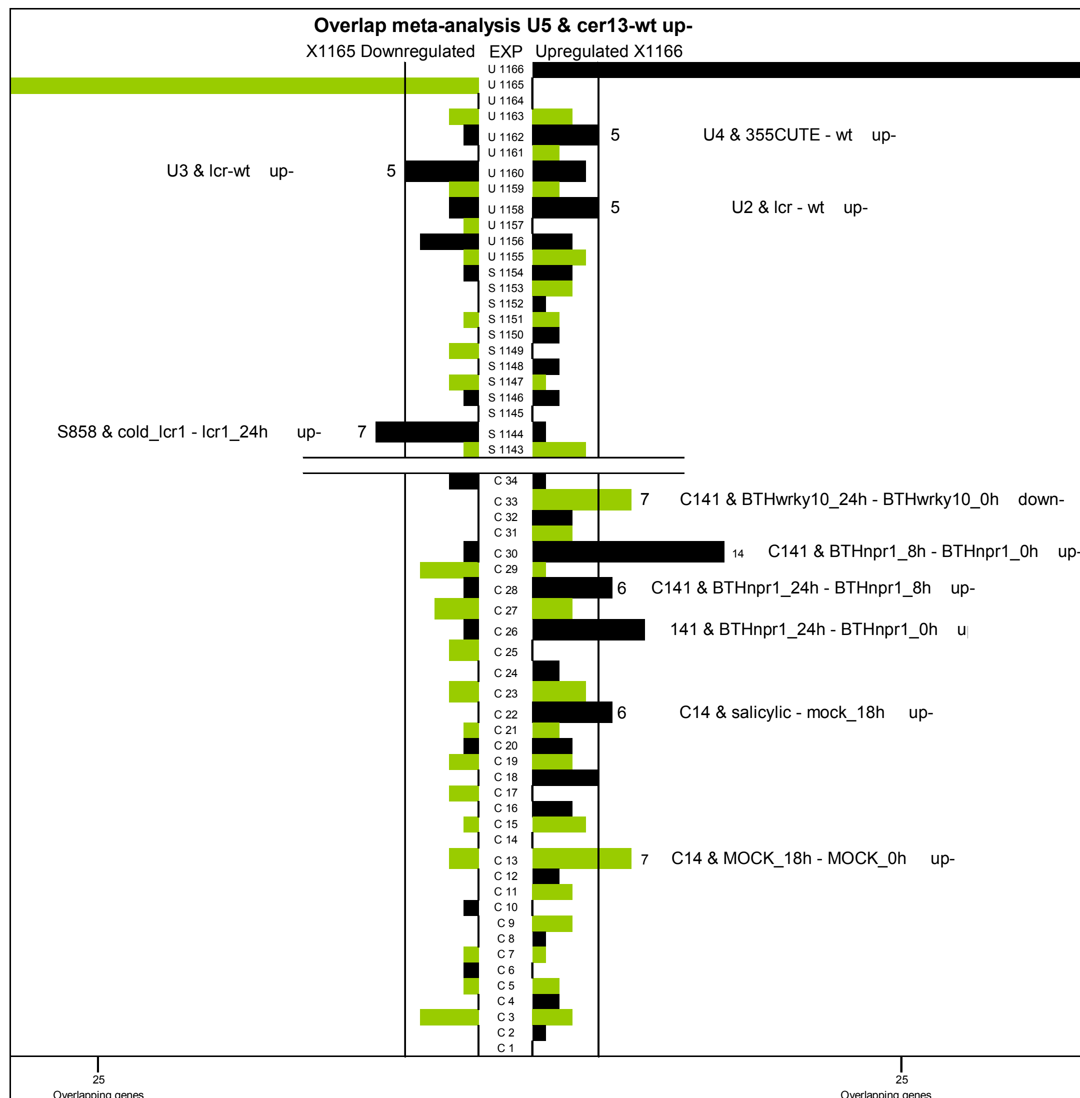

Figure 10. Fragment from a graphical output of MASTA analysis, with the contrast "*cer13* vs WT" as querry (Overlap meta-analysis U5 & cer – wt). EXP, comparison contrast from MASTA database. Bars represent the number of overlapping differentially expressed genes (DEGs). Bars on the left side of the graph: number of DEGs overlapping with down-regulated genes in querry contrast. Bars on the right side of the graph: number of DEGs overlapping with up-regulated genes in querry contrast. Black bars: up-regulated genes in the comparison contrast; pink bars: down-regulated genes in the comparison contrast.

EMS mutagenesis revealed one dominant supressor of the *cer13* phenotype

Since CER13 might be a regulator of wax biosynthesis, targeting the expression of CER3, it became interesting to search for a CER13 suppressors. Potential

suppressors of the *cer13* phenotype would bring new insight into the biological function of the CER13 protein. To find possible suppressors, an EMS mutagenesis was performed as described in methods. From five thousand M1 seeds, approximately 80% survived, out of which several plants exhibited morphological abnormalities. One plant exhibited a suppressed *cer13* wax phenotype (figure 11) and PCR genotyping revealed the presence of the expected T-DNA insertion. Segregation analysis in the M2 generation will be the biological confirmation of whether this is indeed a dominant suppressor of *cer13*. Pollen of this putative dominant suppressor was used to backcross it to *rst1-3* in order to obtain a mutant that can be further characterized and mapped.
The M2 generation resulting from the M1 EMS-mutagenized population will be screened further for recessive suppressors of the *cer13* phenotype.

Figure 11. Putative suppressor of *rst1-3*. From left to right: *rst1-3*, putative suppressor, WT Col-0. Scale bar, 2mm.

PROTEIN INTERACTIONS INVOLVED IN CUTICLE DEVELOPMENT

ALE1 (ABNORMAL LEAF SHAPE 1) is a putative interactor of CSN5

Epidermal differentiation and implicitly cuticle formation is essential for the general development of the whole plant, starting from the very early embryo stage. This fact is supported by the characterization of the *abnormal leaf shape 1* (*ale1*) mutant of *Arabidopsis*, which shows impaired cuticle formation, adhesion of endosperm and embryo, as well as fusion of cotyledons and leaves. The corresponding *ALE1* gene encodes a member of the subtilisin-like serine protease family and it is expressed during seed development, showing a weak transcript expression in young embryo and a strong one within the endosperm cells closely surrounding the developing embryo (Tanaka et al., 2001). Three aminoacid residues (D, H and S) are consistently conserved in the catalytic regions of subtilisin-like serin proteases. In animals, such proteases activate precursors of hormones, growth factors, or receptors involved in the control of various developmental processes, including embryonic patterning and proper epidermal differentiation. Although many members of this family of proteases were reported in plants (Siezen, 1997; Schaller, 2004), little is known, with few exceptions, about their precise role. As it was implied that *ALE1* is an extracellular protein involved in causing a signal from the endosperm that is required for the formation of epidermis and cuticle around the embryo, finding the molecular nature of such a signal would give more insight as to the regulation of cuticle development at the very first stage of a plant’s ontogenesis. Thus, ALE1 was selected for a Y2H cDNA library screening, in order to find putative interactors.

The cDNA library Y2H screening, performed with ALE1 H252A as bait revealed a total of eight putative interactors. The identity of the eight initial ALE1 interactors and their respective LacZ pehnotype is shown in table 2. CSN5A, CSN5B and a nodulin-related gene result from the screening as putative interactors of ALE1.

Table 2. Putative interactors of ALE1. +, growth of yeast colonies; -, no growth of yeast colonies; QDO, quadruple drop-out.

No	Accession No.	Predicted protein function	LacZ+	Growth after re-streaking on QDO
1	AT1G71230.1	CSN5B	+	+
2	AT1G43170.3	*Arabidopsis* ribosomal protein 1 (structural constituent of ribosome)	-	-
3	AT5G25940.1	Early nodulin-related gene	+	+
4	AT1G71230.1	CSN5B	+	+
5	AT5G11670.1	*Arabidopsis thaliana* NADP-ME2 malate dehydrogenase	-	+
6	AT2G30570.1	Photosystem II reaction centre W (PsbW) family protein	-	+
7	AT1G22920.2	CSN5A	+	+
8	AT3G15340.1	Proton pump interactor 2 (PPI2)	-	+

To test whether the interactors activate the LacZ reporter gene, each interactor was re-transformed in the corresponding yeast strain and mated with the empty bait vector and the bait, respectively, as indicated in figure 12 below. This result indicates that, although CSN5A, CSN5B and the nodulin-related gene activate the β-Galactosidase expression in the presence of DBD without bait, this activation is visibly stronger in the presence of ALE1-DBD. Thus, the most likely interactors for ALE1 are CSN5 and the nodulin-related gene.

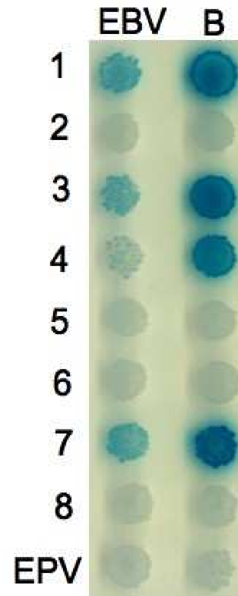

Figure 12. LacZ phenotype of ALE1 interactions. EBV, empty bait vector; EPV, empty pray vector; B, ALE1H252A in bait vector; 1, 4, CSN5b; 2, *Arabidopsis* ribosomal protein 1; 3, Early nodulin-related gene; 5, NADP-ME2 malate dehydrogenase; 6, Photosystem II reaction centre W (PsbW) family protein; 7, CSN5A; 8, Proton pump interactor 2 (PPI2)

Interactors of CER2 (ECERIFERUM 2) and PPT (PALMITOYL PROTEIN THIOESTERASE)

The putative acyl-transferase *CER2* is the second gene chosen for Y2H cDNA library screening, due to its very peculiar nuclear localization, surprising for a protein with such a function. The cDNA library Y2H screening, performed with CER2 as bait revealed a total of five putative interactors.

Table 3. Putative interactors of CER2. Int. Test (colonies), test mating of each interactor – results given in number of colonies; EBV, empty bait vector; B, bait: CER2.

No	Accession No.	Predicted protein function	LacZ+	Growth after re-streaking on QDO	Int. Test (colonies)	
					EBV	B
1	AT5G15090.1	putative porin / putative voltage-dependent anion-selective channel protein	+	+	5	10
2	AT1G20810.1	immunophilin / FKBP-type peptidyl-prolyl cis-trans isomerase family protein	+++	+	8	17
3	AT5G15090.1	putative porin / putative voltage-dependent anion-selective channel protein	+	+	3	8
4	AT5G15090.1	putative porin / putative voltage-dependent anion-selective channel protein	+	+	8	8
5	AT3G55520.1	immunophilin, putative / FKBP-type peptidyl-prolyl cis-trans isomerase, putative	++	+	22	7

To test whether the interactors activate the LacZ reporter gene, each interactor was re-transformed in the corresponding yeast strain and mated with the empty bait vector and the bait, respectively, The identity of the putative interactors as well as their LacZ phenotype are listed in table 3 above.

The most probable interactor of CER2 is the immunophilin AT1G208810, due to the fact that the test-mating of this putative interactor resulted in a higher number of colonies when mated with the bait, as compared to the mating with the empty bait vector.

PPT is a third gene, which might provide valuable insight into the mechanisms that a plant uses to compensate for a deficient cuticle formation. It was found to be up-regulated in three independent cuticular mutants (Voisin, 2008) and putative interactors could bring new insight into its implication in cuticle development.

Two putative interactors were isolated from the cDNA library by Y2H, using PPT as bait. After testing each interactor by mating with the empty bait vector and respectively with the bait, both interactors seem to be false positives, due to the fact that both of them give a higher number of colonies when mated with the empty bait vector, as compared to the mating with the bait (table 4).

Table 4. Putative interactors of PPT. Int. Test (colonies), test mating of each interactor – results given in number of colonies; EBV, empty bait vector; B, bait: PPT.

No	Accession No.	Predicted protein function	LacZ+	Growth after re-streaking on QDO	Int. Test (colonies)	
					EBV	B
1	AT1G20960.1	ATP-dependent helicase	+	+	18	14
2	AT1G23490.1	ADP – ribosilation factor	+++	+	9	2

The results of the Y2H cDNA library screening require further in vivo confirmation.

DISCUSSION

CER13/RST1 COULD FUNCTION AS A RECEPTOR OR AS A TRANSPORTER IN WAX BIOSYNTHESIS

The present study has shown that *cer13* is allelic to *rst1*. The *RST1* gene was previously reported to encode a predicted 1,841-amino acid protein with a molecular mass of 203.6 kD and a theoretical pI of 6.21. Transcript of *RST1* was found in leaves, flowers, roots, stems, and siliques, but accumulation levels were not correlated with the degree to which different organs appeared affected by the mutation (Chen et al., 2005). Furthermore, the same study conducted by Chen et al. (2005) indicated that the RST1 protein does not show high identity to any protein of known function. However, it was 34% (636/1,841) identical and had 51% (964/1,841) positives to the 1,842-amino acid annotated rice protein OJ1276_B06.27 (GenBank BAB92518) and no integral membrane domain was found in the RST1 protein by the TMHMM program. RST1 was predicted to target the mitochondria with TargetP score of 0.550 and probable signal sequence length of 78 amino acids (Chen et al., 2005). When using the ARAMEMNON database (Schwacke et al., 2003), the present study indicates that CER13/RST1 could have up to 9 trans-membrane domains when aligned with its rice homolog (OS01G07490). Thus, CER13/RST1 may function as receptor in the regulation of wax biosynthesis.

CER13/RST1 MODULATES WAX BIOSYNTHESIS THROUGH CER3/WAX2/YRE

Microarray analysis conducted in this study revealed a three-fold down-regulation of *CER3/WAX2/YRE* and this data was confirmed by qRT-PCR. In previous studies, the *cer13* mutant was reported to have decreased amounts of C28 free fatty acids, C28 and C30 aldehydes, C27 and C29 alkanes, C29 secondary alcohols and C29 ketone on the stem. These decreases were coupled with increased amounts of longer

chain length components for both primary alcohols (C30) and alkanes (C31) compared to wild-type. Based on this data, it has been proposed that CER13 is involved in C30 fatty acid to C30 aldehyde reduction (Rashotte et al., 2001). However, the possibility that *cer13* could also have defects in a C30 fatty acid elongase release mechanism were not ruled out and it was also proposed that this would provide fatty acids to the alkane flux of the pathway (Rashotte et al., 2001; Jenks et al., 1995). Furthermore, it was noticed that the *cer3* and *cer7* mutants have a highly similar biochemical pattern of stem cuticular wax constituents (Jenks et al., 1995; Rashotte et al., 2001). *CER3* encodes a protein related to the aldehyde decarbonylase encoded by *CER1* (Aarts et al., 1995). Although it is known that CER3 is required for acyl-CoA reduction to aldehydes, its function is still uncertain. *CER7* encodes the RNase PH-type subunit RRP45B exosome. Interestingly, *CER7* seems to act by degrading the transcript of a transcriptional repressor, since its mutation was found to reduce transcript levels of endogenous *CER3* and of *E. coli* β-glucuronidase (GUS) in pYRE:GUS transgenic lines (Hooker et al., 2007).

Since the *cer13* mutation does not cause a complete loss of C30 aldehyde or subsequently derived pathway products, a hypothesis was already formulated that more than one gene product is needed for the conversion of C30 fatty acid to C30 aldehyde in *A. thaliana* (Rashotte et al., 2001). Due to the highly similar biochemical pattern in the components of epicuticular wax on the stems of *cer13*, *cer3* and *cer7*, coupled with the down-regulation of *CER3* in *cer13* and in *cer7*, at least one of the other gene products mentioned above could be CER3. The data from wax analysis is consistent with the expression analysis data and constitute a strong indicator that CER3/WAX2/YRE acts in the same pathway as CER13. Thus, CER3 is a key player in the wax biosynthesis and it is regulated by both CER13 and CER7.

CER13/RST1 PROVIDES A LINK BETWEEN WAX BIOSYNTHESIS, IMMUNITY AND LIGHT SIGNALING

The *cer13* mutant was previously reported to show organ fusion (Jenks et al., 2002), a phenotype that varies according to the growth conditions, especially humidity (Lolle et al., 1997). Seeds of *cer13* were also reported as shrunken and to have

decreased constituents of the seed coat (Faust, 2006). In L*er*-0 background, the *cer13* allele exhibits a pleiotropic phenotype that includes a decreased wax load on stems and siliques and stunted stature, both responsive to photoperiod. Such phenotypes indicate that CER13 could play a role in regulation. The *ressurrection1* (*rst1*) mutant alleles were reported to have a decreased wax load on the shoots and siliques, coupled with a biochemical pattern of the wax components similar to that of *cer13* (Chen et al., 2005).

The microarray results obtained in this study revealed three up-regulated genes that are involved in jasmonate (JA) signaling. Several transcription factors involved in plant defense against pathogens are also up-regulated in *cer13* transcripts. The MASTA analysis revealed numerous coupling-phase overlaps between up-regulated genes in *cer13* and up-regulated genes involving pathogen-related mutants, as well as BTH treatments. Although the mechanisms are not yet clear, this data does indicate that wax biosynthesis is linked to JA- and SA-mediated defence.

In the plant's interaction with non-biotic factors, the cuticle acts as a protective layer, shielding the plant so that these factors would not become stressors. However, when the shield itself is dysfunctional, the plant perceives certain environmental factors as stressors and responds accordingly. Interestingly, according to the MASTA analysis, the incomplete epicuticular wax layer present on *cer13* seems to mimic transcriptionally the cold and drought stresses. Additionally, the variability in the stunted stature of *cer13* as well as the variation in wax load as responses to day length are indicators that light is a factor that contributes actively to the regulation of wax biosynthesis.

Thus, one can propose that CER13 integrates signals from light and defense responses with those involved in wax biosynthesis and regulation.

CUTICLE DEVELOPMENT IS GOVERNED BY A COMPLEX REGULATORY NETWORK

Transgenic *Arabidopsis* plants that expressed and secreted a cutinase from *Fusarium solani f. sp. pisi* resulted in altered ultrastructure of the cuticle, enhanced permeability to solutes and strong postgenital organ fusions. In addition, pollen can

germinate on fully differentiated leaves of the cutinase-expressing plants (Sieber et al., 2000). From such an extreme reaction, as well as from the numerous mutants characterized up to date it was inferred that an intact cutin layer is needed for normal epidermal differentiation and organ formation (Nelson, 2004; Yephremov and Schreiber, 2005; Nawrath, 2006).

The *ABNORMAL LEAF SHAPE1* (*ALE1*) gene of *Arabidopsis* is expressed during the very first developmental stage of the plant (Tanaka et al., 2001) and was used in the present study as bait in a Y2H cDNA library screening. The results obtained with the screening indicate subunit 5 of the constitutively photomorphogenic 9 (COP9) signalosome (CSN) as main ineractor of ALE1. CSN is a multisubunit protein complex located in the nucleus and is highly conserved throughout evolution. Initially defined as a repressor of photomorphogenesis in *Arabidopsis*, it has now been found to participate in the regulation of a variety of signaling and developmental processes, including embryogenesis, cell cycle, circadian rhythms, DNA repair, and plant responses to light and hormones (Wei and Deng, 2003). CSN is highly homologous to the lid sub-complex of the 26S proteasome and it is composed of eight distinct subunits called CSN1 to CSN8; six contain the PCI domain (proteasome, COP9 signalosome, initiation factor3) and two the MPN domain (Mpr1-Pad1-N-terminal). CSN5 contains an MPN domain and it has over 60% identity between its animal and plant counterparts. In *Arabidopsis*, two conserved genes, named *CSN5A* and *CSN5B* encode two isoforms of CSN5. The two genes (At1g22920 and At1g71230) are located on the opposite arms of chromosome one and sequence analyses indicated that *CSN5A* and *CSN5B* share 86 and 88% identity at the nucleotide (cDNA) and protein levels, respectively (Wei and Deng, 2003). Mutations in the *Arabidopsis CSN5A* but not *CSN5B* have resulted in multifaceted developmental defects at vegetative and reproductive stages (Gusmaroli et al., 2007). In plants and mammals, several CSN subunits are unstable in an unbound form (Wei and Deng, 2003). Furthermore, Gusmaroli et al., (2007) showed that the MPN subunits are essential for the CSN holocmplex assembly and stability and they are inactive in an unbound form. It is possible therefore that *ALE1* would hydrolyze the unbound CSN5, preventing thus the formation of the CSN holocomplex. The interaction of *CSN5A* and *CSN5B* with *ALE1* would thus indicate

that the differentiation of epidermal cells at embryo stage might require the repression of all processes that are mediated by the COP9 signalosome.

The high diversity of the proteins involved in cuticle development, many with unknown function, indicates that a broad regulatory network governs the formation of this active protective layer. Although still far from being elucidated, such a network is present at all key developmental stages of the plant.

CONCLUSION

The plant cuticle is no longer considered only an inert physical barrier that protects the plant against biotic and non-biotic stress factors. Instead, it is required for normal development of organs and constitutes an active signaling interface throughout all developmental stages of the plant in its constant interaction with the environment (Martin, 1964; Chassot et al., 2008; Reina-Pinto and Yephremov, 2009). In plant-biotic interactions, the cuticle is involved in a cross talk signaling between the plant and the potential pathogen, in both directions. Thus, when sensing breakdown producs of the cuticle, fungi would secrete cutinase and form appresoria (Lin and Kolattukudy, 1978; Kolattukudy, 1985; Woloshuk and Kolattukudy, 1986). In turn, plans would sense products resulting from a breakdown of the plant cell wall and these would act as elicitors of defense mechanisms (Boller, 1995).

From the results available this far, one could infer that a highly complex metabolic network is involved in cuticle development. CER13 modulates wax synthesis via CER3 and when the protective shield is disrupted or does not function properly, the plant recurs to defense mechanisms that are normally used in response to biotic and abiotic stress factors. Thus, the plant uses a chemical barrier to compensate for the lack of its mechanical barrier.

MATERIALS AND METHODS

MATERIALS

Plant material and growth conditions

Arabidopsis thaliana ecotypes Columbia (Col-0) and Landsberg *erecta* (L*er*-0) were used as wild types (WT). Seeds of the following lines were ordered from the Nottingham Arabidopsis Stock Centre (NASC): Col-0 (N1093); L*er*-0 (N8581); T-DNA insertion lines *rst1-2* (SALK_070359) and *rst1-3* (SALK_129280) (Chen et al., 2005). Seeds of *eceriferum13* (*cer13-1*, CS95) mutant were ordered from the Arabidopsis Biological Research Center (ABRC).

A. thaliana plants were grown in the greenhouse or in growth chambers, under standard conditions (120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light, 60% relative humidity, 21°C), 16 hours light (long day, LD) or 8 hours light (short day, SD), on commercially available soil. The cultivation on plates was done on medium, containing ½ MS salt (2.21 g/l), Bacto agar (8 g/l), and cysteine (400 mg/l) for 1 l distilled H_2O, pH 5,7 (adjusted with KOH) and sterilized by autoclaving before use. In the case of selective growth, the medium was supplemented with the appropriate antibiotic.

Bacterial strains and cultivation conditions

Cultivation of *E. coli DH10B* (Invitrogen) was usually performed in LB medium (Luria-Bertani Medium) as described by Sambrook *et al*., (1989). LB medium contains: Bacto-tryptone (20 g/l), Bacto-yeast-extract (5 g/l) and NaCl (0.5 g/l) added to 950 ml H_2O. The pH was adjusted to 7.0 with 5N NaOH and the volume was subsequently brought to 1l (in the case of solid medium, 15 g/l Bacto Agar was added). The medium was sterilized by autoclaving for 20 minutes at 120°C.

Agrobacterium tumefaciens strain *GV3101* (pMP90) cells (Koncz and Schell, 1986) were grown in YEB medium (Yeast Extract Broth) containing: Bacto-tryptone (5 g/l); Bacto-yeast-extract (1 g/l); Bacto-peptone (1 g/l); sucrose (5 g/l), in 950 ml H_2O. The pH was adjusted to 7.0 with 5 M NaOH and the volume was brought to 1l

(in the case of solid medium, Bacto Agar (15 g/l) was added). The medium was sterilized by autoclaving for 20 minutes at 120°C.

Antibiotics and selection solutions

The solvent used for the stock solutions is sterilized double distilled H_2O, unless otherwise stated.

Solution	Final concentration
Ampicillin	100 µg / ml
Kanamicin	50 µg / ml
Chloramphenicol	34 µg / ml
Gentamycin	50 µg / ml
X-Gal, in dimethylformide (DMFA)	80 µg / ml
Rifampicin, in DMFA	100 µg / ml
IPTG	0,5 mM

Enzymes and reaction kits

Enzymes

RedTaq Genomic DNA polymerase, Sigma-Aldrich (Germany)

PrimeSTAR™ HS DNA Polymerase, TaKaRa BIO INC. (Japan)

Phusion High-Fidelity DNA Polymerase, Finnzymes (Finland)

KOD XL DNA Polymerase, Novagen (Germany)

T4 DNA ligase: Promega (Germany)

One-step RT PCR kit, Qiagen, (Germany)

IQ™ SYBR Green Supermix, Bio-Rad Laboratories (Germany)

Restriction endonucleases: New England Biolabs (Germany)

Reaction kits

DNeasy® Plant Mini Kit, Qiagen (Germany)

QIAprep® Spin Miniprep Kit, Qiagen (Germany)

RNeasy® Plant Mini Kit, Qiagen (Germany)

GenElute™ mRNA Miniprep Kit, Sigma-Aldrich (Germany)

QIAquick PCR purification kit Qiagen, Hilden, Germany

Yeastmaker^{TM} Yeast Plasmid Isolation Kit, Clontech (Germany)

Phusion® Site-Directed Mutagenesis Kit, Finnzymes (Finland)

Synthetic oligonucleotides

Primers for the genetic markers used in the *cer13* mapping process

Lists of polymorphisms between the ecotypes Col-0 and L*er*-0 were downloaded from "Monsanto Arabidopsis Polymorphysm and Ler Sequence Collections" in "The Arabidopsis Information Resource" (TAIR) database: http://www.arabidopsis.org/browse/Cereon/index.jsp. Single nucleotide polymorphisms (SNPs) and small insertions/deletion (InDel) DNA polymorphisms between the Columbia and Landsberg *erecta* ecotypes were selected for primers design. Primers for InDel markers were designed manually, whereas primers for SNP markers were designed using the SNAPER program (Drenkard et al., 2000): http://ausubellab.mgh.harvard.edu/.

Primers used for T-DNA insertion lines analysis

The sequences of oligonucleotides for T-DNA insertion lines analysis were obtained by using the "T-DNA Primer Design" tool: http://signal.salk.edu/tdnaprimers.2.html.

Oligonucleotides used to amplify genes, for cloning

The primers used for the amplification of genes with the purpose of cloning were designed containing cleavage sites for restriction enzymes that are non-cutters of the respective fragment.

Oligonucleotides for qRT-PCR

All the synthetic oligonucleotides used for qRT-PCR were designed with the Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast), developed from Primer3 (Rozen and Skaletsky, 2000). Full length CDS of the selected gene was inserted as template and the following conditions were different from the default settings: PCR product size of minimum 80 and maximum 105 nucleotides; Tm: minimum 61°C, optimum 62°C, maximum 63°C; GC content:

minimum 45%, maximum 55%; primer pair specificity was set for the organism *Arabidopsis thaliana* (taxid: 3702).

Equipment

Standard equipment for molecular biology laboratory was used throughout this project. All non-standard equipment is indicated in the description of the respective method.

METHODS

Observation of plant material

The assessment of the wax phenotype was made by direct observation and by using light binoculars or scanning electron microscopy. Transgenic plants expressing fluorochrome markers were observed by confocal microscopy.

Scanning electron microscopy (SEM)

Fragments of the main shoot, from 2 cm above the rosette level as well as siliques with pedicel, of L*er*-0, *cer13*, *rst1-3*, F1:*cer13* x *rst1-3* and Col-0 were used as observation material. The samples were deep-frozen and sputtered with palladium using the K1250X cryogenic preparation system (Emitech, Ashford Kent, England). The observation of the samples was carried out with a Zeiss SUPRA™ 40VP scanning electron microscope.

Confocal microscopy

Leaves of five weeks old *Arabidopsis* plants as well as transverse sections of shoots from eight weeks old plants expressing *CER13*-DsRED were placed in water, between microscope slide and cover glass. A Zeiss LSM 510 META confocal laser scanning microscope (Zeiss, Germany) was used for the analysis, with the settings as recommended by the manufacturer, according to the sample requirements.

Isolation of nucleic acids

Nucleic acids were mostly isolated with QIAGEN kits. Genomic DNA for map-based cloning was isolated from leaf tissue (approximately 0,25 cm^2 leaf material) by 15 minutes boiling in extraction buffer (0,2M Tris-HCl ph9; 0,4M LiCl; 25mM EDTA; 1% SDS) followed by precipitation with 2-propan-diol (v/v), 10 minutes centrifugation at maximum speed and re-suspension of the resulting DNA pellet in TE buffer (10mM Tris pH 8; 1mM EDTA). The concentration of nucleic acids was determined using the NanoDrop® ND-1000 spectrophotometer (PeqLab, Germany); RNA quality was also assessed by electrophoresis on 1% agarose gel.

Amplification of DNA fragments via polymerase chain reaction (PCR)

For mapping and checking the presence of inserts in specific vectors, a standard PCR reaction was performed, using the RedTaq® DNA Polymerase (Sigma), according to the producer's instructions.

Standard PCR mixture:

Component	Volume (µl)
10 µM Forward Primer	1
10 µM Reverse Primer	1
2mM dNTP mix (Fermentas)	2
10x Buffer with $MgCl_2$ (Sigma)	2,5
RedTaq® Genomic DNA Polymerase (Sigma) (1U)	1
Sterilized, ddH_2O	Until a total of 25 µl
DNA template	10-20 ng

Standard programme for a PCR reaction:

Step	Temperature (°C)	Time	Number of repetitions
Denaturation	95	3 min	1
Denaturation	95	30 sec	
Annealing	58[1]	30 sec	35
Extension	72	30 sec[2]	
Final extension	72	10 min	1
Store	4	∞	-

PCR amplifications of DNA fragments to be subsequently used for cloning were performed using either KOD XL DNA Polymerase (Novagen) or PrimeSTAR™ HS DNA Polymerase (TaKaRa), using the conditions specified by the respective producer.

[1] Annealing temperature was calculated for each primer pair as Tm minus 5°C

[2] Extension time was 1 minute per 1kb DNA

DNA cloning methods

Standard cloning methods were performed as described by Sambrook and Russell (2001). The presence of the desired insert was checked in the isolated plasmid DNA by restriction analysis as well as sequencing.

Site-directed mutagenesis (SDM)

As *ALE1* encodes a protein with hydrolytic function, I mutagenized the H aminoacid of the catalytic triad from position 252 into alanine (A), by site directed mutagenesis (Finnzymes), in order to avoid the hydrolysis of a putative interactor. After sequencing analysis, four out of 12 separate mutagenized clones (ALE1H252A) could be used further for cloning into the pGBKT-T vector containing the DNA binding domain (DBD), for the Y2H cDNA library screening.

Transformation of electro-competent bacteria by electroporation

Electrocompetent *Escherichia coli* cells were prepared according to Sambrook *et al*., (1989) and *Agrobacterium tumefaciens* cells were prepared as described by Nagel et al. (1990). Transformation of electro-competent bacteria *E. coli* was performed according to Dower (1988) and of *A. tumefaciens* strain GV3101 (pMP90RK) was carried out as described by Mersereau *et al*., (1990). An amount of 50 – 100 ng DNA (in less than 10% of the cells volume) was mixed with a 50µl aliquot of electro-competent cells on ice and the mixture was transferred to a cold 1 mm electroporation cuvette. The electroporation was carried out at 1.6 kV for *E.coli* and 1.8 kV for *A. tumefaciens*, with 5 msec pulse length, in the electroporator (Electroporator 2510, Eppendorf). After electroporation, the cells were suspended in 950 µl of YEB medium (with no antibiotic), transferred to 15 ml falcon tubes and incubated one hour at 37°C in the case of *E.coli* and at 28°C for 2 – 3 hours in the case of *A. tumefaciens*, with 250 rpm shaking (Shaker Innova 44, New Brunswick Scientific). Subsequently, all the transformed cells were plated using 100 µl per LB plate with selective antibiotics (in the case of *E.coli* transformed cells) or per YEB plate with selective antibiotics (Rifampicin, Gentamicin and the selection antibiotic carried by the respective vector in the case of *A. tumefaciens* transformed cells).

Plates with *E.coli* transformed cells were incubated for 16 hours at 37°C and plates with *A. tumefaciens* cells were incubated for 48 hours at 28°C.

Transformation of *Saccharomyces cerevisiae* yeast cells

S. cerevisiae competent yeast cells (strains AH109 and Y187) were prepared and transformed according to "Matchmaker^{TM} Library Construction & Screening Kits User Manual", protocol number PT3955-1, version number PR742237.

Y2H cDNA library screening

The bait plasmid was transformed into *Saccharomices cerevisiae* yeast strain Y187, whereas the empty activating domain (AD) vector, as well as a mixed *Arabidopsis* cDNA library of polyA-tailed cDNAs from total plant tissues harvested at different developmental stages, cloned into the AD vector, were transformed into the yeast strain AH109. Prior to the cDNA library screening, a test mating between the yeast strain containing the bait and the one containing the empty AD vector proved that the bait is not toxic for the yeast cells and that it does not interact with the AD of the library vector. The same mating test was performed to establish the most suitable selection medium, which proved to be the quadruple drop-out (QDO). The identity of the putative interactors was established by sequence analysis on PCR products obtained according to the "Matchmaker™ Library Construction & Screening Kit User Manual", protocol PT3955-1, version PR742237. β-Galactosidase expression tests were performed as described by (Gusmaroli et al., 2007).

To check whether the putative interactors bind to the DBD, as compared to the bait, each of the possible interactors, as well as the empty prey vector, were transformed into the yeast strain AH109 and mated with the Y189 strain containing empty DBD vector (pGBKT7) and the bait vector, respectively.

Agrobacterium-mediated transformation of *Arabidopsis thaliana*

Agrobacterium tumefaciens strain GV3101 (MP90) was used for transformation of *Arabidopsis thaliana (Clough and Bent, 1998)*, following the protocol described by (Logemann et al., 2006). Plants resistant to glufosinate (commercially known as Basta® - Bayer CropScience, Germany) were grown on soil and selected by

spraying 0,01% Basta® at eight days after germination. Kanamicin-resistant transformants of T1 generation were selected by the non-sterile method described by (Hadi et al., 2002) as well as on MS plates containing 37,5 µg/ml Kanamicin.

Generation of transgenic plants for fluorescence expression studies

A region containing the 5'UTR as well as the ORF of CER13, excluding the stop codon was amplified by PCR using primers FP_T175_XhoI (TTTTTTCTCGAGGTGATCGGCGTGTGCATGTAGA) and RP_T201_ScaI (AAAAAAAGTACTGCAGCAAGACATGTCCATAGAAGCAAGTCTAAGC) (XhoI and ScaI sites are underlined in the primer sequences). The XhoI/ScaI fragment was cloned into the pBHSGFP62 and respectively pBctDsRED binary vectors (Efremova et al., 2004). *Arabidopsis thaliana* WT Col-0 and L*er*-0, mutants *cer13* and *rst1-3*, as well as marker lines expressing YFP and GFP fluorochromes were transformed with the resulting plasmid containing CER13-DsRED (Logemann et al., 2006). Transgenic plants were selected using 0,01% Basta® and analyzed by confocal laser-scanning microscopy.

In situ hybridization

Two PCR fragments were generated with the following primers containing SP6 or T7 regions, as indicated in the respective primer name. FP_T202_CER131-SP6 (CTCGAGTTTAGGTGACACTATAGAACTGGAGGGATTCCGCTTTCAGATTCGTC) was used with the RP_T203_CER132-T7 (CTCGAGTAATACGACTCACTATAGGGAGCGCACCAGGTAGTTCTCCAGTC), to generate one PCR fragment. The second PCR fragment was generated using primers FP_T204_CER133-SP6 (CTCGAGTTTAGGTGACACTATAGAACTGGAGCACTCTTGTAAAAGGAGCCTGTGG) and primer RP_T205_CER134-T7 (CTCGAGTAATACGACTCACTATAGGGAGTTCCAATGAAGTCCATAGAAACGAG). Using these PCR fragments, the probe preparation, the hybridization procedure and the immunohistochemical detection were conducted by Dr. Nadia Efremova, as described previously (Zachgo et al., 2000; Efremova et al., 2004).

Seed surface sterilization

The seeds were washed with 70% ethanol (v/v) for approximately 40 seconds and after a brief centrifugation step, the supernatant was removed by pipetting. Two consecutive washing steps were subsequently performed with sterilized ddH_2O. The seeds were re-suspended in distilled water and were thus ready for cultivation on sterile agar plates, using a P20 pipette with the tip cut off (allowing the seeds to pass).

Positional cloning and identification of *CER13*

For the identification of *cer13* locus, a map-based cloning approach was used (Lukowitz et al., 2000). An F2 generation of *cer13* crossed to Colombia (Col-0) was generated and used as mapping population. Single nucleotide polymorphisms (SNPs) and small insertions/deletion (InDel) DNA polymorphisms between the Columbia and Landsberg *erecta* ecotypes were used as markers for mapping the *cer13* locus. InDel markers were used to set an initial border for fine-mapping, whereas SNP markers were used for further positional cloning. A candidate gene approach was subsequently used, followed by the sequencing of the candidate gene using *cer13* genomic DNA, compared to L*er*-0 genomic DNA. For PCR amplification and sequencing, a genomic-DNA region of the candidate gene AT3G27670.1 was considered as follows: 5' UTR: nucleotides (nt) 1 – 1471; ORF: 1472 – 9292; 3' UTR: 9293 – 9797. Due to the large size of the candidate gene, it was amplified in two fragments using KOD XL DNA polymerase (Novagen), for sequencing purpose. The first fragment consisted of the 5'UTR and 1472-4990 nt from the ORF and it was amplified using primers FP: T175 (5'-TTTTTCTCGAGGTGATCGGCGTGTGCATGTAGA-3') and RP: T178 (5'-TTTTTAGTACTGATTACAAGCTCTATACCTGTGCTCGTGAA-3'), with an expected size of 5014 base pairs (bp) PCR product. The second PCR fragment consisted of a region from nucleotides 4903 – 9292 of the ORF, plus the 504 bp region of 3' UTR and was amplified using the primers FP: T177 (5'-TTTTTCTCGAGCGGAGACAAATGCGGAAATTCTGAA-3') and RP: T176 (5'-TTTTTAGTACTTCATAATAATAGCATCAACAAAAATAATCAGAAC-3') with an expected size of 4918 nt. The PCR fragments were cleaned by

MICROCON (Milipore) and were sequenced. For the biological confirmation of *CER13* identity, an allelism test was performed by crossing *cer13* to *rst1-3*.

Ethyl methanesulfonate (EMS) mutagenesis on *rst1-3*

EMS mutagenesis was performed as previously described (Kim et al., 2006), on 2,05 g (approximately 75 000) seeds of *rst1-3*. Approximately 5100 freshly mutagenized seeds were sown on soil. The resulting M1 population was screened for supressors of the *rst1-3* wax phenotype.

Transcriptome analysis

Total RNA was extracted from pieces of stems of approximately 1,5 cm length, located immediately below the inflorescence of 4 weeks old plants, using the RNeasy Plant Minikit (QIAGEN). Three biological replicates for each genotype (*cer13* and L*er*-0) were sent to the Integrated Functional Genomics (IFG, Muenster, Germany), platform of the Westfalian-Wilhelms-University (Muenster, Germany; http://ifg-izkf.uni-muenster.de/Genomik/) for a further quality checking, concentration optimisation, preparation of biotin-labeled cRNA probes, hybridization to GeneChip Arabidopsis ATH1 Genome Arrays (Affymetrix, 900385), washes and scanning of the slides. There were no technical replicates. Data from CEL files was analyzed using a script based on the rank-prod method and written to run in the R-environment (http://www.r-project.org/). Two lists of differentially expressed genes (DEGs) ranked according to false discovery rate (FDR) were generated, containing the top five thousand up- and respectively down-regulated genes. To estimate FDR, pfp (prediction of false positive) values have been calculated from 100 permutations and the predicted differentially expressed genes (DEGs) have been ordered by increasing pfp value. A 5% (0.05) pfp cutoff has been applied to define DEGs in the *cer13* mutant.

The top-DEGs with a fold-change of at least 1.9 and a p-value smaller than 0.003 were considered for confirmation with qRT-PCR, excluding the genes encoding putative proteins of unknown function. The two lists of DEGs were used for in silico suppressor/enhancer screen.

In silico suppressor/enhancer screen by MicroArray overlap Search Tool and Analysis (MASTA)

The script for the meta-analytic software MASTA (MicroArray overlap Search Tool and Analysis) was written by Dr. Alexander Yephremov to run in R (http://www.r-project.org) and its purpose is to find similarities between a certain number of top DEGs from a query contrast (*cer13* versus WT) and the same number of top DEGs from contrasts (mutant vs. wild type or treatment vs. control) of a database.

To date, the MASTA database comprises DEGs for over 600 contrasts calculated from CEL files that were downloaded from the publicly available databases Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo), ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/), TAIR AtGenExpress (http://www. arabidopsis.org/index.jsp) and Integrated Microarray Database System (http://ausubellab.mgh.harvard.edu/imds) or via the NASC Affywatch subscription service (http://nasc.nott.ac.uk/). Several CEL files in the MASTA database have been obtained from authors' websites or directly from authors. The top one hundred of the RankProd-selected DEG lists containing up- and down-regulated genes were taken for the overlap analysis in this report. PDF files obtained as an output of the MASTA analysis were imported to Adobe Illustrator (Adobe Systems, San Jose, CA) for assembly. The statistical significance of the overlap between two DEG lists was determined using the online program available at http://elegans.uky.edu/MA/progs/overlap_stats.html.

When two contrasts are considered (query contrast and compared contrast), there are four possibilities of overlaps: two "coupling-phase" overlaps (up-regulated genes in the query contrast overlap with up-regulated genes in the compared contrast, down-regulated genes in the query contrast overlap with down-regulated genes in the compared contrast) and two "repulsion-phase" overlaps (up-regulated genes in one contrast overlap with down-regulated genes in the other contrast and the other way around).

qRT-PCR

Total RNA from one of the biological replicates in the microarray experiment was used for qRT-PCR. First strand cDNA was generated with Superscript II (Invitrogen) reverse transcriptase, from 3μg of total RNA. IQ SYBR® Green

Supermix (Bio-Rad), containing SYBR Green I dye, hot-start iTaq DNA polymerase, optimized buffer and dNTPs qualified for quantitative PCR. The real-time PCR detection was carried out using a MyIQ detection system (Bio-Rad). The reaction was carried out with the parameters listed below.

Standard qRT-PCR mixture:

Component	Volume (µl)
10 µM Forward Primer	1,25
10 µM Reverse Primer	1,25
SYBR® Green	12,5
cDNA	10
Total volume	25

Standard programme for a qPCR reaction:

Step	Temperature (°C)	Time	Number of repetitions	Data acquisition
Denaturation	95	3 min	-	-
Denaturation	95	30 sec	50 X	-
Annealing	58[3]	30 sec		PCR
Extension	72	30 sec[4]		RT
Denaturation	95	1 min	-	-
Annealing	55	1 min		-
Annealing	55	10 sec	81 X	Melting curve
Store	4	∞	-	-

[3] Annealing temperature was calculated for each primer pair as Tm minus 5°C
[4] Extension time was used as 30 seconds for all reactions

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2004- 2006	Master-Studium, "Al. I. Cuza" Universität von Iasi, Fakultät Biologie, Fachrichtung Molekulargenetik
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Teodor, R.-I., Yephremov, Al. (2008). Genes of Arabidopsis thaliana involved in wax metabolism. *Analele ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi*, **LIV**, fasc. 2, s.II a. Biologie vegetală, 109

Reina-Pinto, J.-J., Voisin, D., Teodor, R.-I. and Yephremov, Al. (2009). Probing differentially expressed genes against a microarray database for in silico suppressor/enhancer and inhibitor/activator screens. *The Plant Journal* **61(1)**, 166

ERKLÄRUNG

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Roxana-Iuliana Teodor