The Plant Cell, Vol. 12, 1795, October 2000, www.plantcell.org

IN THIS ISSUE

Complex Waxes

A mechanism that plants have adopted to survive in a terrestrial, aerial environment is the production of waxes deposited into and on the cuticular layer. The cuticle acts as the first protective barrier against UV radiation and bacterial and fungal attacks, and it regulates nonstomatal water loss. Cuticular waxes are a mixture of several compounds, such as very-long-chain fatty acids (VLCFA; >18C), fatty aldehydes, primary and secondary alcohols, ketones, and esters. The proportion of these compounds differs among plant species and even among the different tissues of an individual plant. VLCFA, the precursor of most wax components, is produced by elongation of fatty acids (C16 or C18) through the addition of two-carbon moieties from the donor malonyl-CoA at each step in a series of four reactions (condensation, reduction, dehydration, and second reduction) catalyzed by the fatty acid elongation (FAE) system. Other components of the wax are produced by modifications of elongated fatty acids, either through decarbonylation, which produces aldehydes, secondary alcohols, alkanes, and ketones, or through acyl reduction, which forms primary alcohol, which in turn combines with free fatty acids to give wax esters (reviewed in Post-Beittenmiller, 1996).

In addition to epidermal cells, on which a cuticle is generally present, specialized cells of reproductive organs also produce fatty acids. For example, cuticle is present on the cell walls of differentiated epidermal cells, such as the stigmatic papillae (Lolle and Pruitt, 1999). The outer wall of the pollen, or the exine, is composed of sporopollenin, a very stable polymer containing long-chain fatty acids and phenylpropanoids (Guilford et al., 1988), which are probably synthesized by the activity of a FAE complex. Exine deposition must be under the control of a highly regulated mechanism that determines a specific pattern characteristic for each plant species. Although both tapetum and microspores contribute to exine formation, genes encoding lipid biosynthetic enzymes were found mainly in the tapetum (Piffanelli et al., 1997).

ms2 mutants of Arabidopsis form aborted pollen without an exine. The *MS2* gene is expressed in the tapetum and encodes a putative fatty acid reductase (Aarts et al., 1997). In some plants, a lipidic pollen coat or tryphine is laid onto the exine wall. This is made up of substances secreted or released in the locule when tapetal cells disintegrate, and it fills the cavities between the baculae of the exine. The pollen coat has been well characterized in Brassicaceae. It includes proteins and is highly enriched in neutral esters containing mostly medium- and long-chain saturated fatty acids, free fatty acids, waxes, and other hydrocarbons (Piffanelli et al., 1997). In Arabidopsis, lipid analysis of the tryphine has also shown the presence of very-long-chain wax esters that are essential for pollen hydration (Preuss et al., 1993). The pollen coat, therefore, is considered essential for hydration but not for adhesion, because pollen mutants deprived of a tryphine adhere by means of an exine to the papillar cells (Zinkl et al., 1999).

MUTANTS AND GENES FOR WAX BIOSYNTHESIS

Defects in the wax layer are easily detectable because mutant plants have a glossy appearance. This characteristic has become an accurate genetic marker, and several mutants affected in wax biosynthesis have been isolated and studied in some plant species. In Arabidopsis, 21 independent *eceriferum* (*cer*) loci seem to be involved in regulating wax production, and for 20 *cer* mutants, relative changes in the amount of the wax compounds were compared with levels in the wild type (Koornneef et al., 1989; Hannoufa et al., 1993). Interestingly, the *cer1*, *cer3*, *cer6*, *cer8*, and *cer10* mutants also exhibited reduced fertility if they were kept under conditions of low humidity. In the past few years, some *CER* genes have been cloned, and sequence analysis of these clones has further elucidated their function in wax biosynthesis.

Novel Arabidopsis *cer1* mutant alleles were generated using the *I/dSpm* transposon tagging system (*cer1-m*; Aarts et al., 1995) or by ethyl methanesulfonate treatment (*cer1-147*; Huelskamp et al., 1995). Both mutants showed the typical glossy phenotype characteristic of the *cer* mutants as well as conditional male sterility. The latter phenotype has been thoroughly described by Preuss et al. (1993) for another *cer* mutant, *cer6-2* (or *pop-1*), which is allelic to the first *cer6-1* mutant identified by Koornneef et al. (1989). *cer1-m*, *cer1-147*, *cer6-1*, and *cer6-2* are all male sterile if grown under relatively dry conditions, but fertility is restored if plants are grown under high humidity.

In wild-type Arabidopsis plants and other members of Brassicaceae, a conversion of the tryphine forms a foot of lipids and proteins between the pollen grain and the papilla; this occurs when the pollen lands on the stigma. Through this foot, hydraulic contact is established, and pollen hydrates and consequently germinates a pollen tube. Pollen from *cer1* and *cer6* mutant lines did not germinate on wild-type stigmas, but wild-type pollen germinated perfectly on the stigmas of mutant plants.

IN THIS ISSUE

Furthermore, when grown in vitro, mutated pollen germinated a tube that grew similar to that of the wild type. The inability of *cer6-2* to germinate on the stigma was interpreted as a failure in hydration caused by the lack of tryphine in this mutant, particularly by the lack of C29 and C30 VLCFAs. When wild-type mentor pollen was used in a mixed pollination with mutant pollen, the tryphine carried by the former could rescue the functionality of the latter (Preuss et al., 1993). Interestingly, *cer1-m*, *cer6-1*, and another *cer6* mutant, *cer6-2654* (Huelskamp et al., 1995), retain the tryphine, although this has smaller lipid droplets than wild-type pollen, whereas *cer1-147* has a tryphine without lipids. This mutant and *cer6-2654* can also be rescued in a mixed pollination using pollen from wild-type *Brassica rapa*, a species very closely related to Arabidopsis (Huelskamp et al., 1995).

The *cer1-m* mutant tagged with *I/dSpm* was used to clone the genomic flanking DNA corresponding to the *CER1* gene. Sequence analysis revealed homology with fatty acid desaturases and an alkane hydroxylase and the presence of an iron binding motif. This, together with the fact that *cer1* mutants are blocked in the conversion of C30 aldehydes to C29 alkanes, strongly supports the idea that the CER1 protein acts as a catalytic iron-containing component of a fatty aldehyde decarbonylase enzyme.

The analysis of waxes of several *cer* mutants (Hannoufa et al., 1993) established that in *cer2* Arabidopsis plants, the total amount of wax was ~40% of that found in wild-type plants, with a particular reduction in aldehydes, alkanes, secondary alcohols, and ketones that was two or four carbons shorter than that in wild-type plants. This finding is in agreement with a block in elongation from C28 to C30. These characteristics are also found in *cer6* mutants. In addition, Preuss et al. (1993) showed that *cer2*, like *cer6-1* and *cer6-2*, is a conditional male-sterile mutant affected in pollen hydration. Unlike *cer6-2* but like *cer6-1*, *cer2* has a tryphine with reduced lipid droplets.

The *CER2* gene has been cloned by chromosomal walking (Xia et al., 1996) and by T-DNA tagging (Negruk et al., 1996). The deduced CER2 protein shares sequence similarity with GL2, the product of the *Glossy2* gene of maize, which is also involved in cuticular wax accumulation (Tacke et al., 1995). On the basis of the wax composition in *cer2* mutants, it was suggested that *CER2* encodes an elongase activity. Unfortunately, the sequence analysis in this case does not help to elucidate the function of this gene, because no similarities were found with enzymes required for fatty acid biosynthesis.

Among all of the *cer* mutants, *cer3* and *cer6* show the most extreme defects in leaf wax accumulation, and a novel *cer3* mutant, *cer3-2186* (Huelskamp et al., 1995), was shown to be defective in hydration, like *cer1* and *cer6*. This phenotype was also investigated by cross-pollinating *cer1*, *cer3*, and *cer6* mutant plants with one another. If the *CER1*, *CER3*, and *CER6* genes produce different end products required for pollen hydration, hydration should be rescued in some combinations. This was not the case, demonstrating that all three genes are required for one class of products. Moreover, the *cer3-2186* mutant exhibited a tryphine indistinguishable from that of the wild type, which indicates that conditional male sterility is not related to the structure of the tryphine. The Arabidopsis *CER3* gene has been cloned by T-DNA tagging (Hannoufa et al., 1996), and its deduced protein contains a nuclear localization signal, indicating that CER3 is a regulatory protein.

THE *CER6* GENE

The *cer6* mutant was the first to be characterized as being defective in pollen hydration, and it was originally named *pop1* (for *pollen−pistil interaction1*; Preuss et al., 1993). To date, it is also the only mutant that has been characterized with respect to the lipid composition of the pollen tryphine. Some of the characteristics of this mutant have been discussed above in connection with *cer1*. On pages 2001–2008 of this issue of THE PLANT CELL, **Fiebig et al.** describe the cloning by positional mapping of the *CER6* gene from Arabidopsis. In this article, the authors unequivocally demonstrate that the *CER6* gene is located on chromosome 1 at 107 centimorgans (cM), and they show by sequence comparison that it is identical to a previously cloned gene, *CUT1*, that was mapped erroneously to 35.98 cM on chromosome 1 (Millar et al., 1999). *CUT1* was identified among a group of expressed sequence tags corresponding to a family of *FAE1*-related genes, and when silenced in transformed Arabidopsis plants by cosuppression, it produced a phenotype identical to that of the *cer1*, *cer2*, and *cer6* mutants. Despite the wrong map position, the characterization of this gene strongly supported the idea that *CUT1* encodes a condensing enzyme.

Fiebig et al. also show here that on chromosome 1 at 38 cM, there is instead a novel gene that shares sequence homology with exons 1 and 2 of *CER6*. Sequence analysis of the *cer6-1* and *cer6-2* mutant alleles also highlighted an interesting point. *cer6-1* is produced by the deletion of a codon, whereas *cer6-2* is produced by an A-to-C transversion that changes a His residue to a Pro in a peptide region that is not conserved among condensing enzymes. Moreover, the phenotype of the *cer6-2* mutant plants was stronger than that of other *cer6* mutants, suggesting the *cer6-2* may be a null allele (Preuss et al., 1993).

The most surprising result described in this article derives from the complementation analysis. The authors observed that the glossy phenotype and male sterility were not rescued equally after the introduction of the *CER6* gene in *cer6-2* mutants. Some of the trans-

formants with restored fertility still had defective stem wax. This is the first report that the two phenotypic effects of the *cer* mutations can be separated. Even more interesting is the finding of a dominant intragenic suppressor mutation that converts the Pro residue in the *cer6-2* mutated allele to a Ser residue. Again, this mutation is in a nonconserved region among condensing enzymes, thus diminishing the possibility that the active site of the enzyme is affected. It would be interesting to visualize a folding model for these three peptides. Pollen of suppressor plants regains an abundant tryphine with lipid droplets that contain the C29 and C30 VLCFAs, although not at the wild-type level.

This brings us back to the question of whether (and how much) tryphine and lipids are necessary to recover pollen fertility, because some alleles of four independent *CER* loci have tryphine and some lipids, yet are male sterile.

A NEW ROLE FOR EXTRACELLULAR LIPIDS

The *Fiddlehead* gene, which is implicated in lipid biosynthesis but is not related to the *CER* genes, was recently cloned and shown to share similarities with *FAE1* and *CHS* (Pruitt et al., 2000). *fdh* mutants are disturbed in epidermal fusions and interactions, exhibiting fused organs, pollen germination, and tube growth on epidermal cells. Epidermal cell walls and cuticles in these mutants are more permeable and show an altered composition of lipids in crude cell wall fractions (Lolle et al., 1997). Based on these observations, the authors formulated a model in which localized changes in the permeability of the cell wall and the cuticle modify the interaction between epidermal cells. This model is supported by a recent report (Sieber et al., 2000) that describes alterations in the cuticle, organ fusions, and pollen tube growth on leaves of transformed Arabidopsis plants expressing a fungal cutinase. Interestingly, though, the chemical wax composition of transgenic plants was identical to that of wild-type plants.

Pruitt et al. (2000) suggested that "the properties of the cuticle are modified to accommodate cell−cell interactions by altering its lipid composition." The long-chain lipids of the tryphine may indirectly serve a similar function in pollen−pistil interactions. For example, they may act as a solvent to release particular compounds of the tryphine or the papillar cells. We have demonstrated that a class of triacylglycerols (which are absent in the tryphine of *Brassica* spp and Arabidopsis) are sufficient to establish pollen interaction with pistils and leaves (Wolters-Arts et al., 1998). Nevertheless, in our experiments, we always used untreated wild-type pollen, which carries its own components for the interaction. One of these components could be a protein such as oleosin, which has been shown to be necessary for the hydration of Arabidopsis pollen (Mayfield and Preuss, 2000). We discussed the possibility that triacylglycerols (and perhaps other lipids) act as a matrix in which water is distributed or that they modify the membrane permeability of the pollen (at the site of tube formation) and/or the stigma. The latter hypothesis (modification of permeability) is closer to the model of Pruitt et al. (2000) and does not attribute a specific action to the lipids; rather, it creates a suitable physicochemical environment for the interaction to occur.

C. Mariani
M. Wolters-Arts
Department of Botany
University of Nijmegen
Nijmegen, The Netherlands

REFERENCES

Aarts, M.G.M., Keijzer, C.J., Stiekema, W.J., and Pereira, A. (1995). Molecular characterization of the *CER1* gene of Arabidopsis involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell **7,** 2115–2127.

Aarts, M.G.M., Hodge, R., Kalantidis, K., Florack, D., Wilson, Z.A., Mulligan, B.J., Stiekema, W.J., and Pereira, A. (1997). The *Arabidopsis MALE STERILITY 2* protein shares similarity with reductases in condensation/elongation complexes. Plant J. **12,** 615–623.

Fiebig, A., Mayfield, J.A., Miley, N.L., Chau, S., Fischer, R.L., and Preuss, D. (2000). Alterations in *CER6*, a gene identical to *CUT1*, differentially affect long-chain lipid content on the surface of pollen and stems. Plant Cell **12,** 2001–2008.

Guilford, W.J., Schneider, D.M., Labovitz, J., and Opella, S.J. (1988). High resolution solid state ^{13}C NMR spectroscopy of sporopollenins from different plant taxa. Plant Physiol. **86,** 134–136.

Hannoufa, A., McNevin, J.P., and Lemieux, B. (1993). Epicuticular wax of *Arabidopsis thaliana eceriferum* (*cer*) mutants. Phytochemistry **33,** 851–855.

Hannoufa, A., Negruk, V., Eisner, G., and Lemieux, B. (1996). The *CER3* gene of *Arabidopsis thaliana* is expressed in leaves, stems, roots, flowers and apical meristems. Plant J. **10,** 459–467.

Huelskamp, M., Kopczak, S.D., Horejsi, T.F., Kihl, B.K., and Pruitt, R.E. (1995). Identification of genes required for pollen-stigma recognition in *Arabidopsis thaliana*. Plant J. **8,** 703–714.

Koornneef, M., Hanhart, C.J., and Thiel, F. (1989). A genetic and phenotypic description of *eceriferum* (cer) mutants in *Arabidopsis thaliana*. J. Hered. **80,** 118–122.

Lolle, S., and Pruitt, R.E. (1999). Epidermal cell interactions: A case for local talk. Trends Plant Sci. **4,** 14–20.

Lolle, S.J., Berlyn, G.P., Engstrom, E.M., Krolikowski, K.A., Reiter, W.-D., and Pruitt, R.E. (1997). Developmental regulation of cell interactions in the *Arabidopsis fiddlehead-1* mutant: A role for the epidermal cell wall and cuticle. Dev. Biol. **189,** 311–321.

Mayfield, J.A., and Preuss, D. (2000). Rapid initiation of *Arabidopsis* pollination requires the oleosin-domain protein GRP17. Nat. Cell Biol. **2,** 128–130.

IN THIS ISSUE

Millar, A.A., Clemens, S., Zachgo, S., Giblin, E.M., Taylor, D.C., and Kunst, L. (1999). *CUT1*, an Arabidopsis gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. Plant Cell **11,** 825–838.

Negruk, V., Yang, P., Subramanian, M., McNevin, J.P., and Lemieux, B. (1996). Molecular cloning and characterization of the *CER2* gene of *Arabidopsis thaliana*. Plant J. **9,** 137–145.

Piffanelli, P., Ross, J.H.E., and Murphy, D.J. (1997). Intra- and extracellular lipid composition and associated gene expression patterns during pollen development in *Brassica napus*. Plant J. **11,** 549–562.

Post-Beittenmiller, D. (1996). Biochemistry and molecular biology of wax production in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 405–430.

Preuss, D., Lemieux, B., Yen, G., and Davis, R.W. (1993). A conditional sterile mutation eliminates surface components from *Arabidopsis* pollen and disrupts cell signaling during fertilization. Genes Dev. **7,** 974–985.

Pruitt, R.E., Vielle-Calzada, J.-P., Ploense, S.E., Grossniklaus, U., and Lolle, S. (2000). *Fiddlehead*, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. Proc. Natl. Acad. Sci. USA **97,** 1311–1316.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P.E., Metraux, J.-P., and Nawrath, C. (2000). Transgenic Arabidopsis plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12,** 721–738.

Tacke, E., Korfhage, C., Michel, D., Maddaloni, M., Motto, M., Lanzini, S., Salamini, F., and Doering, H.-P. (1995). Transposon tagging of the maize *glossy2* locus with the transposable element *en/spm*. Plant J. **8,** 907–917.

Xia, Y., Nikolau, B.J., and Schnable, P.S. (1996). Cloning and characterization of *CER2*, an Arabidopsis gene that affects cuticular wax accumulation. Plant Cell **8,** 1291–1304.

Wolters-Arts, M., Lush, W.M., and Mariani, C. (1998). Lipids are required for directional pollen-tube growth. Nature **392,** 818–821.

Zinkl, G.M., Zwiebel, B.I., Grier, D.G., and Preuss, D. (1999). Pollen–stigma adhesion in *Arabidopsis*: A species-specific interaction mediated by lipophilic molecules in the pollen exine. Development **126,** 5431–5440.