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Analysis of *Arabidopsis thaliana* transgenic plants transformed with *CER2* and *CER3* genes in sense and antisense orientations

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Abstract A number of genes involved in the biosynthesis of the epicuticular wax (EW) of *Arabidopsis thaliana* have recently been isolated through genetic approaches. In view of the evidence in favor of the importance of EW compounds in the adaptation of higher plants to a number of physiological and ecological stresses, we have used clones of some of these genes to genetically engineer constructs with which to manipulate EW biosynthesis in transgenic *A. thaliana* plants. All our constructs were placed under the control of the near constitutive CaMV 35S promoter. We were able to complement mutant plants with the construction in the sense orientation as well as induce phenocopies of the *eceriferum* phenotype by transforming wild-type plants with both the sense and antisense constructs. We observed reduced fertility in the wild-type plants transformed with the 35SCER3sense or 35SCER3antisense constructs but not in those transformed with the 35SCER2sense or 35SCER2antisense constructs.

Key words *Arabidopsis thaliana* · Epicuticular wax biosynthesis · *Eceriferum* mutants · *Glossy* mutants · Transgenic plants

Introduction

The aerial surface of plants are covered with a mixture of extractable lipids referred to as epicuticular waxes (EWs), which are derived from very long-chain fatty acids. EW biosynthesis is readily amenable to genetic analysis because plants without an epicuticular wax layer are easily detected by the naked eye as bright green glossy plants compared with the glacous normal plants. Such mutants have been reported in *Brassica oleracea* (Baker 1974; Macey 1974), *Brassica napus* (Holloway et al. 1977), *Zea mays* (Bianchi 1979), *Pisum sativum* (Macey and Barber 1970), barley (von Wettstein-Knowles 1995) and *Arabidopsis thaliana* (Delleart et al. 1979). Some of these mutants have been useful in dissecting the pathways through which EW is synthesized. More recently, five *Arabidopsis* and maize genes involved in wax biosynthesis have been cloned through a number of genetic approaches (Aarts et al. 1995; Tacke et al. 1995; Deng et al. 1996; Hannoufa et al. 1996; Negruk et al. 1996 a,b; Xia et al. 1996).

In *Arabidopsis*, at least 22 different eceriferum (*cer*) loci are involved in the regulation of EW biosynthesis (Koornneef et al. 1989; McNevin et al. 1993). Some of these EW biosynthesis mutants also have a partially reduced fertility phenotype when grown under arid conditions (Koornneef et al. 1989). Koornneef et al. (1989) used reduced fertility as a criterion for grouping the *cer* mutants into different classes; i.e., *cer1*, *cer3*, *cer6*, *cer8*, and *cer10* were reported to have reduced fertility and were arranged into group I, while the *cer2*, *cer4*, *cer5*, *cer7*, and *cer9* mutants were described as fertile and were arranged into group II. Recent studies have shown that waxes on the pollen coat of *A. thaliana* may play an important role in regulating pollen-stigma recognition (Preuss et al. 1993; Hulskamp et al. 1995). Preuss et al. (1993) proposed that the reduced amount of pollen surface waxes in the tryphine layer leads to a premature breakdown of this layer such that unknown compound, essential for pollen-pistil recognition, is prematurely removed from the pollen surface of these mutants.

Hulskamp et al. (1995) suggested that a slight modification in the chemical composition of the tryphine

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layer of these mutants is sufficient for an interruption in the cell–cell communication between the pollen grain and stigmatic papillae. The developmental control of EW chemical composition suggests these lipids may serve a variety of ecological and biological functions. The hydrophobic nature of waxes has been used to impart these with a number of roles in resistance to a number of enviromental stresses ranging from drought (Chatterton et al. 1975; Jordan et al. 1984) to frost (Thomas and Barber 1974). These hydrophobic characteristics are also thought to protect plants from fungal pathogens (Hargreaves et al. 1982; Jenks et al. 1994). The reflective properties of hydrocarbons have also been used to implicate waxes in tolerance to UV radiation (Reicosky and Hanover 1978; Mulroy 1979). Some wax compounds have been shown to play a role in the recognition of plant hosts by phytophagous insects as well as the predators and parasitoids of these herbivores (Eigenbrode and Espelie 1995). For example, 1-hexacosanol (a C_{26} primary alcohol) has been shown to be a possible feeding inhibitor of the neonate larva of the diamondback moth (Eigenbrode et al. 1991). Therefore, the manipulation of plant EW chemical composition through genetic engineering may lead to a number of potentially useful agronomic traits.

Here we report some results of transformation experiments designed to alter the chemical composition of EW on the stem of transgenic plants. Constructs of the *CER2* and *CER3* genes in direct and reverse orientations and under the control of the CaMV 35S promoter were transformed into both mutant and wild-type *A. thaliana* plants. With these constructs, we could control either the glossy or glaucous phenotype on the stem of transgenic plants.

Materials and methods

Plant material

A. thaliana, var. Landsberg erecta, as well as the transgenic plants, were grown in growth chambers operating at 20°C, 70–90% RH under a 16 h long-day at a light intensity of 100–200 $C\mu Em^{-2}s^{-1}$. Plants were watered with the nutrient solution described in McNevin et al. (1993). The mutant lines used as a host for the molecular complementation of *cer3* were isolated by Delleart et al. (1979), while the MK1 line used for the molecular complementation of *cer2* is a mapping line produced by Koornneef et al. (1989). Transgenic plants containing a fusion construct of the cauliflower mosaic virus 35S promoter and the *β*-glucuronidase (GUS) structural gene were kindly provided by Tim Caspar (E.I. DuPont de Nemours and Co.).

CER2 constructs under the 35S promoter

The *CER2* gene-construct in antisense orientation was generated by digesting a genomic clone of the BRL9 mutant allele (Negruk et al. 1996a) with *Hin*dIII. A 5.8-kb *Hin*dIII digestion product, which encodes the *CER2* gene sequence, was ligated into the *Hin*dIII site of the pSK bluescript vector (Stratagene) according to Sambrook et al. (1989). A single clone from this bank, pSKCER2-1, was digested with *Hga*I and a 1056-bp fragment, which contains the CER2 coding sequence, was collected by agarose-gel purification. This fragment was blunt-ended and re-ligated into the *Eco*RV site of the pSK vector according to Sambrook et al. (1989). A number of clones were analyzed in order to identify one which had the 3′-end of the *CER2* gene near the *Hin*dIII site of pSK. A *Hin*dIII-*Bam*HI double-digestion of this pSKCER2-2 produces a fragment of 1056 bp which contains 699 bp of the second exon of *CER2*, 100 bp of the intron, as well as 24–36 bp of a T-DNA right-border sequence at the site of T-DNA insertion in the BRL9 mutant allele. This fragment was used to make a construct in the Bin35SCATE9 binary Ti plasmid vector (Kuhlemeier et al. 1987). As the chloramphenicol acetyltransferase (*CAT*) gene of Bin35SCATE9 is flanked by a unique *Hin*dIII site close to 35S promoter, and a unique *Bam*HI site at it's 3′-end, the *Hin*dIII-*Bam*HI fragment from pSKCER2-2 can be directionally cloned into the *Hin*dIII-*Bam*HI sites of the Bin35SCATE9 vector. The orientation of these sites in this Ti plasmid vector is such that the CER2 fragment is inserted in an antisense orientation relative to the 35S promoter. Panel A of Fig. 1 shows a map of this construct. The manipulations used for the production of the 35SCER2sense construct, presented in Fig. 1 B, are described in Negruk et al. (1996 b).

CER3 constructs relative to the 35S promoter

A *Xho*I digestion product of the *CER3* cDNA clone pAHC1 (Hannoufa et al. 1996) was blunt-ended and subcloned into the *Eco*RV site of pSK. A number of clones were characterized by restriction mapping to find one, pSKCER3, which had the 3′-end of the *CER3* cDNA fragment in close proximity to the *Sal*I site of pSK. This clone and the Bin35SCATE9 vector were digested by *Sal*I and *Hin*dIII, respectively. Incubation with these restriction enzymes linearies

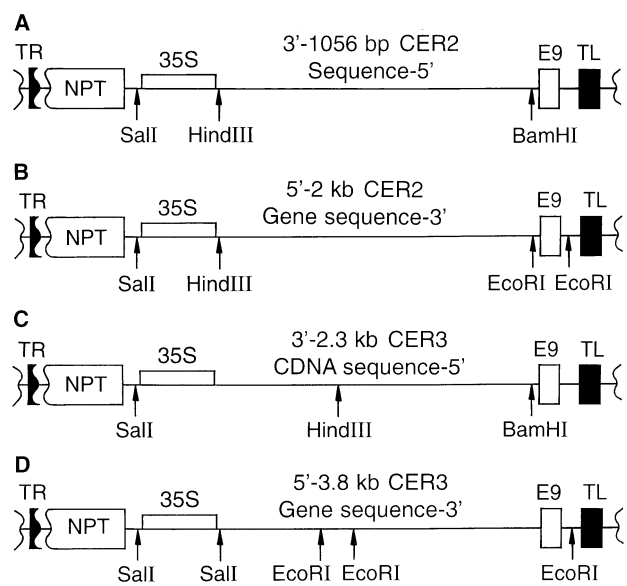

Fig. 1 Partial physical maps of 35SCER2antisense (**A**), 35SCER2 sense (**B**), 35SCER3antisense (**C**), and 35SCER3sense (**D**) constructs. Abbreviations: *TR* right border of T-DNA, *NPT* neomycin phosphotransferase coding sequence, *35S* CaMV 35S promoter, *E9* 3′ end from the pea rbc-E9 gene (Kuhlemeier et al. 1987), *TL* left border of T-DNA

both of these plasmids. These digestion products were filled-in by the Klenow fragment of the DNA polymerase and digested by *Bam*HI. The products of the *Bam*HI digests have a sticky end and a blunt end, such that when these were mixed and ligated the cohesive end will force the insertion of the CER3 fragment in the antisense direction relative to the 35S promoter. A map of this construct is shown in Fig. 1 C.

The creation of the construct used for molecular complementation of the *cer* phenotype, 35SCER3sense, is described in Hannoufa et al. (1996). Contrary to the restriction map of pASK presented by Hannoufa et al. (1996), the 3′-end of the *CER3* gene is in close proximity to the *Xho*I site of pSK in this clone. The partial physical map of the final construct is shown in Fig. 1D.

Transformation

All of the aforementioned constructs were inserted into *Escherichia coli* DH5α. Plasmid DNAs from these clones were used to transform *Agrobacterium tumefaciens* C58/C1 pGV3850 by tri-parental mating (Zambyrski et al. 1983). The resulting strains were used to transform root explants from 9 to 14 day-old plants according to Valvekens et al. (1988). We also transformed the whole plants by vacuum infiltration according to a modification of the protocol of Bechtold et al. (1993) kindly provided to us by Dr. P. Green (Michigan State University). Kanamycin-resistant regenerants were transfered into soil and selfed to produce T_1 seeds. These seeds were tested for kanamycin resistance according to McNevin et al. (1993) and T_2 seeds were collected from the resistant individuals. Stems and siliques from the T_2 plants obtained with these seeds were harvested and wax structures were analyzed by scanning electron microscopy (SEM) according to Negruk et al. (1996 a).

Chemical analysis of EW

The EW was isolated by immersing stems obtained from 1 to 2 g of plant material in 4 ml of chloroform for 30 s. The extracts were evaporated under a N_2 stream and the dried wax residues were prepared for gas chromatography according to Yang et al. (1992). Quantitive analysis was based on the addition of an internal standard (n-pentocosane). The gas chromatography analysis program was the same as that used by McNevin et al. (1993).

Analysis of *A. thaliana* plants transformed with the 35S-GUS construct

Seeds of *A. thaliana* transformed with 35S-GUS construct were kindly provided by Tim Casper (DuPont, Wilmington Del.). Histochemical analysis of GUS activity was done essentially as described by Xia et al. (1996).

Results and discussion

Histochemical analysis of transgenic *A. thaliana* plants bearing the 35S-GUS construct

As previously reported (Jefferson et al. 1987), genes whose transcription is under the control of the 35S promoter of cauliflower mosaic virus are well expressed in a number of different tissues of transgenic plants. We observed staining of part of the flowers (sepals, anthers and pistils), young leaves, and an upper meristematically more-active part of the stems in *A. thaliana* transgenic plants containing the reporter gene 35S-GUS construct (data not shown). GUS activity in petals, older bottom parts of the stem, and in epidermal and mesoderm tissues of older leaves was mainly nearly at background staining levels. Young siliques showed a pronounced staining; however, only upper and bottom parts were stained in old siliques. The expression of the 35S-GUS construct was examined in cross-sections of GUS-stained stems, young siliques, and juvenil anthers. In stems and siliques GUS staining was detected in different tissues including the epidermis. These observations confirm data reported for different plants (wheat, tobacco, tomato, potato, *B. napus* and *A. thaliana*) by Jefferson et al. (1987). Thus, genes under control of the 35S promoter are well transcribed in epidermal tissues of upper parts of the stem and the young siliques. Considering that the 35S promoter-driven GUS gene gave strong staining in epidermal tissues of siliques and stems, we used this promoter in our transformation experiments to control the expression of the CER2 and CER3 genes on the surface of transgenic *Arabidopsis* plant stems and siliques.

Selection of transgenic plants

Several dozen kanamycin-resistant transgenic plants were obtained for each of the four constructs described in Materials and methods. The presence of the construct in the genome of these plants was confirmed by PCR analysis (data not shown). Plants were selected for detailed phenotypic characterization after visual observation of the transgenic plants. The stems of the selected plants were analysed by scanning electron microscopy (SEM) and the chemical composition of the EW of a limited number of representative individuals was further determined by gas chromatography (GC). Results of the visual and SEM analysis of transgenic plants are summarized in Table 1.

Molecular complementation of *cer2* with the 35SCER2sense construct

The *cer2* mutant plants transformed with the 35SCER2sense construct showed different levels of molecular complementation. Results of the SEM analysis of some of these transgenic plants, compared to the wild-type, are shown in Fig. 2. For some of these transgenic plant lines, the density of crystal structures was equal to that found on wild-type plants (Fig. 2, panels A and B) while on others the density of structures was lower than that of the wild-type plants (Fig. 2, panels A and C). In total, eight of these were visually glaucous plants (Table 1). However, the morphology of wax structures on these partially complemented plants was not identical to those found on wild-type plants

Table 1 Summary of the phenotypic analysis of transgenic plants

Name of construct	Host for transformation	Number of Km^R plants	Number of glossy plants	Number of sterile plants
35SCER2	Wild-type[a]	31	2[c]	0
Sense	Wild-type[b]	26	4	0
35SCER2	Wild-type[a]	78	2	0
Antisense	Wild-type[b]	14	3	0
35SCER3	Wild-type[a]	27	0[c]	0
Sense	Wild-type[b]	7	1[c]	3
35SCER3	Wild-type[a]	77	1	0
Antisense	Wild-type[b]	51	22	22
35SCER2	*cer2* plants[a]	24	1	24
Sense	*cer2* plants[b]	18	7	18
35SCER3	*cer3* plants[a]	26	1	26
Sense	*cer3* plants[b]	16	6	16

[a] Vacuum infiltration according to Bechtold et al. (1993)
[b] Root explant transformation according to Valvekens et al. (1988)
[c] Chimeric plants with both *eceriferum* and wild-type branches

(Fig. 2, panel C). Interestingly, the fertility of all of the complemented plants was significantly reduced compared to that of wild-type plants when grown under arid conditions, as was observed for the original *cer2* mutant.

SEM of wild-type plants transformed with the 35SCER2sense construct

Six of the transgenic plants obtained after transformation of wild-type *Arabidopsis* with the 35SCER2sense construct were found to have *glossy* stems compared to wild-type plants (Table 1). SEM of wax structures on these plants is shown in Fig. 3. The pronounced reduction in the number of wax blooms on the surface of these plants suggests that the introduction of the transgene causes co-supression of the expression of the endogenous *CER2* gene (Fig. 3, panel B). The density of wax structures on these plants is as low as that found on the surface of mutants with null alleles of *CER2* (Negruk et al. 1996 a). None of these transgenic plants displayed the reduced-fertile-phenotype characteristics of the *cer2* mutant and the molecular-complementation transgenic plants mentioned above.

SEM of plants transformed with the 35SCER2antisense construct

The introduction of the 35SCER2antisense construct into the wild-type resulted in five plants with *glossy* stems (Fig. 3, panels C and D; Table 1). These *glossy* plants had fully fertile flowers even when grown under arid conditions. Depending on the transgenic line observed, the density of wax crystal structures on the surface of these plants varied from none (Fig. 3, panel C) to levels close to that found on the wild-type stem (Fig. 3, panel D). As a rule, the co-supression-observed transgenic plants generated by the introduction of the 35SCER2sense construct into the wild-type had a stronger mutant phenotype compared to plants produced with 35SCER2antisense construct (Fig. 3, panels B and D). Some of the transgenic lines containing the 35SCER2antisense construct had *glossy* stems and glacous siliques while others had glacous stems and *glossy* siliques (data not shown). In the T_1 generation genetic analysis of the kanamycin resistance marker encoded by the T-DNA element always showed co-segregation with the *cer* phenotype of the transgenic plants containing the antisense construct (data not shown). The T_0 transgenic plants generated by the introduction of the 35SCER2sense construct into the wild-type plant, however, all demonstrated resistance to kanamycin and non-Mendelian segregation of the *eceriferum* phenotype in the T_1 generation. Each of the *glossy* T_1 transgenic plants later gave rise to a homogeneous *glossy* T_2 population. Similar observations have been made by Hart et al. (1992).

Molecular complementation of *cer3* with the 35SCER3sense construct

The stem of seven of the transgenic plants obtained after the transformation of *cer3* mutants with 35SCER3sense constructs were glacous (Fig. 4, panel B; Table 1), indicating that molecular complementation was successful. In agreement with our previous observations with *cer2* molecular complementation, all these

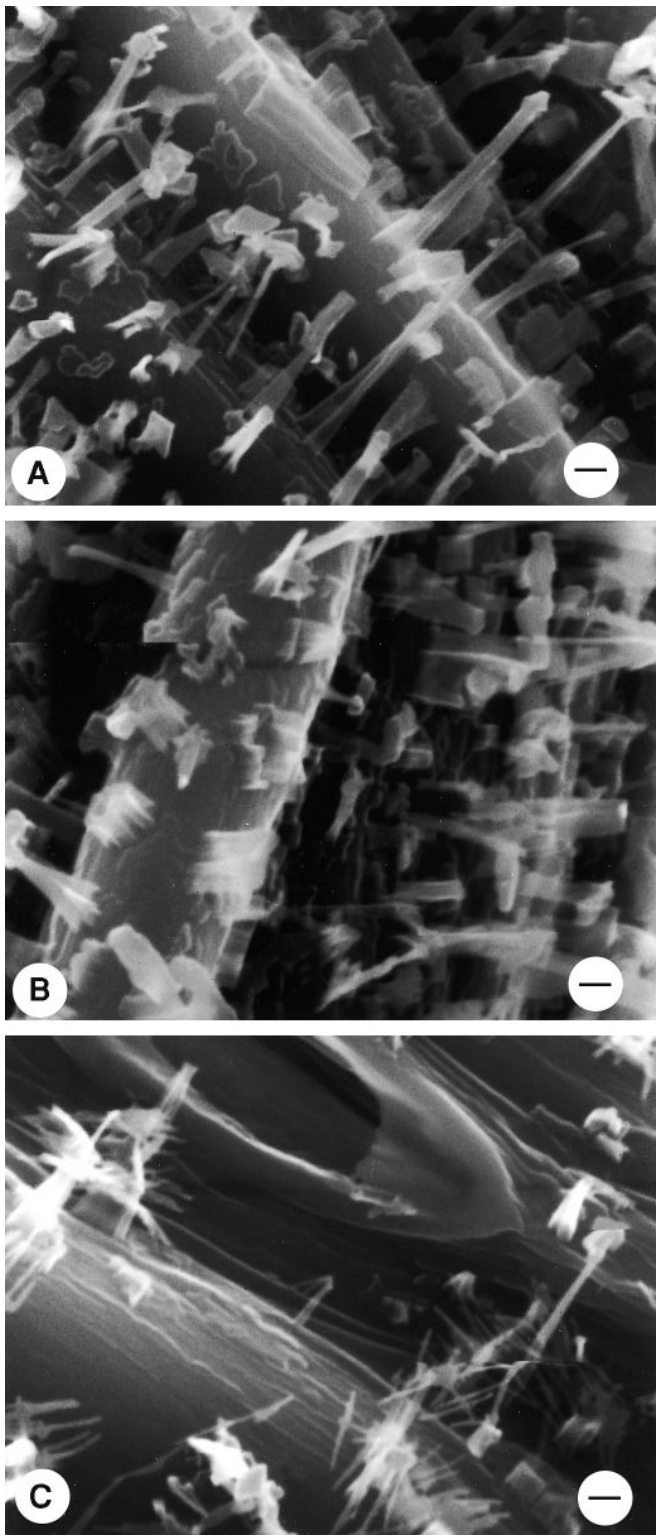

Fig. 2 Scanning electron micrograph of stem wax structures on the surface of the wild-type plant (**A**) and two different *cer2* mutant plants transformed with 35SCER2sense construct (**B** and **C**). Bar = 1 μm

plants were partially sterile when grown under arid conditions. SEM analysis revealed that one of the seven transgenic plants categorized as glaucous had stem sections which were devoid of wax blooms. The siliques of this transgenic plant also had significantly higher densities of wax structures compared to the wild-type plant (Fig. 4, panels C and D).

SEM of wild-type plants transformed with the 35SCER3sense construct

SEM revealed that one of the wild-type plants transformed with the 35SCER3sense construct had a density of wax blooms on their stems which was intermidiate to that of the *cer3* mutants and the wild-type plants (Fig. 2, panel A; Fig. 4, panels A and E), suggesting that the transgene suppresses the expression of the endogenous *CER3* gene. It is interesting to note that co-supression of *CER3* by the 35SCER3sense transgene is not frequent because 34 transgenic plants were needed to find a single example of this phenomenon compared to a frequency of one case of co-supression of *CER2* by the 35SCER2sense construct in ten transgenic plants (Table 1).

SEM of plants transformed with the 35SCER3antisense construct

Transformation with the 35SCER3antisense construct into wild-type plants allowed us to obtain 23 transgenic plants with a *glossy* stem (Fig. 4, panel F; Table 1). Note, only one of the 52 transgenic plants generated according to the procedure of Valvekens et al. (1988) had the same level of glossiness as the *cer3* mutant in the T_1 generation. Eight other T_1 plants had reduced numbers of wax structures in the first 2–3 cm of stem growth, but accumulated wax blooms as they aged. However, in the T_2 generation, progeny from half of these plants segregated the *cer* phenotype. Thirteen plants showed intermediate *eceriferum* phenotypes in both T_1 and T_2 generations. Some of the plants with intermediate densities of wax structures on their stems had wild-type levels of wax blooms on their siliques. Interestingly, most of these plants also displayed reduced fertility when grown under arid conditions. The degree of sterility was proportional to the degree of stem glossiness as estimated by the density of wax structures on the stems.

Gas chromatography analysis of transgenic plants

Results of the gas chromatography (GC) analysis of transgenic plant stems characterized by SEM are presented in Tables 2 and 3. The transgenic plants generated by transformation of wild-type plants with the

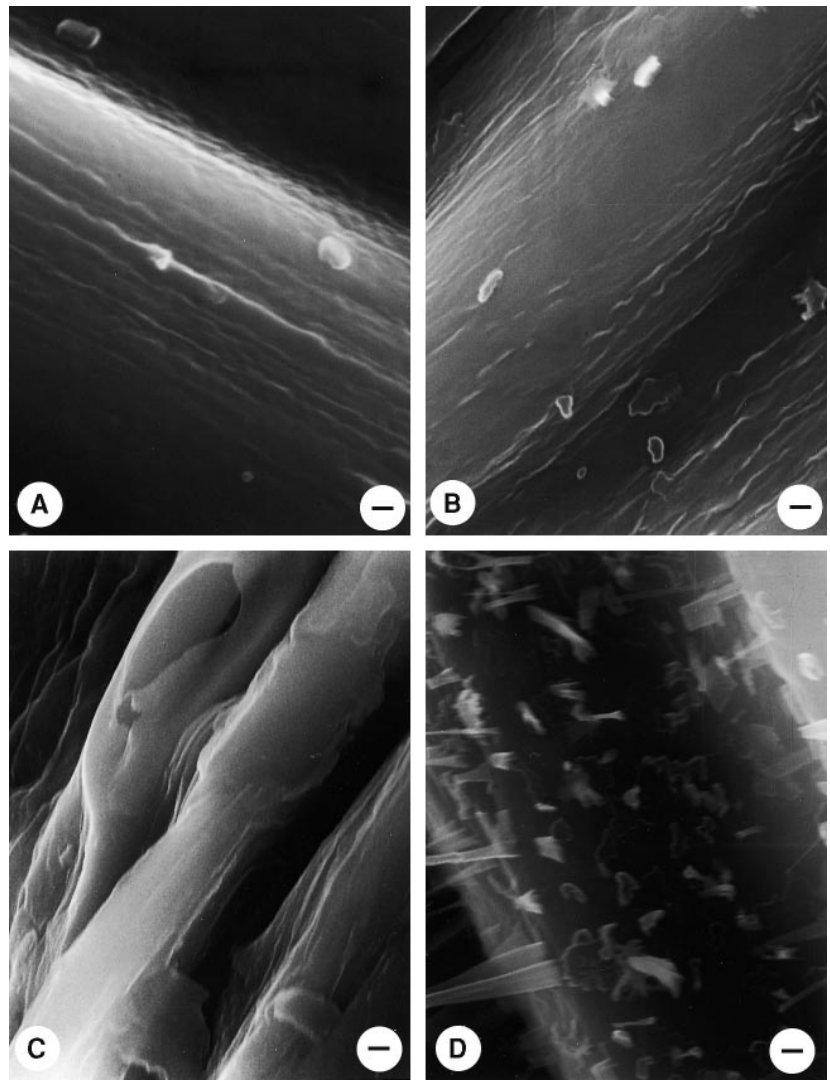

Fig. 3 Scanning electron micrograph of stem wax structures on the surface of the *cer2* mutant plant (**A**), the wild-type plant transformed with a 35SCER2sense construct (**B**), and two different wild-type plants transformed with the 35SCER2antisense construct (**C** and **D**). Bar = 1 μm

35SCER2antisense construct (Fig. 3, panel D) had a reduced level of 15-nonacosanone compared to the other components (Table 2). Interestingly, the 1-hexacosanol level of this plant did not deviate from that of the wild-type in a significant way, despite the fact that the wax load on the stems of these plants was only 46% of that found on wild-type plants. Transgenic plants exhibiting co-supression of *CER2* by the 35SCER2sense transgene (Fig. 3, panel B) had a stem EW chemical composition which is very similar to that of the *cer2* mutant (Table 2). These results suggest that 1-hexacosanol accumulation only occurs with a severe block in the pathway, such as that caused by null alleles. Therefore, the prospects for increasing the level of this compound on the surface of cruciferous vegetables through genetic engineering, as previously suggested by Lemieux et al. (1994), may be difficult to implement. The stem EW of the transformants obtained by introducing the 35SCER2sense construct into the MK1 line have a virtually identical chemical composition to the wild-type stem EW.

A comparison of the chemical composition of the EW of wild-type plants and plants transformed with 35SCER3antisense constructs showed that the latter have a notable increase in the ratio of n-hentriacontane and 1-triacontanol compared to other compounds in the EW (Table 3). This alteration in EW chemical composition was most notable in transgenic plants with low densities of wax structures on their stems, as estimated by SEM. Note, this chemical composition difference is usually mentioned as the most specific feature of the EW of *cer3* mutants (Hannoufa et al. 1993; Jenks et al. 1995). In cases where molecular complementation of the *cer3* mutant by the 35SCER3sense construct was incomplete, the proportion of 1-triacontanol compared to other wax components was similar to that found in

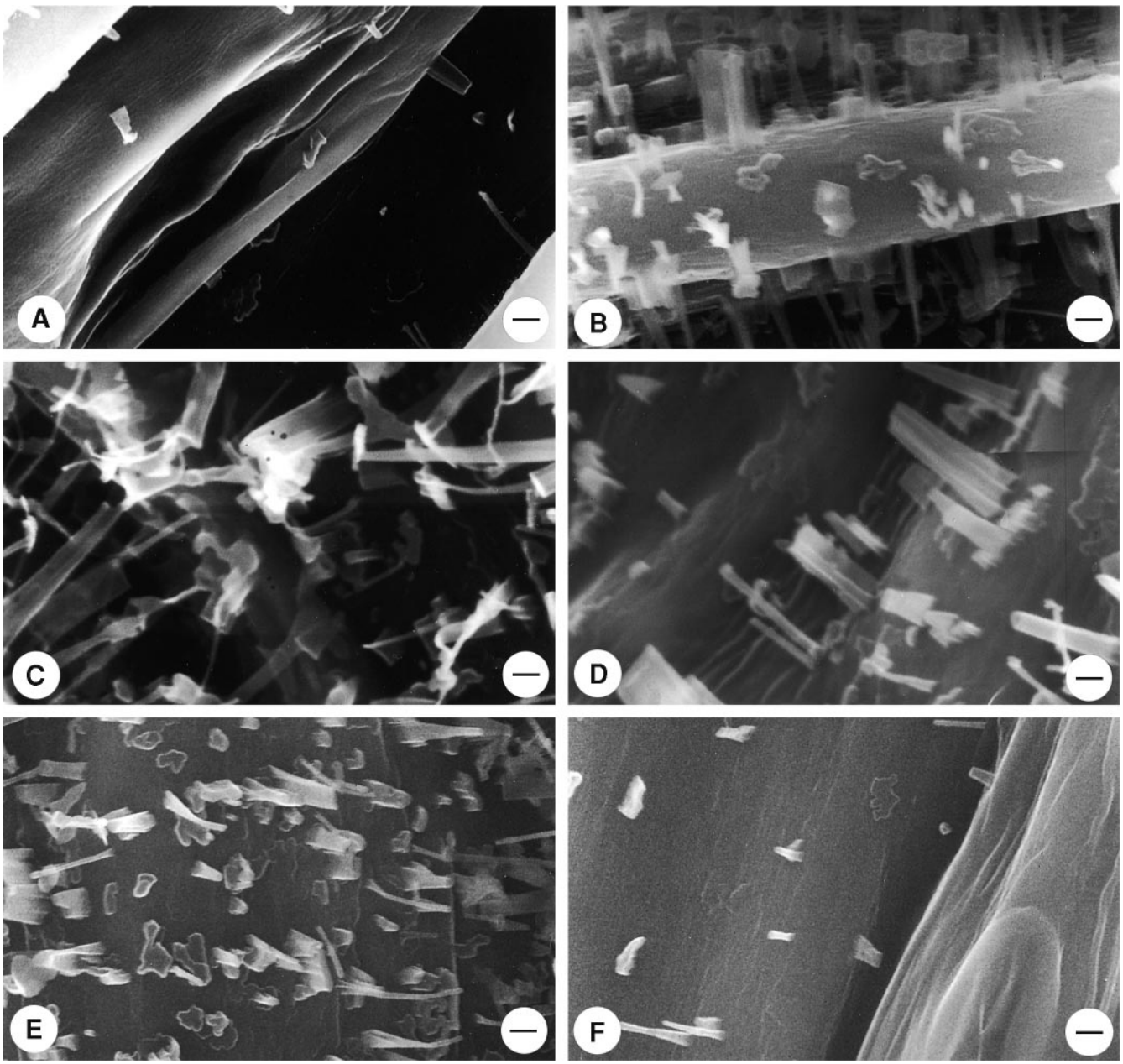

Fig. 4 Scanning electron micrograph of: stem wax structures on the surface of the *cer3* mutant plant (**A**), the *cer3* mutant plant transformed with a 35SCER3sense construct (**B**), the wild-type plant transformed with a 35SCER3sense construct (**E**), the wild-type plant transformed with a 35SCER3antisense construct (**F**), and silique wax structures on the surface of one of *cer3* mutant plants transformed with a 35SCER3sense construct (**C**) and the wild-type plant (**D**). Bar = 1 μm

wild-type stem EW (Fig. 4, panel E; Table 3). Moreover, plants with a density of structures intermediate to that of the *cer3* mutant and the wild-type plant contained almost the same proportion of this compound as wild-type plants (e.g., 35SCER3antisense12), whereas plants with a strong eceriferum mutant phenotype (e.g. 35SCER3antisense15) had a ratio of n-hentriacontane and 1-triacontanol to other wax compounds which was close to that of the mutant. From these observations, we can conclude that manipulating the chemical composition of stem EW will require combinations of different transgenes. To explain the fact that CER3 transgenes affect both the stem epicuticular waxes and plant fertility, whereas CER2 constructs reduced or eliminated stem epicuticular waxes in transgenic plants but did not affect plant fertility, it will be necessary to make a detailed comparative analysis of differential gene expression for both the CER2 and CER3 genes. On the other hand, the fact that a 35S promoter-driven sense or antisense construct of *CER3* can give rise to

Table 2 Chemical composition (in area %) of the epicuticular wax of *A. thaliana* race Landsberg, the *cer2* mutant, and transgenic plants obtained in this study. – less than 1%; WT, wild-type plants; MK1, *cer2* mutant plants

Wax compounds	WT	MK-1	35SCER2 antisense-WT	35SCER2 sense-WT	35SCER2 sense-MK1
n-Heptacosane	1.1	7.1	–	9.5	–
n-Nonacosane	35.9	1.0	40.6	3.0	45.2
1-Hexacosanol	3.3	41.4	2.9	42.8	3.9
14- and 15-Nonacosanol	7.0	–	6.7	2.3	4.5
15-Nonacosanone	15.9	–	9.7	–	17.6
1-Octacosanol	6.8	12.7	8.4	20.5	10.4
1-Triacontanol	3.1	–	2.5	–	4.1

Table 3 Chemical composition (in area %) of the epicuticular wax of *A. thaliana* race Landsberg, the *cer3* mutant, and transgenic plants obtained in this study. – less than 0.5%; WT, wild-type plants; *cer3*, *cer3* mutant plants

Wax compounds	WT	*cer3*	35SCER3 antisense 12-WT	35SCER3 antisense 15-WT	35SCER3 sense-*cer3*
n-Heptacosane	1.1	–	0.5	–	0.8
n-Nonacosane	35.9	8.5	30.6	9.4	36.4
1-Hexacosanol	3.3	1.8	1.9	1.9	3.9
14- and 15-Nonacosanol	7.0	1.6	7.9	2.7	6.0
15-Nonacosanone	15.9	7.0	24.4	8.5	20.5
n-Hentriacontane	–	1.0	2.2	1.0	1.9
1-Octacosanol	6.8	3.1	8.2	3.5	8.9
1-Triacontanol	3.1	25.0	4.0	32.2	3.0

male-sterile plants suggests that clones of this gene could be a useful tool for the production of male-sterile plants.

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