

RESEARCH PAPER

# The *sensitive to freezing3* mutation of *Arabidopsis thaliana* is a cold-sensitive allele of homomeric acetyl-CoA carboxylase that results in cold-induced cuticle deficiencies

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## Abstract

The *sfr3* mutation causes freezing sensitivity in *Arabidopsis thaliana*. Mapping, sequencing, and transgenic complementation showed *sfr3* to be a missense mutation in *ACC1*, an essential gene encoding homomeric (multifunctional) acetyl-CoA carboxylase. Cuticle permeability was compromised in the *sfr3* mutant when plants were grown in the cold but not in the warm. Wax deposition on the inflorescence stem of cold-grown *sfr3* plants was inhibited and the long-chain components of their leaf cuticular wax were reduced compared with wild-type plants. Thus, freezing sensitivity of *sfr3* appears, from these results, to be due to cuticular deficiencies that develop during cold acclimation. These observations demonstrated the essential role of the cuticle in tolerance to freezing and drought.

**Key words:** acetyl-CoA carboxylase, *Arabidopsis*, cuticle, freezing tolerance, wax.

## Introduction

Freezing tolerance in plants is generally acknowledged to be a complex trait, with many mechanisms contributing to the survival of the hardy plant. Comparisons of hardy and non-hardy plants have yielded a bewildering array of differences at physiological, biochemical, and molecular genetic levels. Freezing tolerance can be treated as a developmental trait, as hardy species are freezing sensitive in the summer and develop their freezing tolerance only after exposure to cold, a process known as cold acclimation (Guy, 1990). However, unlike morphological development, the development of freezing tolerance does not reveal qualitatively different stages or components; thus, it has been difficult to distinguish what, if anything, is contributed by each of the many changes during cold acclimation.

Several complementary approaches have been used in an attempt to overcome this problem. The investigation of genes

with altered expression during cold acclimation (reviewed by Thomashow, 1999, 2010) has proved to be an extremely powerful tool, leading to identification of the C-repeat (CRT)-binding factor/dehydration-responsive element (DRE)-binding protein 1 (CBF/DREB1) regulon and a growing understanding of cold acclimation signalling pathways (Stockinger *et al.*, 2001; Thomashow *et al.*, 2001; Seki *et al.*, 2003; Vogel *et al.*, 2005; Doherty *et al.*, 2009). A reporter-based screen was used to identify a large set of mutants specifically altered in their signalling response to cold (and osmotic stress) (Ishitani *et al.*, 1997; Gong *et al.*, 2002), and through this, a number of novel components of the CBF/DREB1 signal transduction pathway were identified (reviewed by Xiong *et al.*, 2002). Through similar approaches, ICE1, an upstream regulator of CBF/DREB1, was isolated and characterized (Chinnusamy *et al.*, 2003; Miura *et al.*, 2007) and

mutants that affect CBF/DREB1-independent regulatory pathways were identified (Zhu *et al.*, 2004; Medina *et al.*, 2005). These studies have not, however, led to an understanding of the role of individual genes in freezing tolerance, except where the effect has been to control the expression of other genes (Xin and Browse, 2000).

Individual genes with clear effects on freezing tolerance can be selected by their mutant phenotypes. Several groups have isolated such mutants and determined the contribution of individual genes or molecular processes to freezing tolerance. Xin and Browse (1998) and Xin *et al.* (2007) isolated mutants that increased tolerance to freezing in the absence of cold acclimation. The mutations may be in components of the signalling pathway(s) responsible for cold acclimation or in genes that make self-sufficient individual contributions to protection against freezing. Mutations that decrease freezing tolerance are likely to be in genes required for freezing tolerance in the wild-type. The *frs1* mutation was found to be an allele of *ABA3* (Llorente *et al.*, 2000) and confirms the importance of abscisic acid in freezing tolerance.

The *Arabidopsis sensitive to freezing (sfr)* mutants (Warren *et al.*, 1996) are impaired in their freezing tolerance following cold acclimation but have near normal (wild-type) levels of freezing tolerance prior to cold acclimation. The corresponding *SFR* genes could be components of the cold-induced gene response whose individual contributions to freezing tolerance are not currently understood. Alternatively, they may be genes essential for freezing tolerance that are not transcriptionally regulated by cold. It has been shown that the *sfr6* mutant is unable to induce genes with CRT/DRE elements in their promoters (Knight *et al.*, 1999; Boyce *et al.*, 2003). The *SFR6* gene was identified as a protein of unknown function that acts post-translationally on CBF function as a key component of the cold acclimation process (Knight *et al.*, 2009). *SFR2* encodes a constitutively expressed chloroplast targeted  $\beta$ -glycosidase (Thorlby *et al.*, 2004; Fourrier *et al.*, 2008) that is involved in galactolipid remodelling of the outer chloroplast envelope (Moellering *et al.*, 2010).

The *sfr3* mutation causes the greatest freezing sensitivity in young incompletely expanded leaves. Expression analysis (Knight *et al.*, 1999) indicates that it is not compromised in its ability to induce genes associated with cold stress signalling pathways. Plants with this mutation are indistinguishable from wild-type plants at normal growth temperatures but display two pleiotropic effects at 4 °C; in the short term (during cold acclimation), mutant plants are deficient in anthocyanin, and in the long term (8 weeks and above), senescence of older leaves is accelerated (McKown *et al.*, 1996). On current knowledge, neither of these phenotypes would be expected to directly cause freezing sensitivity. Therefore, elucidating the function of the wild-type *SFR3* gene will give new insights into the mechanism by which plant cells achieve freezing tolerance. In this paper, we identified *SFR3* and investigated its role in freezing tolerance.

## Materials and methods

### Plant growth, and freeze and drought testing

Plants were grown as previously described (Thorlby *et al.*, 2004). To screen individuals for freezing tolerance, seedlings were grown for 4/5 weeks at 18–20 °C with a 9 h photoperiod at 250  $\mu\text{M m}^{-2} \text{s}^{-1}$ , and then

subjected to 11 d of cold acclimation at 4 °C, with an 8 h photoperiod at 220  $\mu\text{M m}^{-2} \text{s}^{-1}$ . They were placed in a freezer with an air temperature minimum of –6.0 °C for 16 h and then returned to their pre-acclimation growth conditions. Injury was assessed after 5 d. Cold treatments were carried out for the time period stated under the same conditions as for cold acclimation. For drought testing, plants were grown at 18–20 °C as above, with or without a period of cold treatment. Water was withheld and the plants monitored until they wilted. Plants were then watered and recovery assessed.

### Mapping of *SFR3*

From the mapping cross of *sfr3/sfr3* Col (Columbia)  $\times$  *SFR3<sup>+</sup>/SFR3<sup>+</sup>* Ler (Landsberg *erecta*), a total of 557 new F<sub>2</sub> zygotes were screened for crossovers in this region. Among those zygotes showing crossovers, the *SFR3* genotypes were determined by freeze testing the F<sub>3</sub> progeny of each zygote. Details of the markers developed in this work are available on request. DNA samples were isolated from F<sub>2</sub> plants (Thorlby *et al.*, 1999), and analysed for cleaved amplified polymorphic sequence (CAPS) marker genotypes (Konieczny and Ausubel, 1993) and simple sequence length polymorphism marker genotypes (Bell and Ecker, 1994).

### Subcloning for complementation

*In silico* analysis of the genomic sequence indicated that gene At1g36160 was contained on a 15 kb *SpeI* fragment on bacterial artificial chromosome (BAC) F12D14. A genomic fragment was cloned from this BAC into a modified version of the vector pCAMBIA3300 (CAMBIA, Canberra, Australia: <http://www.cambia.org>) that had an *SpeI* site introduced into the multiple cloning site.

### Plant transformation and selection

Plasmids were transferred to *Agrobacterium tumefaciens* GV3101/pM90 (Koncz and Schell, 1986) by electroporation (Cangelosi *et al.*, 1991). Plants were transformed by the floral dip method (Clough and Bent, 1998). Primary transformants were selected for Basta-resistance (the marker in the pCAMBIA vectors) by repeated spraying of the seedlings with a 250 mg l<sup>-1</sup> of a solution of the herbicide Challenge 60 (AgrEvo Ltd, King's Lynn, UK) until the growth differential was clear.

### Visualization of defects in leaf cuticle using toluidine blue staining

The method of Tanaka *et al.* (2004) was used. Seeds were grown on Petri dishes of 0.5  $\times$  MS medium solidified with 0.7% plant culture-tested agar (Sigma). Plants were stratified at 4 °C for 3 d, transferred to a growth chamber at 22 °C with continuous light for 3/4 weeks, and then transferred to a 4 °C growth room for periods of up to 10 d. To stain plants, aqueous 0.05% (w/v) toluidine blue filtered through a 0.2  $\mu\text{m}$  syringe filter (Sartorius, Hannover, Germany) was poured into the culture plate until the plants were submerged and they were incubated for 2 min. The staining solution was poured off and the plants were washed by submerging them twice in distilled water. Stained plants were viewed by light microscopy.

### Measurement of chlorophyll leaching

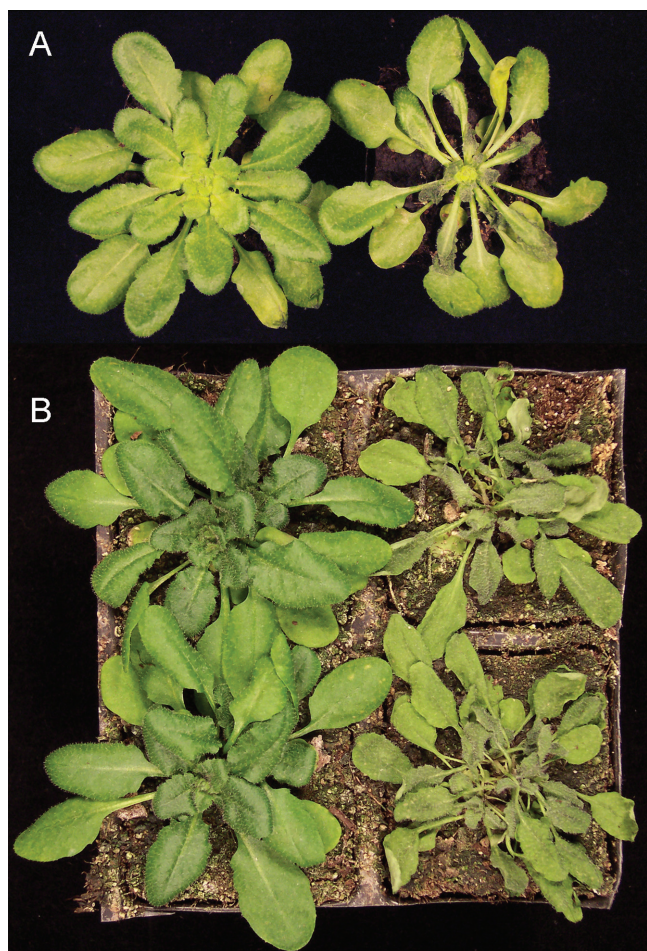
To measure chlorophyll leaching, the aerial parts of 4-week-old plants, grown as described for freeze testing, were weighed and immersed in tubes containing 30 ml of 80% ethanol. Approximately 0.5 g of material was used per assay. Tubes were incubated in the dark with gentle agitation on a rotary shaker, aliquots taken at the indicated times and the absorbance measured at 654 nm. Chlorophyll content was calculated according to the formula  $A_{654} \times \text{extraction volume (ml)} \times 1/39.8 \times 1/\text{weight of tissue sample (g)} = \text{mg chlorophyll g}^{-1} \text{ fresh weight of tissue}$  (Wintermans and de Mots, 1965).

### Scanning electron microscopy

Stems from wild-type and *sfr3* plants were collected, allowed to air dry, mounted on stubs, and coated with gold particles using a Polaron E5100 coating unit. They were examined using a Hitachi S3000N scanning electron microscope at an accelerating voltage of 20kV.

### Analysis of waxes

Detached leaves were immersed with gentle swirling in chloroform for 30 s, carefully removed, and the extracts dried under nitrogen. Metabolite analysis of the extracts was by the gas chromatography/mass spectrometry method of Fiehn *et al.* (2000) but using a greater range of authentic standards for lipophilic metabolite identification. Each analysis was performed with six biological replicates.



**Fig. 1.** The *sfr3* phenotype. (A) Comparison of wild-type (left) and *sfr3* (right) plants after a freezing test. Cold acclimated plants were frozen at  $-6.0\text{ }^{\circ}\text{C}$  for 16h and returned to pre-acclimation growth conditions for recovery. The picture was taken after 4 days recovery. All leaves of the wild-type appeared healthy and green, as did the older, fully expanded leaves of *sfr3* plants. The younger leaves of *sfr3* plants showed severe damage and were discoloured and collapsed. (B) Wild-type (left) and *sfr3* (right) plants after water was withheld for 25 days from plants maintained at  $4\text{ }^{\circ}\text{C}$ . Damage was apparent across the whole rosette but was most severe in young leaves (none fully expanded).

## Results

### Isolation and identification of the *SFR3* gene and *sfr3* mutation

The *sfr3* mutation was isolated from the pedigree ethyl methyl sulfonate mutant set of James and Dooner (1990) as a freezing-sensitive mutant (Warren *et al.*, 1996), previously mapped close to the centromere region on chromosome I between markers AIG1 and T27K12 (Thorlby *et al.*, 1999). It had a clear freezing-sensitive phenotype (Fig. 1A).

The gene was fine mapped to a 220 kb region of the *Arabidopsis* genome between genes At1g36150 and At1g36410 (Supplementary Fig. S1 at JXB online). This interval was examined for gene annotations. As expected, due to the proximity of the chromosome I centromere, this interval had a low gene density and a high frequency of pseudogenes and transposon genes. Genes with homology to transposable elements and genes annotated as pseudogenes are unlikely to encode a non-redundant function and were not initially selected for analysis. The remaining genes in the 220kb interval were analysed by sequencing and/or by the isolation and analysis of knockout mutants, when available.

A total of nine genes were sequenced using DNA amplified from the *sfr3* mutant and compared with their database versions. The gene At1g36160 was different from the database but the rest were identical. The sequence change in At1g36160, G $\rightarrow$ A at nt 910 in the coding sequence (nt 1541 in the genomic DNA), created a missense mutation (Fig. 2A) and a restriction site difference; a *Bse*RI site found in the wild-type was not present in *sfr3*. We exploited this to develop a CAPS marker to distinguish between the wild-type and *sfr3*, and we used this marker to confirm segregation of the mutant phenotype with the sequence change.

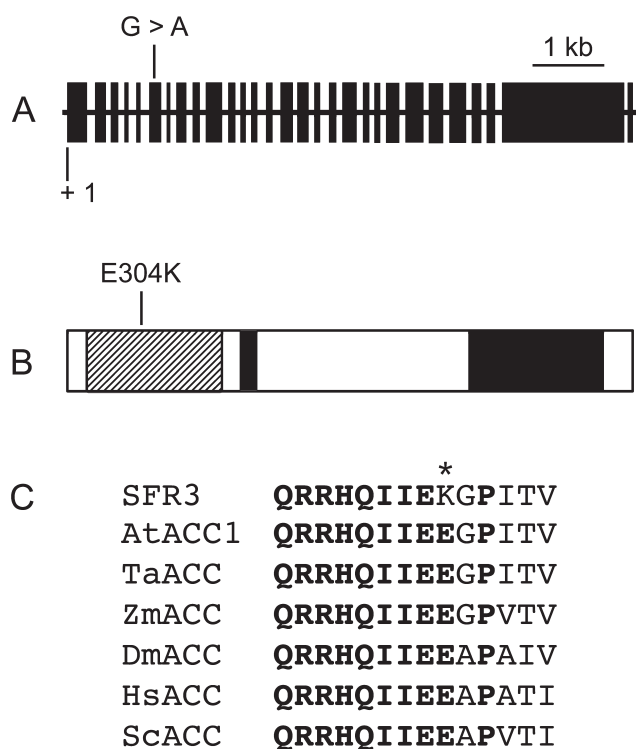
At1g36160 encodes a homomeric acetyl-CoA carboxylase (ACCase), ACC1, shown previously to be essential for embryo survival (Baud *et al.*, 2003). Recently, a weak allele of ACC1, *glossyhead* (*gsd1*), was identified (Lu *et al.*, 2011) that does not cause embryo death (unlike other ACC1 mutants) but rather confers phenotypic changes associated with perturbation in the biosynthesis of cuticular waxes.

### The *sfr3* mutation is complemented by ACC1

The ACC1 gene was cloned from BAC F12D14, which contains the ACC1 gene in its entirety, into a plant transformation vector. A region of 4265 bp upstream of the translation start codon and 1188 bp of the downstream sequence were included with the gene. The incorporation of these regions should give normal control of gene expression in the resulting transgenic lines. No other genes, according to current genome annotations, were present in the cloned fragment.

Out of 24 freeze-tested primary *sfr3* transformant plants, 23 displayed freezing tolerance indistinguishable from the wild-type (Supplementary Fig. S2 at JXB online). A number of freezing-tolerant primary transformants that showed complementation with the wild-type gene were selected for further analysis. The plants were left to self-fertilize and set seed. Testing of plants grown from this seed confirmed that the transgene segregated with the freezing-tolerant phenotype, confirming ACC1 as the site of the *sfr3* mutation.

In *Arabidopsis*, there are potentially two genes, located contiguously within a 25 kb region near the centre of chromosome 1, encoding the homomeric form of ACCase, *ACC1* and *ACC2* (At1g36160 and At1g36180) (Yanai *et al.*, 1995). As the gene At1g36180/*ACC2* was within the area delineated as containing *SFR3*, it was sequenced, but it did not contain a sequence change. A T-DNA knockout homozygous for the T-DNA insert within At1g36180/*ACC2* was also isolated. This line did not show either a pre- or post-freezing phenotype, suggesting that this second homomeric ACCase is not an essential gene and is not necessary for freezing tolerance. Baud *et al.* (2003) obtained similar results, demonstrating that no ACCase protein could be detected in *acc1* knockout mutant embryos, suggesting that *ACC2* is either not expressed or is expressed only at a very low level.



**Fig. 2.** Location of the *sfr3* mutation in the *ACC1* gene. (A) Schematic representation of the *ACC1* gene showing the location of the *sfr3* point mutation. Solid boxes represent exons. (B) Amino acid change in the conceptual translation product of the *ACC1* gene. The hatched box, dotted box, and solid box represent the biotin carboxylase domain, biotin carboxyl carrier domain and carboxyl transferase domain, respectively. (C) Alignment of amino acids in the conserved region of the biotin carboxylase domain of acetyl-CoA carboxylases of various species. Bold letters indicate invariant amino acids while the asterisk identifies the amino acid changed in the *sfr3* mutant. AtACC1, *Arabidopsis thaliana* ACC1 (At1g36160); TaACC, *Triticum aestivum* cytosolic ACCase (A57710); ZmACC, *Zea mays* ACCase (T02235); DmACC, *Drosophila melanogaster* ACCase (CG11198-PB); HsACC, *Homo sapiens* ACCase (S41121); ScACC, *Saccharomyces cerevisiae* ACCase (P11029).

### The *sfr3* mutation is within a highly conserved motif

The nucleotide change in the *sfr3* mutant created a missense mutation that changes aa 304 in the ACC1 protein from a glutamic acid to a lysine (Fig. 2B). This amino acid is part of a highly conserved motif, the carbamoyl-phosphate synthase L chain ATP binding subdomain of the biotin carboxylase domain, which is conserved in plants, animals, and yeast (Fig. 2C). Interestingly, the mutation E86K in *gsd1*, the only other non-embryo-lethal mutation currently identified in *ACC1*, also lies in the biotin carboxylase domain (Lu *et al.*, 2011).

### The *sfr3* mutant is drought sensitive in the cold

As *ACC1* has been implicated in the development and maintenance of the plant cuticle (Baud *et al.*, 2004; Lu *et al.*, 2011), the primary barrier to water loss in plants, the drought tolerance of *sfr3* plants was examined. The recovery of *sfr3* and wild-type plants after water was withheld for a time and then made available was identical when they were grown in the warm (data not shown). Differences between *sfr3* and the wild-type plants were apparent, however, if drought testing was carried out on plants that had been grown in the warm for 4 weeks before transfer to the cold for drought testing. Immediately after transfer to the cold, water was withheld. After 25 d without water, *sfr3* plants wilted while wild-type plants remained vigorous (Fig. 1B). Wild-type plants did not show signs of water stress for an additional 5 d, whereas, at this time, watering of *sfr3* plants did not result in their recovery.

### The *sfr3* mutation results in a cold-induced increase in cuticle permeability

Several assays have demonstrated cuticular deficiencies in cold-grown *sfr3* plants. Toluidine blue stains the epidermis in plants with damaged cuticle but not when the cuticle is intact (Tanaka *et al.*, 2004), and the degree of staining reflects the severity of cuticular damage. Neither wild-type nor *sfr3* plants showed epidermal staining following growth in the warm (data not shown). Wild-type plants that had been transferred from the warm and grown for 10 d at 4 °C also showed no staining (Fig. 3E, 3I), but *sfr3* plants treated in the same way showed distinct staining (Fig. 3B, 3F, and 3J; C, G, and K; and D, H, and L). The youngest leaves stained strongly, while older leaves stained less intensely or not at all. There was very little staining of the leaf pedicle, even in the most intensely stained plants. The toluidine blue staining procedure was also performed on plants that had been grown at 4 °C for shorter periods. No staining could be detected on plants that had spent less than 6 d in the cold (data not shown).

The permeability of the plant cuticle can also be assessed by measuring the release of chlorophyll from leaves immersed in 80% ethanol (Aharoni *et al.*, 2004; Schnurr *et al.*, 2004). No difference in chlorophyll release was detected between wild-type and *sfr3* plants grown in warm conditions (Fig. 4A), but after only 24 h of growth in the cold, differences were apparent (Fig. 4B). Chlorophyll was leached more rapidly from *sfr3* plants, and the amount released increased with longer growth in the cold (Fig. 4C, 4D). Extended growth at low temperature had little effect on chlorophyll leached from wild-type plants.

The loss of water by evaporation from wild-type and *sfr3* plants was compared (Supplementary Fig. S3 at *JXB* online). Loss from whole detached rosettes of warm-grown wild-type and *sfr3* plants were similar. After 10 d of growth in the cold, wild-type plants lost water at a similar rate to warm-grown plants, but *sfr3* plants lost significantly more water (Supplementary Fig. S3).

#### *Epicuticular wax morphology is altered on cold-grown sfr3 inflorescence stems*

Scanning electron microscopy was used to examine epicuticular wax crystals on wild-type and *sfr3* inflorescence stems. These observations were not possible with leaf material due to the lack of visible epicuticular wax crystals on *Arabidopsis* leaves. Plants that developed an inflorescence stem in the warm (data not shown) or that were transferred to 4 °C for a further 21 d of growth after inflorescence stem development showed a dense pattern of crystals over the surface of the stem for both wild-type (Fig. 5A) and *sfr3* (Fig. 5B) plants. Plants that had initiated bolting but had not produced a significant inflorescence stem before transfer to the cold showed a different result; wild-type stems were decorated with a dense pattern of wax crystals (Fig. 5C) similar to those produced in the warm, whereas the short stems that developed on *sfr3* plants in the cold were devoid of wax crystals (Fig. 5D). Thus, wax is deposited normally on *sfr3* stems that develop in the warm but not in the cold.

#### *Wax composition is altered on sfr3 leaves that develop in the cold*

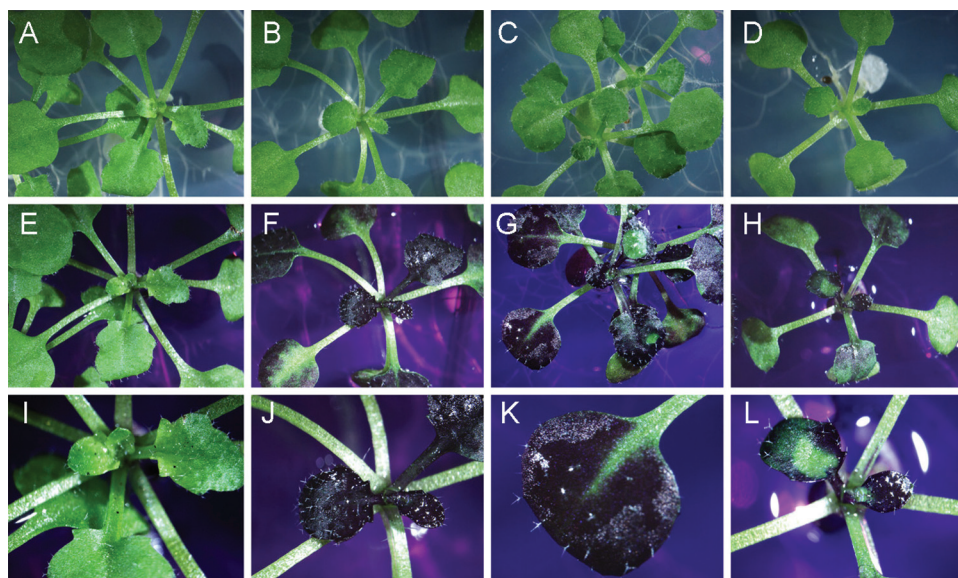
To test whether the cuticular deficiencies of *sfr3* were associated with changes in wax composition, a comparative analysis of the major classes of wax components present on leaves was carried out. A previous analysis of foliar fatty acids (McKown *et al.*, 1996) demonstrated no differences between *sfr3* and wild-type

plants in either the warm or cold. As *Arabidopsis* shows only limited growth at 4 °C, analysis was performed on plants that had been maintained at this temperature for a relatively long period (4 weeks) following initial growth at 20 °C for 3 weeks. The youngest fully expanded leaves were analysed, as these were likely to have had the highest proportion of their wax load deposited under cold growth conditions.

This comparative analysis showed that the leaf wax composition of warm-grown *sfr3* plants was similar to that of wild-type plants (Fig. 6). No significant alterations in the amount of alkanes, the largest component of *Arabidopsis* leaf wax, were found. The relative amounts of primary alcohols and fatty acids were also similar. There was a small increase in the amount of the longer-chain-length varieties of these components on *sfr3* leaves, suggesting that the mutant is not deficient in malonyl-CoA or in its ability to produce cuticular wax in the warm. Substantial differences between *sfr3* and wild-type wax composition became apparent when plants were grown in the cold (Fig. 6). The amount of alkanes, the major wax constituent, was reduced in *sfr3* plants compared with wild-type plants. There was a significant decrease ( $P < 0.05$ ) in the amount of two of the longer alkane components, with C33 reduced to 60% of the amount seen in wild-type plants. There was also a decrease in the amount of longer-chain-length primary alcohol and fatty acid components of the wax, and an increase in the amount of the shorter C20 and C22 fatty acids.

#### *Long-term growth in the cold affects flower development*

To investigate the effect of longer periods of cold on the growth of *sfr3* plants, 4-week-old warm-grown plants were transferred to cold growth conditions and maintained under these conditions for 10 weeks. Previous observations have shown that, after 8 weeks' growth in the cold, accelerated senescence of



**Fig. 3.** Toluidine blue staining of *sfr3* and wild-type seedlings grown in the cold for 10 d. A control wild-type plant before (A) and after (E, I) staining is shown. Three different *sfr3* plants are shown before (B–D) and after (F–H and J–L) staining. The images in the lower panel (I–L) are close ups of the images in the panel above to show details of the different staining patterns.

older leaves occurs (McKown *et al.*, 1996). After a 10-week cold growth period, accelerated senescence of older leaves was again observed, but in other respects the mutant appeared similar to wild-type plants. Differences were, however, observed in the development of flowers. If plants were transferred to cold growth conditions shortly after the initiation of bolting, the inflorescence stem of the *sfr3* mutant was stunted compared with the wild type, and flower development was abnormal (Fig. 7). Shrunken slightly browned structures developed in place of petals, and these senesced without expanding (Fig. 7). Normal siliques developed in wild-type plants, but were not observed in *sfr3* mutants.

## Discussion

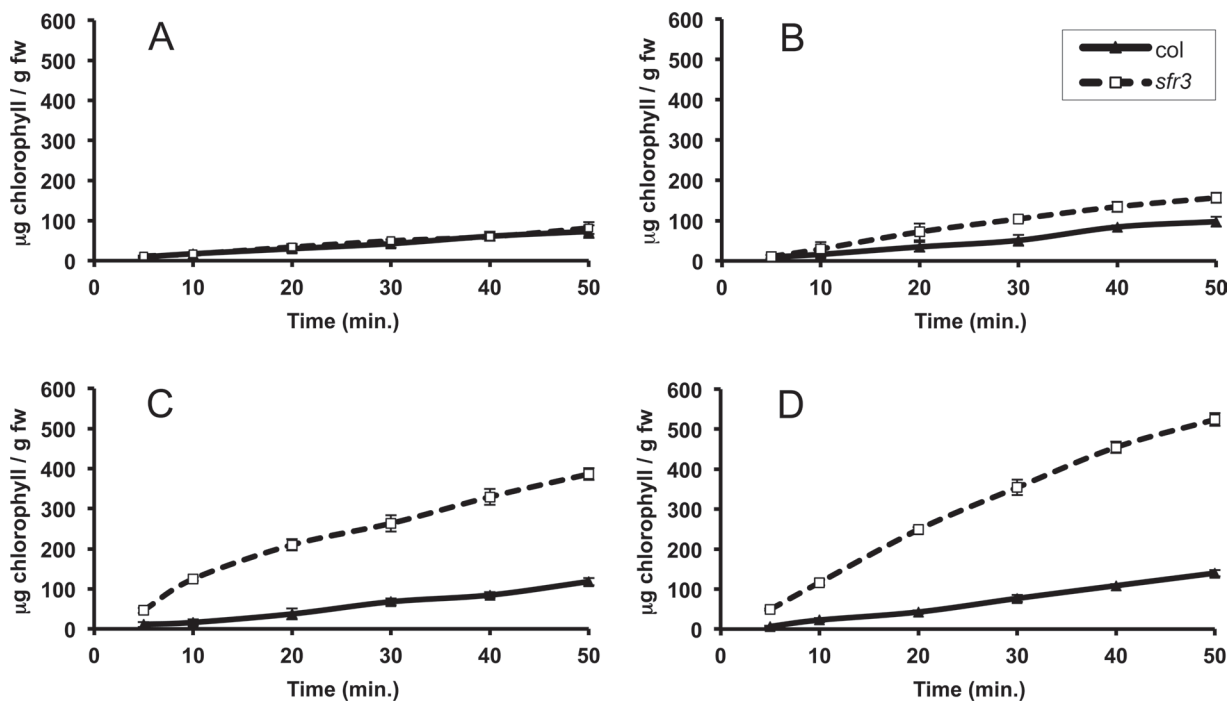
A missense mutation in *ACCI*, a gene encoding a homomeric acetyl-CoA carboxylase (ACCase) is responsible for the freezing sensitivity of *sfr3*. ACCase catalyses the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, the first and committing step in *de novo* fatty acid biosynthesis (Nikolau *et al.*, 2003). Malonyl-CoA is also essential for further elongation of fatty acids to long-chain and very-long-chain fatty acid forms.

ACCases are composed of three distinct functional domains. A structural domain carries the biotin prosthetic group (the biotin carboxyl carrier domain). One catalytic domain, the biotin carboxylase domain, catalyses carboxylation of the biotin prosthetic group, and a second, the carboxyltransferase domain, transfers the carboxyl group from carboxy-biotin to acetyl-CoA (Nikolau

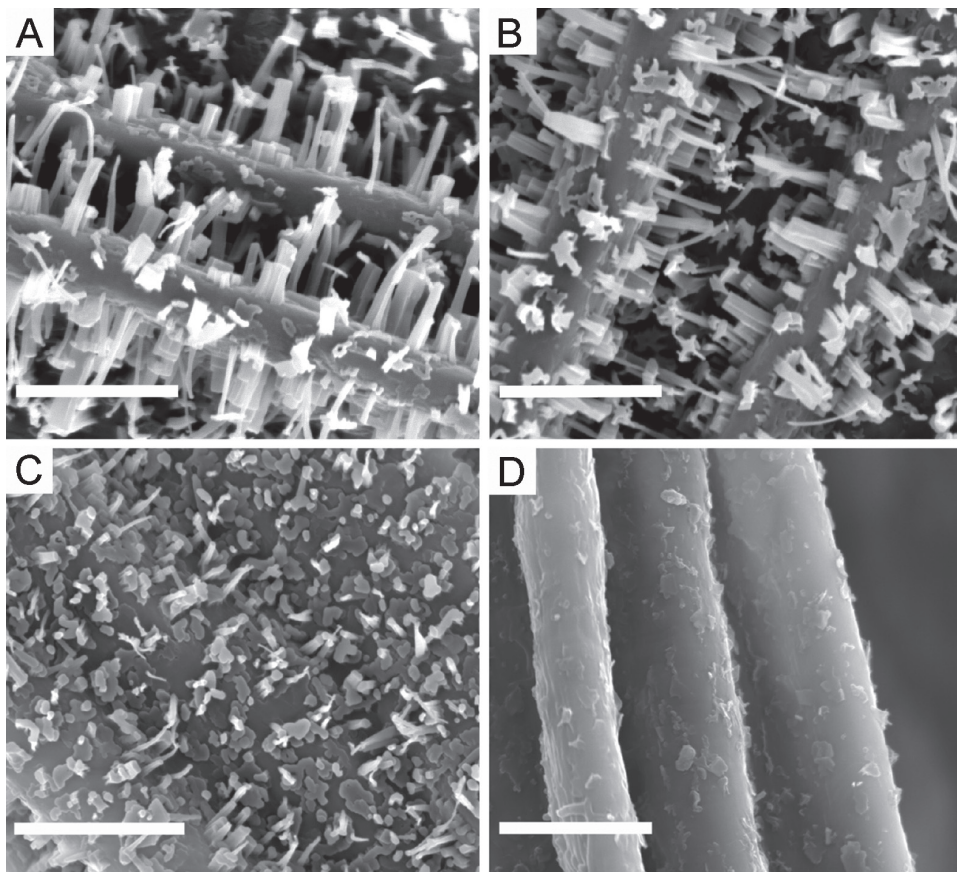
*et al.*, 2003). Each of these domains can be encoded as a separate polypeptide. This is the case for the heteromeric ACCases found in plastids of most non-graminacea plants, which are responsible for the production of the plastidic malonyl-CoA pool required for *de novo* fatty acid synthesis. In the second type of ACCase, the homomeric form, all of the functional domains are encoded on a single polypeptide.

Homomeric ACCase is responsible for the production of the independent cytosolic pool of malonyl-CoA. This pool is separate from the plastid pool because membranes are impermeable to acyl-CoAs (Jacobson and Stumpf, 1972). The cytosolic pool of the malonyl-CoA pool produced by homomeric ACCase is required for a wide range of reactions. These include the elongation of plastid-synthesized C16 and C18 fatty acids to long-chain and very-long-chain fatty acids for seed storage triacylglycerols, waxes, or sphingolipids. Malonyl-CoA is also required for the synthesis of various secondary metabolites, including flavonoids, and the malonation of amino acids and glycosides (Roesler *et al.*, 1994). Mutation of *ACCI* probably reduces (or eliminates) the cytosolic malonyl-CoA pool and thus affects those pathways that utilize it.

A number of plants carrying mutations in *ACCI* have been characterized and the gene has been shown to be essential for the production of very-long-chain fatty acids. The *gurke* and *pasticcino3* mutants of *Arabidopsis* have mutations in *ACCI* (Baud *et al.*, 2003, 2004) and develop abnormal embryos with altered cotyledon primordia. The embryos eventually die, leading to embryo lethality. The *sfr3* mutant is thus unlikely to represent a complete loss of function. More recently, a weak mutant



**Fig. 4.** Graphs showing the rate of leaching of chlorophyll into ethanol from *sfr3* plants compared with wild-type plants. The results from warm-grown plants (A) and warm-grown plants transferred to the cold for 24 h (B), 4 d (C), and 10 d (D) are shown. Experiments were performed in triplicate and results are shown as means  $\pm$  standard deviation. The total amount of chlorophyll present in the leaves of *sfr3* plants, measured by total extraction of chlorophyll in 96% ethanol, was not significantly different from that of wild-type plants (data not shown).



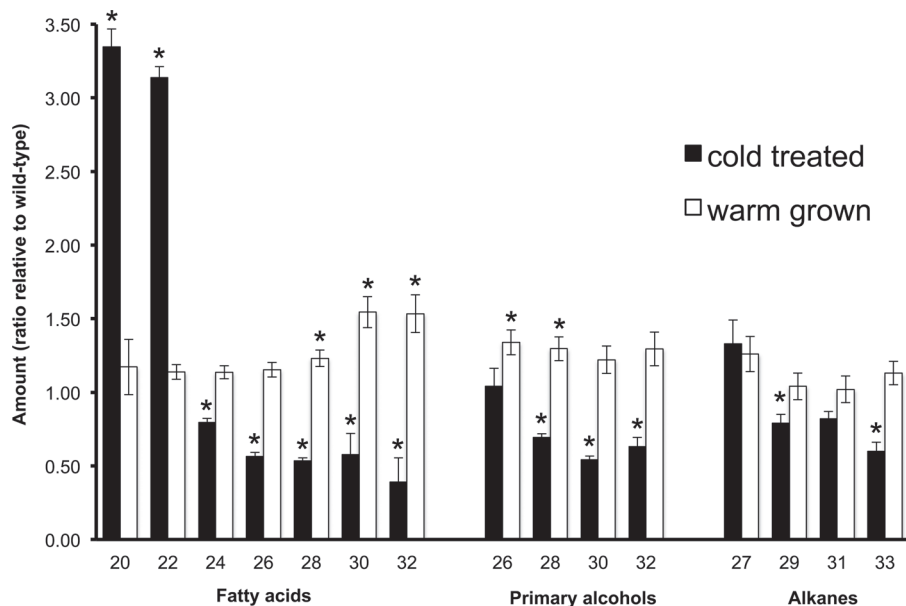
**Fig. 5.** Scanning electron microscopy images showing wax crystals on the inflorescence stem of wild-type (A, C) and *sfr3* (B, D) plants. The surface of wild-type (A) and *sfr3* (B) stems that developed in the warm before transfer to 4 °C for 21 d are decorated by densely distributed columnar-shaped epicuticular wax crystals. Wild-type stems that had initiated bolting in the warm before transfer to the cold showed a dense decoration of wax crystals (C). *sfr3* stems that were grown in the same way (initiation in the warm and growth in the cold) showed a total lack of visible wax crystals (D). Bars, 10  $\mu$ m.

allele of *ACC1*, *gsd1*, has been identified (Lu *et al.*, 2011). This mutant does not confer an embryo-lethal phenotype but produces plants with glossy inflorescence stems, post-genital fusion in floral organs, and reduced fertility. Characterization of *gsd1* has demonstrated the essential role of *ACC1* in the biosynthesis of cuticular waxes and the production of a functioning cuticle.

Several studies have established the ubiquitous expression of the *Arabidopsis ACC1* gene (Roesler *et al.*, 1994; Baud *et al.*, 2003; Lu *et al.*, 2011). This is not surprising, considering the wide range of reactions requiring malonyl-CoA. Transcript levels are highest in flowers and the silique walls of developing embryos, but the gene is also expressed in roots, stems, leaves, and developing seeds. A high proportion of very-long-chain fatty acids are incorporated into triacylglycerols deposited in seeds during maturation, but they are also essential for cuticle and wax synthesis (Baud *et al.*, 2004). Evidence from Nikolau and Wurtele (1998) using Northern blotting and *in situ* hybridization showed that *ACC1* mRNA accumulated in epidermal cells involved in epicuticular wax deposition. Similarly, studies in pea demonstrated that the homomeric form of ACCase is concentrated in the epidermal layer of leaves (Alban *et al.*, 1994). More recently, *Arabidopsis* microarray data (Suh *et al.*, 2005) and GUS

constructs (Lu *et al.*, 2011) have confirmed the preferential expression of *ACC1* in the epidermis of the upper section of inflorescence stems actively involved in cuticle deposition. This is consistent with the major destination of cytosolic malonyl-CoA in the developing plant being for the production of waxes for cuticle deposition. Indeed, even within the mutated embryos of lethal *acc1* alleles, there was evidence of severely altered cuticle formation (Baud *et al.*, 2004).

The broad lipid profiling studies carried out with *gsd1* have confirmed the essential role of *ACC1* in generating malonyl-CoA for wax synthesis (Lu *et al.*, 2011). The *gsd1* mutant exhibits a large reduction in total wax that is due mainly to the decrease in the production of the very-long-chain alkane, secondary alcohol, and ketone precursors (Lu *et al.*, 2011). Synthesis of cutin, the other major cuticle component, is enhanced in *gsd1*, probably because a reduced malonyl-CoA pool leads to the build-up of shorter acyl chains, which are shunted into the shorter-chain-length cutin (Lu *et al.*, 2011). Direct comparison of *gsd1* and *sfr3* wax composition is complicated by the cold-induced nature of the *sfr3* phenotype (see below). A substantial portion of the wax load on *sfr3* plants transferred to the cold is likely to be deposited before transfer, when plants are in the warm and the leaves are growing more actively.



**Fig. 6.** Graph showing a comparison of the relative amounts of the major classes of leaf wax components isolated from warm-grown and cold-treated *sfr3* plants. The ratio of each wax component relative to the amount on wild-type plants grown under the same conditions (warm or cold) is shown. Chemical classes and chain lengths are labelled on the horizontal axis. Statistically significant differences (Student's *t*-test,  $P < 0.05$ ) between wild-type and *sfr3* plants treated in the same way are indicated by an asterisk.

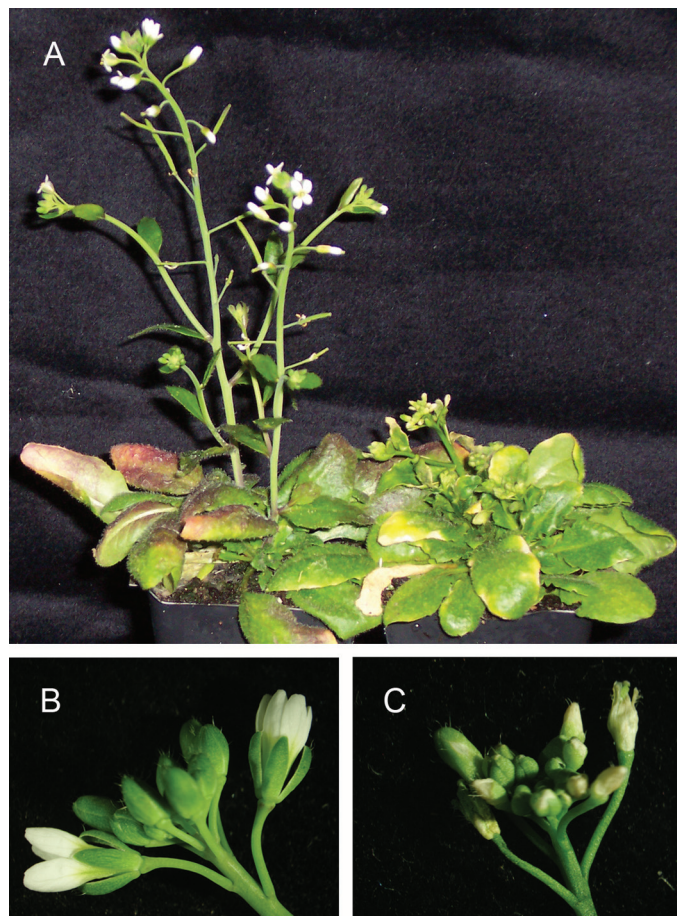
However, a comparative analysis (Fig. 6), using leaves from plants growing in the cold for relatively long periods, showed similar trends. Little difference was observed between *sfr3* and wild-type plants grown in the warm, but clear differences were present after transfer to (and further growth in) the cold. The longer-chain-length components of alkanes, primary alcohols, and fatty acids were all reduced compared with wild-type plants (Fig. 6), as might be expected if the malonyl-CoA pool required for their elongation is limited. The shorter C20 and C22 chain-length fatty acid components of the wax were increased in the cold, probably as a result of insufficient malonyl-CoA being present for further elongation.

The results presented here showed that the *sfr3* mutant cannot be distinguished from the wild-type when grown in the warm. Indeed, it has been shown previously that the ability of non-acclimated (warm-grown) wild-type plants to tolerate only mild freezing temperatures is not further compromised in the *sfr3* mutant (Warren *et al.*, 1996). Cold acclimation, however, has a clear detrimental affect on the ability of *sfr3* to develop the pronounced freezing tolerance associated with this process in wild-type. A number of assays clearly demonstrated that *sfr3* plants had cold-induced cuticular deficiencies that were not detectable in the warm. Evidence from chlorophyll efflux experiments (Fig. 4) suggested that there was some damage to the effectiveness of the cuticle within 24 h of transfer to the cold, and that damage increased with prolonged incubation. The results of the toluidine blue assay, where no staining was visible until 6 d after transfer to the cold, also suggested a gradual degradation of the effectiveness of the cuticle. This was further supported by the images of the rich decoration of wax crystals on the inflorescence stems of *sfr3* plants that developed in the warm and their absence on those that developed in the cold (Fig. 5), as well as by the comparative analysis of leaf cuticular waxes (above).

These observations suggest that, in the *sfr3* mutant, ACCase functions normally in the warm, or that sufficient ACCase activity is present to carry out all required metabolic functions. In contrast, following growth in the cold, the activity of ACCase becomes limiting and leads to clear deficiencies. This suggests that the *sfr3* mutation results in a cold-sensitive allele of *ACC1*. This would explain the normal growth of *sfr3* plants in the warm, while other mutations of *ACC1* have been shown to be embryo lethal (Baud *et al.*, 2004) or, in the case of *gsd1* (Lu *et al.*, 2011), to show clear phenotypic differences. Real-time PCR analysis (Supplementary Fig. S4 at *JXB* online) showed that the transcript level of *ACC1* did not differ between wild-type and *sfr3* plants in either warm or cold growth conditions. Thus, cold sensitivity is unlikely to be a product of transcript instability.

It is possible that *sfr3*, like *gsd1*, is a weak allele of *ACC1* and (unlike *gsd1*) is able to meet the metabolic requirement for cytosolic malonyl-CoA in the warm but not to meet an increased demand necessary for growth in the cold. If the *sfr3* phenotype were a result of an inability to respond to conditions requiring an increased malonyl-CoA supply, it might be expected that *sfr3* would differ from wild-type plants when the plants were subjected to drought stress in the warm as this treatment has been shown to lead to a substantial increase in the deposition of waxes (Kosma *et al.*, 2009) and presumably the demand for malonyl-CoA. However, *sfr3* plants only showed a drought-induced phenotype following growth in the cold. Likewise, *gsd1*, a weak allele of *ACC1*, which shows a clear phenotype in the warm, appears to have a less severe stem wax phenotype (based on comparison with electron microscopy images presented by Lu *et al.*, 2011) than cold-grown *sfr3* plants. If *sfr3* is simply a weaker mutant allele of *ACC1* than *gsd1*, one might expect a less severe phenotype than is present with *gsd1* unless the increased malonyl-CoA demand in the cold is substantial.





**Fig. 7.** Image showing the effects of long-term growth at 4 °C on inflorescence development in *sfr3* plants. Warm-grown 4-week-old plants were transferred to 4 °C growth conditions shortly after the inflorescence stem had initiated and maintained at this temperature for 10 weeks. Wild-type plants (A, left plant) developed normal inflorescence stems supporting flowers and siliques, while *sfr3* plants developed only stunted inflorescence stems (A, right plant). Flowers of normal appearance developed on wild-type plants (B), while those on *sfr3* plants were abnormal (C). Petals were shrunken and brown and did not expand, and no siliques were observed.

The cuticular deficiencies associated with cold-treated *sfr3* (Figs 3 and 4) have been found in a number of mutants with deficient cuticular membranes (Chen *et al.*, 2003; Aharoni *et al.*, 2004; Schnurr *et al.*, 2004), as well as the *gsd1* allele of *ACC1* (Lu *et al.*, 2011). Many of these mutants display developmental defects that are not present in *sfr3* plants. Fusion of leaf surfaces and floral organs is often associated with a defective cuticle, and it has been suggested that this is due to the lack of cuticle allowing the transmission of signals that would not normally occur (Nawrath, 2006). The lack of such abnormalities observed on *sfr3* plants used in freezing tolerance experiments is likely to be a product of the cold-induced nature of the deficiencies that are not present during the normal growth and development of the plant. The abnormal flower development and infertility found following long-term growth in the cold (Fig. 7) suggested that this is the case and provides further evidence of the cold-sensitive nature of the mutation.

This work clearly demonstrates the critical role of the plant cuticle in resisting freezing and drought stress. The cuticle becomes compromised during cold acclimation (when plants are held at 4 °C), and plants are then sensitive to freezing in subsequent freezing assays. The primary function of the cuticle is to control water loss from leaves and other aerial parts of the plant (Riederer and Schreiber, 2001). The clear lack of tolerance to drought in cold-treated *sfr3* plants (Fig. 1B) suggests that the cold-treated mutant has an inability to control the loss of water. This alone may be sufficient to explain the freezing sensitivity of the *sfr3* mutant. The mutant may not be able to resist increased dehydration stress associated with recovery from freezing.

Evidence from other genes involved in cuticular wax deposition has indicated that wax deposition is associated with regions undergoing elongation (Xia *et al.*, 1996) and the biosynthetic flux into waxes is tightly coordinated with surface area expansion (Nawrath, 2006), i.e. wax deposition occurs as the leaf expands. Leaves of the *sfr3* mutant that have developed in the warm would be expected to have normal cuticle development and be less sensitive to a freezing challenge. However, leaves that develop further in the cold, after transfer to cold acclimation conditions, would have a compromised cuticle and be more sensitive to freezing. The greater sensitivity of younger leaves of *sfr3* plants, which mature in the cold, to freezing damage (Fig. 1A) is indicative of this, as is the more intense staining seen in younger leaves in the toluidine blue staining assay (Fig. 3). As the more mature leaves, which developed in the warm but have also experienced cold, are also damaged in freezing, and particularly in drought stress, it is clear that there is a need for continued wax deposition after leaves have matured. This may be associated with the repair of damaged cuticle or increased deposition occurring after transfer to the cold. Previous studies have suggested that alterations in stomatal density (Aharoni *et al.*, 2004) affect the ability of cuticle mutants to resist water loss. However, this does not appear to be a factor in *sfr3* plants, as no differences were detected between the stomatal density of wild-type and *sfr3* plants that were either grown in the warm or cold acclimated for 10 d (Supplementary Table S1 at *JXB* online).

The *sfr3* mutant provides a tool for further investigations of the role of the plant cuticle in plant development and resistance to both biotic and abiotic stresses. The ability to compromise the function of a previously effective cuticle by incubation of intact plants in the cold will facilitate further studies of cuticle function. The ability to inactivate (or at least reduce the activity of) *ACC1* using the same treatment will allow investigation of the role of this enzyme in plant metabolism and gene expression.

## Supplementary data

Supplementary data are available at *JXB* online.

**Fig. S1.** Mapping of the *sfr3* gene.

**Fig. S2.** Complementation of the *sfr3* mutation with the wild-type *ACC1* gene.

**Fig. S3.** Water loss from excised rosettes of *sfr3* and wild-type plants.

**Fig. S4.** Expression levels of *SFR3* in wild-type and *sfr3* plants in both cold and warm growth conditions.

**Table S1.** Stomatal density in wild-type and *sfr3* plants.

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