

# An Arabidopsis Mutant Impaired in Coenzyme A Biosynthesis Is Sugar Dependent for Seedling Establishment<sup>1</sup>

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Once the plant coenzyme A (CoA) biosynthetic pathway has been elucidated by comparative genomics, it is feasible to analyze the physiological relevance of CoA biosynthesis in plant life. To this end, we have identified and characterized Arabidopsis (*Arabidopsis thaliana*) T-DNA knockout mutants of two CoA biosynthetic genes, *HAL3A* and *HAL3B*. The *HAL3A* gene encodes a 4'-phosphopantothenoil-cysteine decarboxylase that generates 4'-phosphopantetheine. A second gene, *HAL3B*, whose gene product is 86% identical to that of *HAL3A*, is present in the Arabidopsis genome. *HAL3A* appears to have a predominant role over *HAL3B* according to their respective mRNA expression levels. The *hal3a-1*, *hal3a-2*, and *hal3b* mutants were viable and showed a similar growth rate as that in wild-type plants; in contrast, a *hal3a-1 hal3b* double mutant was embryo lethal. Unexpectedly, seedlings that were null for *HAL3A* and heterozygous for *HAL3B* (*aaBb* genotype) displayed a sucrose (Suc)-dependent phenotype for seedling establishment, which is in common with mutants defective in  $\beta$ -oxidation. This phenotype was genetically complemented in *aaBB* siblings of the progeny and chemically complemented by pantetheine. In contrast, seedling establishment of *Aabb* plants was not Suc dependent, proving a predominant role of *HAL3A* over *HAL3B* at this stage. Total fatty acid and acyl-CoA measurements of 5-d-old *aaBb* seedlings in medium lacking Suc revealed stalled storage lipid catabolism and impaired CoA biosynthesis; in particular, acetyl-CoA levels were reduced by approximately 80%. Taken together, these results provide in vivo evidence for the function of *HAL3A* and *HAL3B*, and they point out the critical role of CoA biosynthesis during early postgerminative growth.

CoA is a cofactor for a multitude of enzymatic reactions, including the oxidation of fatty acids, carbohydrates, and amino acids, as well as many synthetic reactions (Begley et al., 2001). CoA is synthesized in five steps from pantothenate, and recently all the biosynthetic enzymes have been cloned in both prokaryotes and higher eukaryotes (Begley et al., 2001; Daugherty et al., 2002; Kupke et al., 2003; Leonardi et al., 2005). Indeed, both in humans and plants, the complete biosynthetic pathway from pantothenate to CoA has been reconstituted in vitro using recombinant enzymes (Daugherty et al., 2002; Kupke et al., 2003). The universal pathway for biosynthesis of CoA from pantothenate is initiated by phosphorylation of this precursor to

generate 4'-phosphopantothenate, which is catalyzed by pantothenate kinase (PK). Then, the addition of Cys to 4'-phosphopantothenate gives rise to 4'-phospho-N-pantothenoil-cysteine (PPC), which is catalyzed by PPC synthetase. In the next step, PPC is decarboxylated to 4'-phosphopantetheine by PPC decarboxylase (PPCDC). Finally, 4'-phosphopantetheine is converted to CoA by the sequential action of the enzymes 4'-phosphopantetheine adenyltransferase and dephospho-CoA kinase. In humans, the two latter activities are encoded in a single bifunctional enzyme (Daugherty et al., 2002). In *Escherichia coli*, both the addition of Cys to 4'-phosphopantothenate and the subsequent decarboxylation of PPC are catalyzed by the bifunctional enzyme Dfp (mnemonic for DNA and flavoprotein; Kupke et al., 2000; Strauss et al., 2001; Kupke, 2002).

In plants, every step of CoA biosynthesis from pantothenate is catalyzed by single monofunctional enzymes, and the pathway has been reconstituted in vitro by combining the recombinant enzymes PK (AtCoaA, At1g60440), PPC synthetase (AtCoaB, At1g12350), PPCDC (HAL3A, AtCoaC, and At3g18030), 4'-phosphopantetheine adenyltransferase (AtCoaD, At2g18250), and dephospho-CoA kinase (AtCoaE, At2g27490). However, many aspects of the pathway are not well understood and, to our knowledge,

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the physiological consequences for plant life of impairing CoA biosynthesis have not been addressed by genetic analysis. In particular, the subcellular location of the Arabidopsis (*Arabidopsis thaliana*) CoA biosynthetic enzymes has not been studied in detail. It is known that the last enzyme of the pantothenate biosynthesis pathway (pantothenate synthetase) is found in the cytosol (Ottenhof et al., 2004), whereas PK activity appears to be predominantly localized in the chloroplast in spinach (*Spinacia oleracea*; Falk and Guerra, 1993), with some activity observed in cytosol. However, the five Arabidopsis members of the PK family (At1g60440, At4g32180, At2g17320, At2g17340, and At4g35360) as well as AtCoaC, AtCoaD, and AtCoaE do not show chloroplast-targeting sequences according to prediction programs such as ChloroP or PSORT. In any case, CoA itself is found in all cellular compartments and this multicompartmentation implies that there must be transporters present for shuttling intermediates. However, to our knowledge, currently only one transport activity for import of CoA into mitochondria has been reported in potato (*Solanum tuberosum*), although the corresponding gene has not been cloned (Neuburger et al., 1984).

As CoA plays an essential role in metabolism, null mutations in CoA biosynthetic genes are presumed to be lethal, unless there is some degree of genetic redundancy in the organism. For instance, in both yeast (*Saccharomyces cerevisiae*) and fly (*Drosophila melanogaster*), each with only one PK gene, the null mutant is nonviable (Winzeler et al., 1999; Afshar et al., 2001). To identify plant mutants impaired in CoA biosynthesis, we took advantage of the fact that some steps of this pathway are catalyzed by more than one gene product in Arabidopsis (Kupke et al., 2003). Plant mutants partially impaired in CoA biosynthesis offer the possibility to genetically test the function of CoA in plant biology. For instance, in oilseed plants the use of storage lipids during early seedling establishment is a key process for plant survival that is CoA dependent (Graham and Eastmond, 2002). Additionally, as CoA biosynthesis appears to be a sensitive step in plants under salt stress (Espinosa-Ruiz et al., 1999; Yonamine et al., 2004), an improved knowledge of this pathway might help our understanding of how plants cope with abiotic stresses. Finally, as the CoA biosynthetic pathway is evolutionarily conserved, its study in plants might have clinical relevance, as defects in this pathway lead to a human neurodegenerative disease (Zhou et al., 2001).

The plant CoA biosynthetic pathway has been recently defined in plants (Kupke et al., 2003), and the biochemistry of one of the biosynthetic enzymes, HAL3A (AtCoaC, PPCDC), has been studied in detail (Albert et al., 2000; Kupke et al., 2001; Hernandez-Acosta et al., 2002; Steinbacher et al., 2003). HAL3A is a flavoprotein that catalyzes the decarboxylation of PPC to 4'-phosphopantetheine (Kupke et al., 2001; Hernandez-Acosta et al., 2002), and overexpression of this enzyme leads to improved plant tolerance to salt and

osmotic stress (Espinosa-Ruiz et al., 1999; Yonamine et al., 2004). Two highly homologous genes, HAL3A (At3g18030) and HAL3B (At1g48605), are present in the Arabidopsis genome. Expression of HAL3A and HAL3B mRNAs was previously analyzed in seeds and different organs of adult plants (root, shoot, leaf, flower, and silique), as well as in 12-d-old seedlings (Espinosa-Ruiz et al., 1999). As a general result, transcript level of HAL3B mRNA was found to be lower than HAL3A mRNA. Therefore, according to their relative transcript levels, HAL3A appears to play a predominant role over HAL3B. For instance, HAL3B mRNA was expressed to very low level in seeds, whereas strong expression was observed for HAL3A mRNA. During embryogenesis, in situ hybridization of HAL3A mRNA revealed that the transcript was mainly detected in the cotyledons and hypocotyl of mature seeds, and to a lower level in the seed coat outer integument (Espinosa-Ruiz et al., 1999). Finally, according to northern-blot analysis, HAL3B mRNA was expressed 3- to 4-fold less than HAL3A mRNA in 12-d-old seedlings (Espinosa-Ruiz et al., 1999).

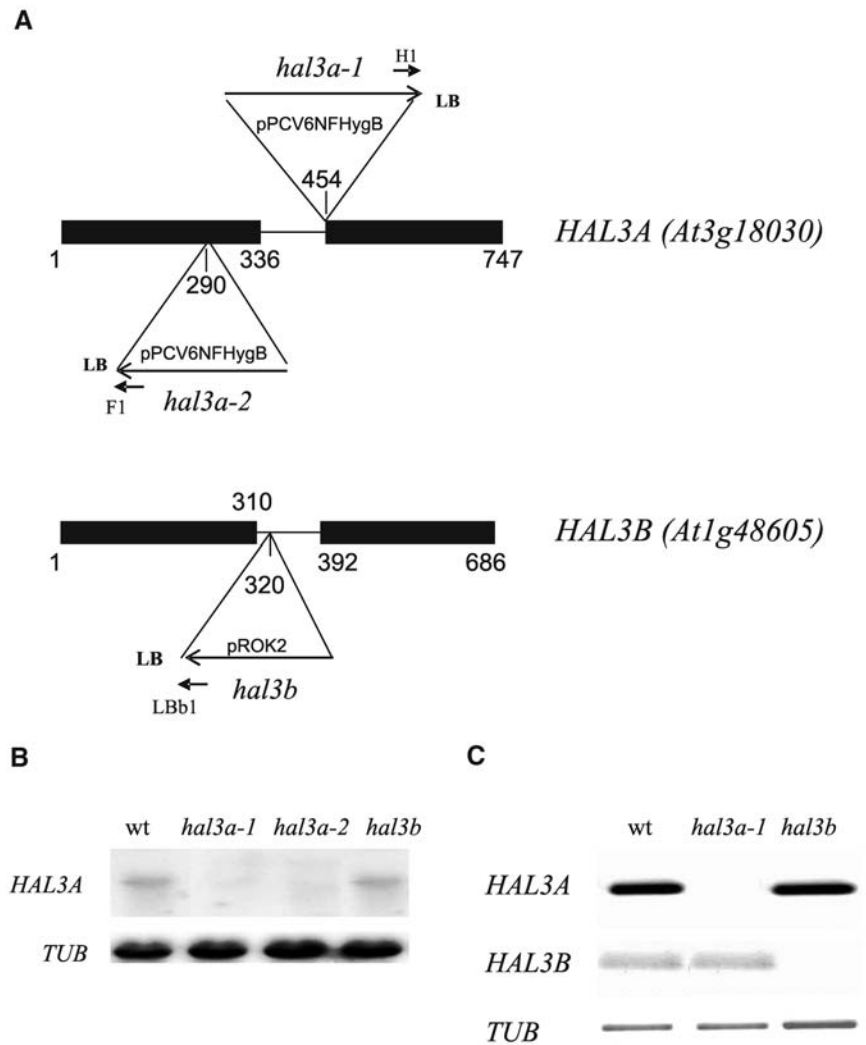
Even though the function of HAL3B has not been experimentally addressed (Leonardi et al., 2005), taking into account the high sequence similarity between both genes, we hypothesized that HAL3B might partially play a redundant role to HAL3A. Keeping in mind the crucial role of CoA biosynthesis for plant life, a certain degree of genetic redundancy would allow the identification of viable *hal3a* and *hal3b* mutants. Thus, T-DNA-disrupted alleles of HAL3A and HAL3B were identified in Arabidopsis T-DNA collections, and the corresponding homozygous mutants were isolated and found to be viable. Indeed, the single *hal3a* and *hal3b* mutants showed similar phenotypes to wild-type plants; however, a *hal3a hal3b* double mutant was embryo lethal, proving the expected crucial role for CoA biosynthesis. Unexpectedly, a Suc-dependent seedling establishment phenotype was found for *hal3a* plants that were heterozygous for the T-DNA-disrupted *hal3b* allele (*aaBb* individuals), which were unable to surpass the heterotrophic growth phase that occurs upon germination.

## RESULTS

### Isolation of T-DNA Insertional Mutations in the Arabidopsis HAL3A and HAL3B Genes

Two T-DNA-disrupted alleles of HAL3A were identified from the T-DNA collection of the Max-Planck Institute (Cologne, Germany) through PCR-based screening of Arabidopsis plants containing random T-DNA insertions (Rios et al., 2002; Fig. 1A). The first allele, *hal3a-1*, is a T-DNA insertion in the intron of the gene, and it is localized 454 nucleotides downstream from the ATG start codon (Fig. 1A). The second one, *hal3a-2*, is a T-DNA insertion in the first exon of the gene, and it is localized 290 nucleotides downstream from the ATG start codon. No HAL3A transcript was

**Figure 1.** Molecular characterization of *hal3a* and *hal3b* mutants. A, Scheme of the *HAL3A* and *HAL3B* genes and localization of the respective T-DNA insertions in the different alleles. The numbering begins at the ATG translation start codon. The T-DNA left border (LB) primers that were used to localize the T-DNA insertion are indicated. B, Northern-blot analysis of wild-type, *hal3a-1*, *hal3a-2*, and *hal3b* mRNAs probed with a cDNA probe specific for *HAL3A* transcript. C, RT-PCR analysis of wild-type, *hal3a-1*, and *hal3b* mRNAs prepared from 5-d-old seedlings. Primers specific for *HAL3A*, *HAL3B*, and *TUB* transcripts were used.



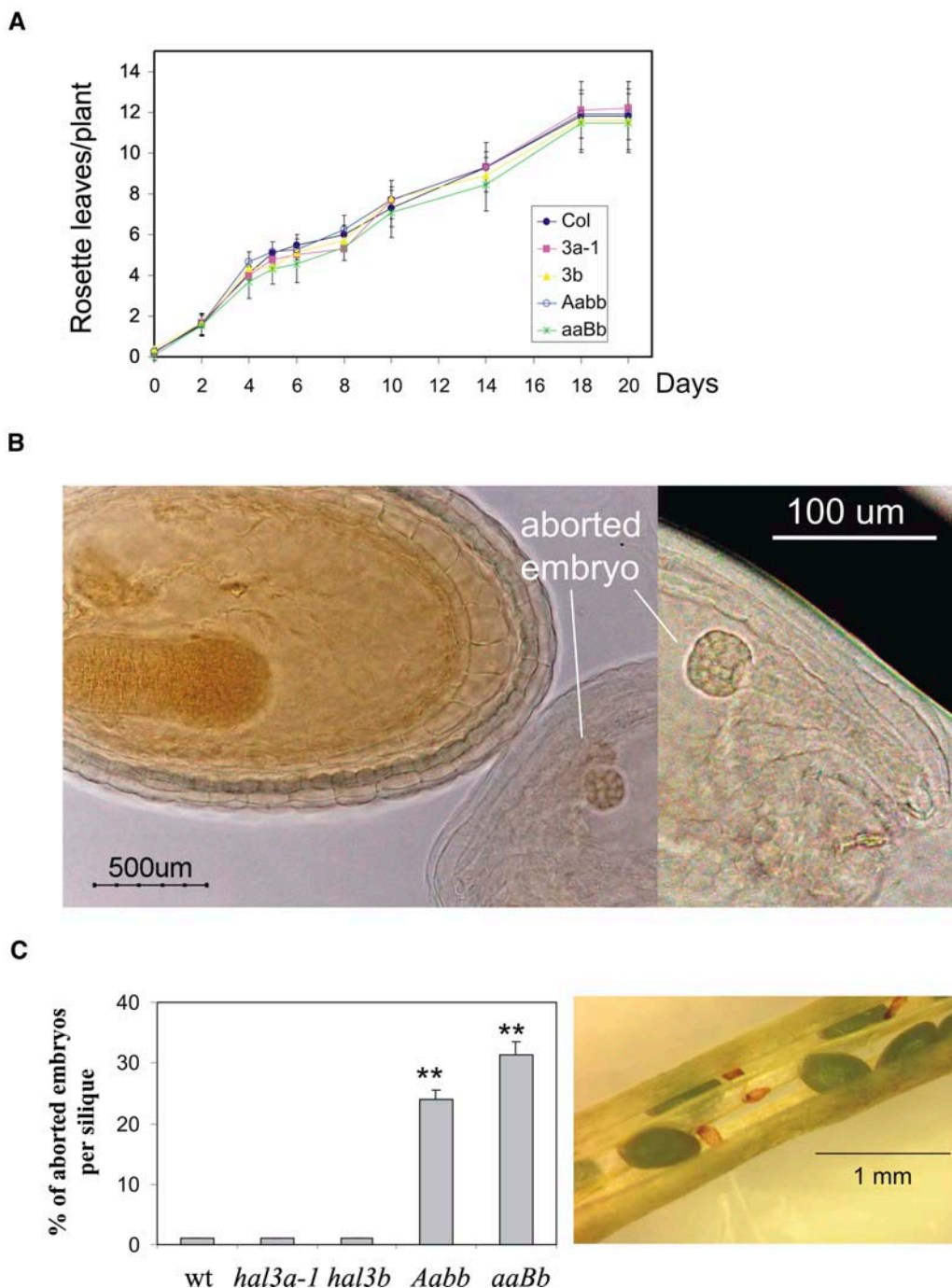
detected in seedlings of either mutant (Fig. 1B). In the case of *HAL3B*, no T-DNA-disrupted allele could be recovered from the Max-Planck collection. Moreover, only one T-DNA line from the currently available collections leads to disruption of the transcription unit (<http://signal.salk.edu/cgi-bin/tdnaexpress>), corresponding to donor stock number SALK\_045607 (Fig. 1A). This *hal3b* allele is a T-DNA insertion in the intron of the gene, and it is localized 320 nucleotides downstream from the ATG start codon (Fig. 1A).

Reverse transcription (RT)-PCR analysis was performed both for *HAL3A* and *HAL3B* mRNA expression in 5-d-old seedlings from wild-type, *hal3a-1*, and *hal3b* mutants (Fig. 1C). The expression of *HAL3B* was found to be lower than *HAL3A* in wild type (Fig. 1C), which confirms that *HAL3A* is the predominant isoform of *HAL3* present during seedling establishment (Espinosa-Ruiz et al., 1999). Indeed, 5-d-old seedlings showed a 4-fold higher expression level for *HAL3A* compared to *HAL3B*, as measured by RT-quantitative-PCR analyses (data not shown). Expression of *HAL3A* and *HAL3B* was abolished in the *hal3a-1* and *hal3b*

mutants, respectively (Fig. 1C). RT-quantitative-PCR analyses of *HAL3A* in *hal3b* or *HAL3B* in *hal3a-1* revealed a similar expression level to the one observed in wild type for each gene (data not shown).

#### *hal3a-1 hal3b* Double Mutant Is Embryo Lethal

Transgenic Arabidopsis plants (two lines) that express an antisense full-length cDNA of *HAL3A* were reported to show a delayed growth rate and impaired osmotic stress tolerance compared to wild-type plants (Espinosa-Ruiz et al., 1999). In contrast, the null mutant *hal3a-1* failed to show those phenotypes, as growth rate (Fig. 2A), salt sensitivity (see later Fig. 4C), and osmotic stress tolerance (data not shown) were quite comparable to wild-type plants. Similar results to those of *hal3a-1* were obtained for the allelic *hal3a-2* mutant (data not shown). Whereas a decrease in *HAL3A* transcript amount was measured in the two antisense lines constructed by Espinosa-Ruiz et al. (1999), *HAL3B* expression was not investigated. Antisense expression of full-length *HAL3A* cDNA might lead to a decreased *HAL3B* transcript

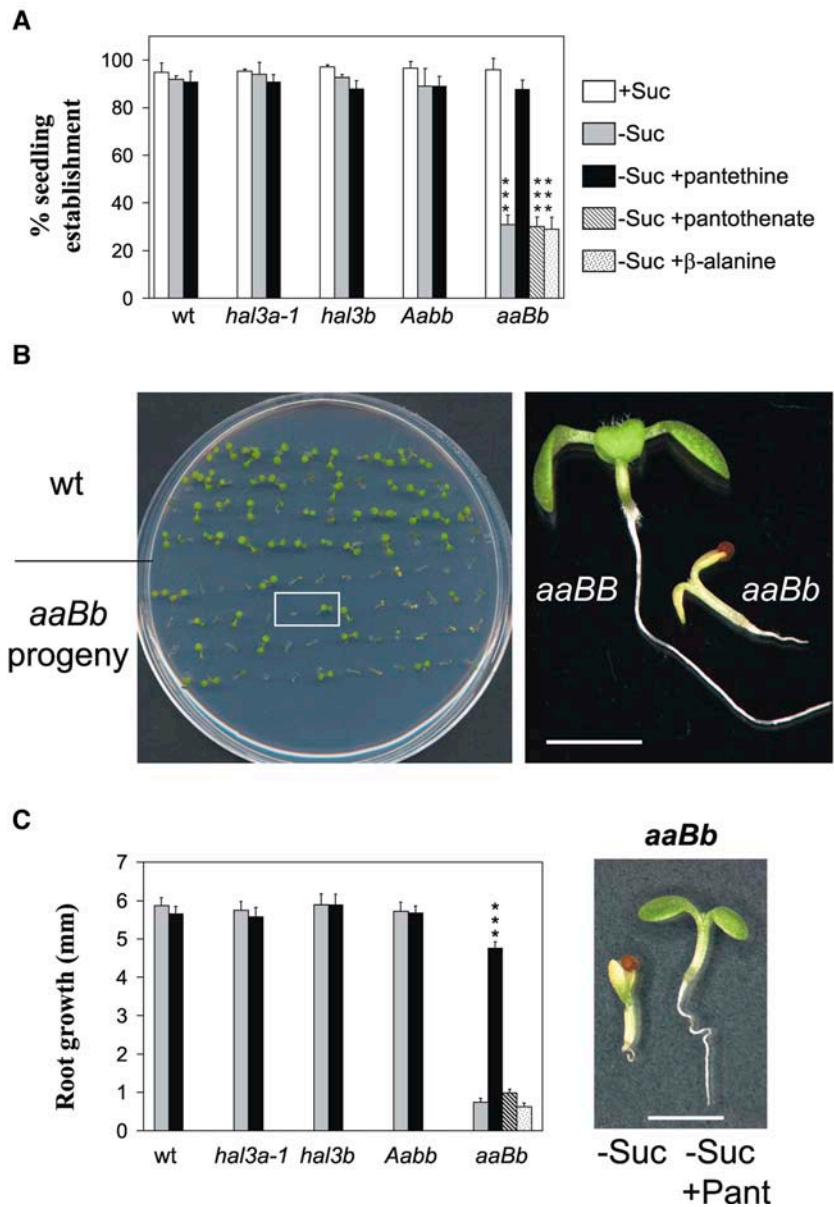


**Figure 2.** A, Growth rate of wild-type, *hal3a-1*, *hal3b*, *Aabb*, and *aaBb* plants. Values are averages  $\pm$  SD ( $n = 20$ ). B, The *hal3a-1 hal3b* double mutant is embryo lethal. Nomarski image of chemically cleared seed progeny from *aaBb* plants showing a viable embryo at the torpedo stage and an aborted embryo arrested at the early/midglobular stage. C, Percentage of aborted embryos per silique. Siliques of wild-type, *hal3a-1*, *hal3b*, *Aabb*, and *aaBb* plants were examined under a Nikon SMZ800 binocular glass. A representative silique from *aaBb* individuals is shown. Values are averages  $\pm$  SD ( $n = 20$ ). Double asterisks (\*\*) indicate  $P < 0.03$  (Student's *t* test) with respect to wild type (wt).

amount because of the high sequence identity between both genes at the nucleotide level. This explanation might reconcile the discrepancy between our results for *hal3a-1* and *hal3a-2* mutants, and the antisense approach of Espinosa-Ruiz et al. (1999).

No visible phenotype for the single *hal3a* or *hal3b* mutant was observed under our experimental conditions (Figs. 2, 3, and 4). As the predicted *HAL3A* and *HAL3B* gene products show 90% amino acid similarity, we reasoned that some functional redundancy might

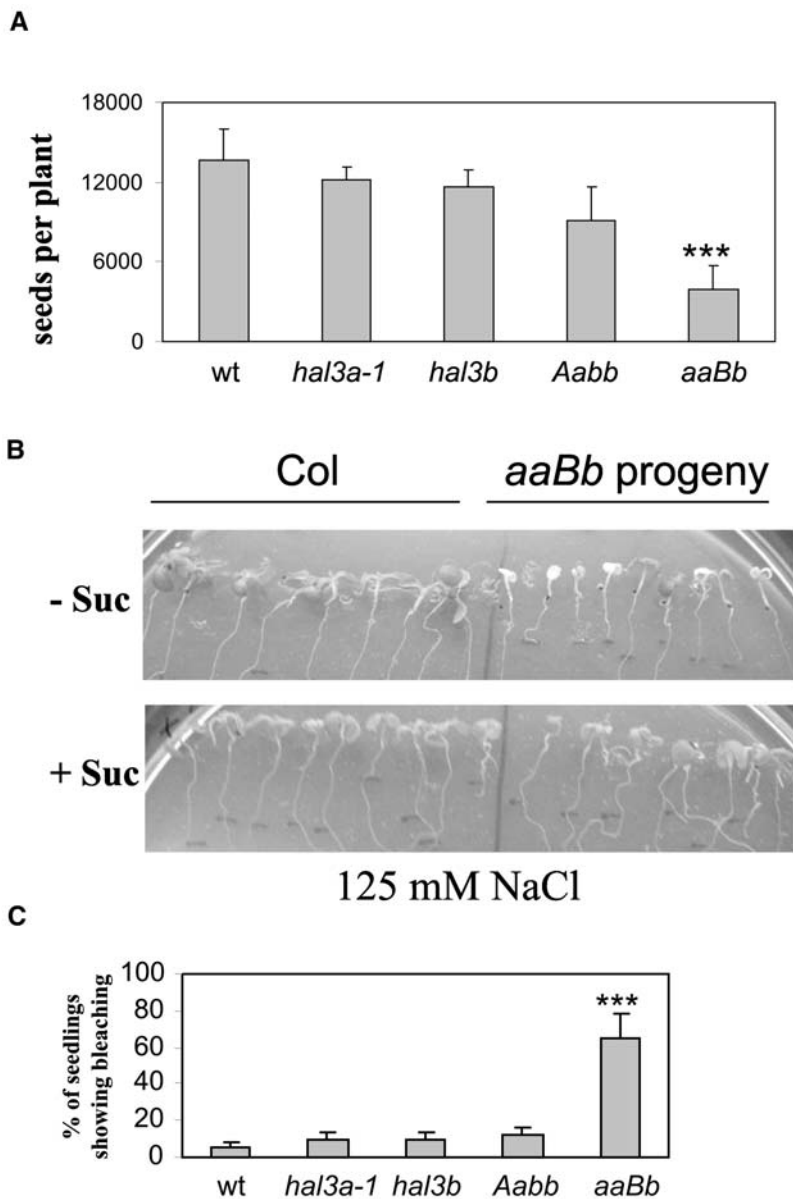
**Figure 3.** Suc-dependent phenotype of *aaBb* seedlings. Genetic and chemical complementation. **A**, Seedling establishment of the seed progeny from wild-type, *hal3a-1*, *hal3b*, *Aabb*, and *aaBb* plants in medium supplemented with 1% Suc (+Suc), lacking Suc (-Suc), lacking Suc and supplemented with 50  $\mu\text{g}/\text{mL}$  of pantethine (-Suc + pantethine), pantothenate (-Suc + pantothenate), or  $\beta$ -Ala (-Suc +  $\beta$ -Ala). Values are averages  $\pm$  sd for three independent experiments (200 seeds each). Triple asterisks (\*\*\*) indicate  $P < 0.01$  (Student's *t* test) with respect to *aaBb* + Suc. **B**, Segregating phenotype of the seed progeny from *aaBb* plants in medium lacking Suc. Representative *aaBB* and *aaBb* seedlings were removed from the medium at 7 d after sowing and they were photographed under a Nikon SMZ800 binocular glass. Bar = 2 mm. **C**, Root growth at 7 d after sowing from wild-type, *hal3a-1*, *hal3b*, *Aabb*, and *aaBb* individuals in medium lacking Suc (gray bars) or lacking Suc but supplemented with 50  $\mu\text{g}/\text{mL}$  of pantethine (black bars), pantothenate (cross-hatched bar), or  $\beta$ -Ala (stippled bar). Root growth in medium with 1% Suc was not statistically different for the seedlings. Values are averages  $\pm$  sd for two independent experiments (40 seedlings each). Triple asterisks (\*\*\*) indicate  $P < 0.01$  (Student's *t* test) with respect to *aaBb* in medium lacking Suc. Representative *aaBb* seedlings were removed from medium lacking Suc (-Suc) or lacking Suc but supplemented with 50  $\mu\text{g}/\text{mL}$  pantethine (-Suc + Pant) and rearranged on agar plates (right). Photograph was taken at 5 d after sowing. Bar = 2 mm. wt, Wild type.



exist between both genes. To establish whether *HAL3A* function is partially redundant with *HAL3B*, we tried to generate a double mutant by crossing *hal3a-1* and *hal3b* homozygous mutants in both directions. We could not recover double-homozygous mutants in spite of genotyping more than 200 plants of the  $F_2$  progeny. However, it was possible to identify *hal3a-1* plants that were heterozygous for the T-DNA-disrupted *hal3b* allele (*aaBb* genotype). Chemically cleared preparations from young fruits of *aaBb* plants showed that embryogenesis was arrested in approximately one quarter of the seeds, which degenerated into brown aborted seeds during maturation (Fig. 2, B and C). Indeed, no *aabb* double mutant was obtained in the progeny of self-fertilized *aaBb* plants, and the ratio of heterozygous *HAL3B/hal3b* to homozygous *HAL3B/HAL3B* plants was close to 2:1 (188:95,  $\chi^2 = 0.012$ ,  $P > 0.9$ ). This finding

suggests that viable embryos of the *aaBb* seed progeny must represent either the *aaBB* or *aaBb* genotype, whereas the aborted embryo must correspond to a putative *aabb* double mutant (Fig. 2B). The *aabb* embryo was arrested at the early/midglobular stage (Fig. 2B), and the number of aborted embryos per silique was in agreement with the expected lethality of the *aabb* genotype (Fig. 2C). These results indicate that homozygous *hal3b* embryos are not viable in the *hal3a-1* background. Additionally, the fact that one or two wild-type copies of *HAL3B* (in a *hal3a-1* background) support the growth of viable embryos reflects a partial functional redundancy between *HAL3A* and *HAL3B* genes.

During the analysis of the  $F_2$  progeny described above, we also identified *hal3b* plants that were heterozygous for the T-DNA-disrupted *hal3a-1* allele (*Aabb* genotype). Siliques of these plants also revealed



**Figure 4.** Reduced seed production and enhanced salt sensitivity of *aaBb* plants. **A**, Seed production of wild type, *hal3a-1*, *hal3b*, *Aabb*, and *aaBb* plants. Values are averages  $\pm$  SD ( $n = 20$ ). Triple asterisks (\*\*\*) indicate  $P < 0.01$  (Student's *t* test) with respect to wild type. **B**, Salt hypersensitivity of *aaBb* seedlings. Seedlings grown in medium containing 1% Suc were transferred to medium supplemented with 125 mM NaCl and lacking (–Suc) or containing 1% Suc (+Suc). The photograph was taken after 7 d in medium containing NaCl. **C**, Percentage of seedlings showing bleaching when transferred to medium supplemented with 125 mM NaCl and lacking Suc. Values are averages  $\pm$  SD for three independent experiments (40 seedlings each). Triple asterisks (\*\*\*) indicate  $P < 0.01$  (Student's *t* test) with respect to wild type (wt).

approximately one quarter of aborted embryos (Fig. 2C), likewise indicating that *hal3a-1* embryos are not viable in the *hal3b* homozygous background. Growth rate of *Aabb* plants was quite similar to wild type, whereas *aaBb* plants showed a slight delay with respect to wild type (Fig. 2A).

#### *aaBb* Plants Require Exogenous Suc for Seedling Establishment

According to the main reserve compound, Arabidopsis qualifies as an oilseed plant, and mutants severely impaired in the ability to catabolize storage lipid require an exogenous supply of Suc for seedling establishment (Hayashi et al., 1998, 2002; Germain et al., 2001; Zolman et al., 2001; Footitt et al., 2002; Fulda et al., 2004). We reasoned that mutants impaired

in CoA biosynthesis might have compromised fatty acid  $\beta$ -oxidation, leading to a requirement for Suc supplementation in the heterotrophic growth phase of the plant. Therefore, we analyzed seedling establishment of the different mutant backgrounds described above on media supplemented with or lacking exogenous Suc (Fig. 3A). Wild-type seeds germinated and grew normally, regardless of the presence or absence of Suc in the growth medium. Similar behavior was observed for seeds of *hal3a-1* and *hal3b* single mutants (Fig. 3A). In contrast, seedling establishment of the progeny of *aaBb* plants was notably impaired in the absence of Suc (Fig. 3A), whereas germination was not severely compromised under the conditions used.

Figure 3B shows that approximately two thirds of the progeny were represented by stunted individuals with a very short root, whereas one third of the

seedlings were similar to wild-type plants (188:95,  $\chi^2 = 0.012$ ,  $P > 0.9$ ). Stunted individuals became senescent after 2 weeks in the absence of Suc, but they could be rescued to normal growth by transfer to a medium supplemented with Suc. Genotyping of these individuals revealed they had an *aaBb* genotype, whereas those seedlings that did not require Suc for postgerminative growth had an *aaBB* genotype. Thus, a single copy of the *HAL3B* gene (in a *hal3a-1* background) was not able to support postgerminative growth in medium lacking Suc. These results also show genetic complementation of the Suc-dependent phenotype of *aaBb* seedlings by an additional copy of the *HAL3B* gene (right section of Fig. 3B, compare *aaBb* and *aaBB* siblings).

In contrast to the phenotype reported above for *aaBb*, the progeny of *Aabb* plants did not show a segregating phenotype in medium lacking Suc (Fig. 3A). Genotyping of this progeny revealed no double *hal3a-1 hal3b* mutant but *hal3b* individuals that had either one or two wild-type copies of the *HAL3A* gene. This result indicates that a single copy of *HAL3A*, in a *hal3b* background, is enough to support postgerminative growth in medium lacking Suc. Thus, whereas *aaBb* seedlings were Suc dependent, this was not the case for *Aabb* genotype. This observation can be explained by the fact that *HAL3A* expression in seedlings is higher than *HAL3B* (Espinosa-Ruiz et al. 1999; Fig. 1C). Additionally, this finding confirms that *HAL3A* function is particularly crucial for seedling establishment.

#### The Suc-Dependent Phenotype of *aaBb* Seedlings Is Complemented by Pantethine

*HAL3A* catalyzes the decarboxylation of 4'-phosphopantothenoyl-cysteine to 4'-phosphopantetheine, and therefore, this step of the CoA biosynthetic pathway must be severely impaired in *aaBb* individuals. The 4'-phosphopantetheine compound is not commercially available and additionally, phosphorylated precursors of CoA or CoA itself are not able to efficiently cross the plasma membrane (Shibata et al., 1983). However, we could obtain pantethine, which is the dimer resulting from the oxidation of the thiol group of pantetheine and subsequent formation of a disulfide bond. Thus, we were interested in examining whether supplementation of the media with pantethine might complement the Suc-dependent seedling establishment phenotype of the *aaBb* mutant. Figure 3A shows that seedling establishment in medium lacking Suc was recovered in *aaBb* upon pantethine supplementation, which was also reflected by measurements of root growth (Fig. 3C). In contrast, supplementation of the medium with other CoA precursors such as pantothenate or  $\beta$ -Ala, which are upstream of *HAL3A* function, was not able to complement the Suc-dependent phenotype of *aaBb* seedlings (Fig. 3, A and C). Pantethine complementation of the Suc-dependent phenotype indicates that impaired CoA biosynthesis is responsible for the observed phenotype in the *aaBb* mutant.

#### Seed Production and Salt Tolerance Are Severely Impaired in *aaBb* Plants

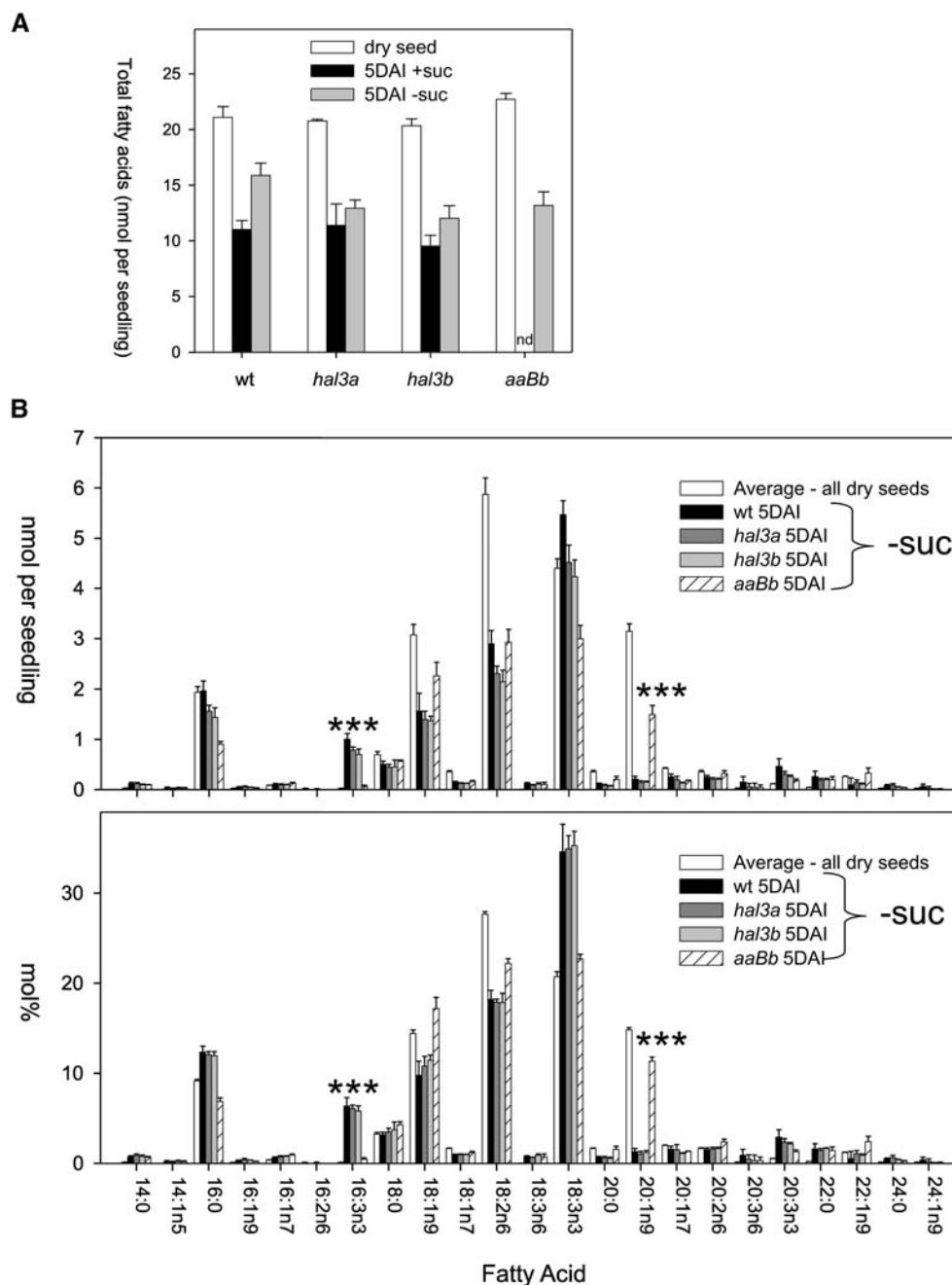
During photoautotrophic growth both *Aabb* and *aaBb* plants did not show obvious vegetative phenotypes, suggesting that a single gene copy of either *HAL3A* or *HAL3B* provides enough CoA for this growth phase. However, reproductive growth was particularly impaired in *aaBb* plants, where less inflorescence stems were present, although inflorescence and floral development were comparable to those of wild-type plants (data not shown). As a result, seed production was severely impaired in *aaBb* plants and to a lesser extent in *Aabb* plants, resulting in a reduction of approximately 70% compared to wild type (Fig. 4A).

Finally, as overexpression of *HAL3A* improves plant tolerance to salt stress (Espinosa-Ruiz et al., 1999; Yonamine et al., 2004), we decided to test salt sensitivity of the different mutant backgrounds. To this end, 7-d-old seedlings grown in a medium containing 1% Suc were transferred to a medium lacking Suc and supplemented with 125 mM NaCl. Compared to wild-type plants, *aaBb* individuals were hypersensitive to salt stress, as they bleached after 7 d in medium supplemented with NaCl (Fig. 4B). However, a similar salt sensitivity as that in wild-type plants was observed after transfer to medium supplemented with 125 mM NaCl and 1% Suc (Fig. 4B). Salt sensitivity of *hal3a-1*, *hal3b*, and *Aabb* mutants was quite similar to wild type both in the presence (data not shown) or absence of exogenous Suc (Fig. 4C).

#### Fatty Acid and Acyl-CoA Profiling

Dry seed from wild-type, *aaBB*, *Aabb*, and *aaBb* plants all had total fatty acid yields between 20 to 23 nmol seed<sup>-1</sup> (30% w/w), as determined by gas chromatography-flame ionization detector analysis (Fig. 5A), with a similar molar percent distribution for individual fatty acids (Fig. 5B). Therefore, although seed yield per plant was lower for *aaBb* plants, normal oil deposition within individual seeds occurred during seed development. Dry seed of an *aaBb* plant contains, respectively, two thirds and one third of *aaBb* and *aaBB* seeds, therefore only a relative comparison with data obtained for *aaBb* seedlings can be made (see below).

In addition, total content of fatty acids was measured for wild type, *hal3a-1*, and *hal3b* in 5 d after imbibition (DAI) seedlings that were grown in medium either supplemented with or lacking Suc. Total content of fatty acids for *aaBb* and *Aabb* genotypes in medium supplemented with Suc could not be determined because their seed progeny consists of a segregating population. However, we could select (and measure total fatty acid content in) *aaBb* seedlings in medium lacking Suc by the stunted phenotype described above. In germinated seedlings of this line, some lipid catabolism took place. However, although total lipid levels decreased in 5-DAI *aaBb* seedlings germinated in the absence of Suc relative to dry seeds



**Figure 5.** Altered fatty acid profile in *aaBb* seedlings. Dry seeds or seedlings grown on medium in the presence or absence of 1% Suc were extracted at 5 DAI for fatty acid profiling. Total fatty acids (A) and profile data (B) are shown. Data in B are expressed both as nmol/seedling as well as mol percentage (nmol fatty acid/total nmol fatty acids). Data in B refer to seedlings that were obtained in the absence of Suc (–Suc). Total fatty acid content of *aaBb* and *Aabb* genotypes in medium supplemented with Suc could not be determined because their seed progeny consists of a segregating population. Dry seeds of an *aaBb* plant contain approximately two thirds and one third of *aaBb* and *aaBB* seeds, respectively. For profile data, an average value for dry seeds from all lines is shown as these were very similar. Values are averages  $\pm$  SD for five separate determinations, expressed on a per seed(ing) basis. Triple asterisks (\*\*\*) indicate  $P < 0.01$  (Student's *t* test) when compared to data from *aaBb* and wild type (wt).

(Fig. 5A), the fatty acid profile of these seedlings indicated incomplete storage lipid catabolism (Fig. 5B). Notably, the level of the storage triacylglycerol-specific fatty acid, eicosenoic acid (20:1n9), decreased only approximately 50% in *aaBb* seedlings, whereas a 95% reduction was observed in the other lines (Fig. 5B). The

accumulation of 20:1n9 in *aaBb* suggests that storage triacylglycerol-derived fatty acid catabolism was stalled, in contrast to the sharp decay observed in wild type and the other genetic backgrounds (Fig. 5B, mol percentage). Additionally, the photosynthetic membrane-specific hexadecatrienoic acid (16:3n3) did not increase,



as would be expected in established seedlings (Fig. 5B). Therefore, this result reflects that fatty acids are not properly mobilized from the lipid body to reach the chloroplast in *aaBb* mutant. In contrast, the other lines all had fatty acid profiles that indicated their storage lipids were almost completely catabolized by 5 DAI, with the balance of lipids made up of membrane-specific fatty acids expected in actively growing tissue.

Measurement of the total acyl-CoA pool was obtained as described by Larson and Graham (2001). Total acyl-CoA content was quite similar in wild type, *hal3a-1*, and *hal3b* seedlings grown in medium supplemented with Suc (Fig. 6A). In medium lacking Suc, total acyl-CoA content was reduced in *hal3a-1*, *hal3b*, and *aaBb* seedlings to 65%, 79%, and 44% of wild-type levels (540, 657, 364, and 828 fmol/seedling, respectively). The reduction in total acyl-CoA content reported for *hal3a-1* and *hal3b* did not lead to a visible phenotype, whereas the more than 50% reduction measured in *aaBb* led to a severe phenotype for seedling establishment (see Fig. 3, A and B). Particularly noticeable in this line was the low level of acetyl-CoA (2:0), approximately 20% of wild type (Fig. 6B, fmol/seedling), and the accumulation of 4:0, 6:0, and 20:1 CoA with respect to wild type (Fig. 6B, mol percentage). A low CoA supply in *aaBb* appears to limit  $\beta$ -oxidation and hence acetyl-CoA production as, for instance, the thiolysis step requires the input of a new CoA molecule for every 2-carbon cleavage.

## DISCUSSION

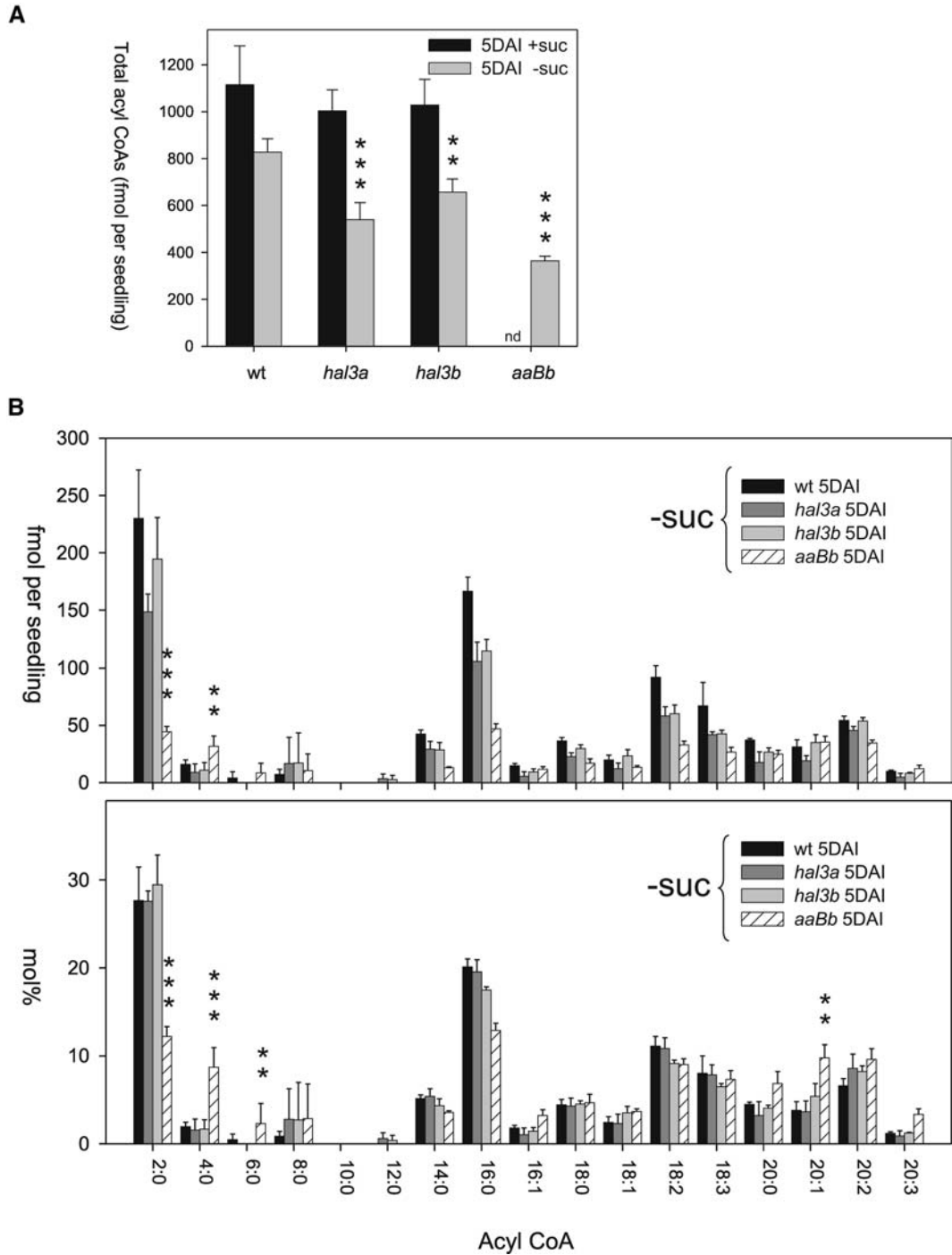
Comparative genomics in both prokaryotic and eukaryotic organisms has been fruitful in the discovery of genes of universal metabolic pathways. In particular, elucidation of the human and plant genes involved in the CoA biosynthetic pathway has greatly benefited from this approach (Daugherty et al., 2002; Kupke et al., 2003). A step forward in plant physiology should be the analysis of reduction-of-function mutants impaired in CoA biosynthesis. In this work we provide an initial effort in that direction, by reporting the identification and characterization of plant knockout mutants impaired in CoA biosynthesis.

Two allelic Arabidopsis mutants with a lesion in the *HAL3A* (*AtCoaC1*, *PPCDC*) gene, *hal3a-1* and *hal3a-2*, did not reveal major phenotypical differences compared to wild-type plants. The Arabidopsis genome encodes a second gene, *HAL3B* (*AtCoaC2*), whose gene product shows 86% amino acid sequence identity to that of *HAL3A*. Therefore, we suspected the corresponding gene products might be able to complement each other. A reverse genetics approach was used to isolate a knockout mutant for *HAL3B*. As it happened with *hal3a-1*, the *hal3b* mutant behaved quite similarly to wild-type plants. Analysis of the progeny of *hal3a-1/+ hal3b/+* plants failed to identify a *hal3a-1 hal3b* double mutant, however we could identify *aaBb* and *Aabb* individuals. The percentage of aborted embryos in

siliques of these plants was in agreement with the expected nonviability of *hal3a-1 hal3b* double mutants. Moreover, the results of the  $\chi^2$  test in the progeny of *aaBb* plants was in agreement with the 2:1 ratio (*HAL3B* heterozygous to homozygous) expected if *aaBb* embryos were lethal. The *aaBb* embryos were arrested to the early/midglobular stage (Fig. 2B), which represents an early step of embryogenesis (36–48 h after flowering). It can be speculated that once the residual CoA present in the *aaBb* zygote is titrated below a certain threshold by early cell divisions, further development is arrested. Additionally, this result shows that embryogenesis arrest in *aaBb* occurs before the synthesis of fatty acids and lipid deposition take place (Baud et al., 2002), revealing a crucial role for CoA at early stages of embryo development. Moreover, de novo CoA biosynthesis by the embryo is required for embryogenesis, as either *aaBb* or *Aabb* mother plants do not support growth of *aaBb* embryos. A maternal effect on the seed by CoA supply is unlikely, as this compound as well as CoA precursors are phosphorylated and therefore do not efficiently cross plasma membrane. Pantothenate is the most advanced CoA precursor taken up by cells (Begley et al., 2001). Indeed, whereas exogenous pantothenate complements mutants lacking de novo pantothenate biosynthesis, exogenous CoA does not complement mutants impaired in CoA biosynthesis (Begley et al., 2001; Leonardi et al., 2005).

The progeny of *aaBb* mother plants showed a segregating phenotype with respect to seedling establishment in medium lacking Suc, which was not present in the progeny of *Aabb* plants. Thus, root elongation, expansion, and greening of the cotyledons, as well as production of true leaves from the apical meristem, were severely impaired in *hal3a-1* individuals that were heterozygous for *HAL3B* (Fig. 3B). An additional wild-type copy of *HAL3B* in *aaBB* siblings of the progeny restored normal growth (Fig. 3B), which provides genetic complementation of the phenotype and proves that the phenotype is due to impaired *HAL3* function. Additionally, chemical complementation of the phenotype was obtained by supplementation of the media with pantethine (Fig. 3C), which is a direct precursor of the CoA metabolite synthesized by *HAL3A*. Taken together, these results indicate that impaired *HAL3* function is responsible for the Suc-dependent seedling establishment phenotype and that adequate CoA biosynthesis is required for seedling establishment. Additionally, these results provide in vivo evidence on *HAL3B* function, and they confirm that *HAL3A* is predominant over *HAL3B* at this stage.

The Suc-dependent phenotype during postgerminative growth of *aaBb* is similar to that of various mutants affected in fatty acid breakdown and subsequent utilization of the resulting acetyl-CoA units. These include the *pxa1/ped3/cts* mutant, which shows a lesion in a peroxisomal ATP-binding cassette transporter involved in uptake of fatty acids into the peroxisome (Zolman et al., 2001; Footitt et al., 2002; Hayashi



**Figure 6.** Altered acyl-CoA profile in *aaBb* seedlings. Seedlings grown on medium in the presence or absence of 1% Suc were extracted at 5 DAI for acyl-CoA profiling. Total fatty acids (A) and profile data (B) are shown. Data in B are expressed both as fmol/seedling as well as mol percentage (fmol acyl-CoA/total fmol acyl-CoAs). Data in B are from seedlings grown in the absence of Suc (–Suc). Values are averages  $\pm$  SD for five separate determinations, expressed on a per seedling basis. Double (\*\*) and triple (\*\*\*) asterisks indicate  $P < 0.03$  or  $P < 0.01$  (Student's *t* test), respectively, when compared data from *aaBb* and wild type (wt).

et al., 2002) and a long-chain acyl-CoA synthetase (LACS) *lacs6-1 lacs7-1* double mutant affected in peroxisomal LACS responsible for activation of fatty acids to fatty acyl-CoAs, i.e. the substrates for  $\beta$ -oxidation (Fulda et al., 2004). Disruption of  $\beta$ -oxidation itself also

results in a Suc-dependent seedling establishment phenotype as demonstrated by the *acx1 acx2* double mutant disrupted in the first step of the pathway (Adham et al., 2005; Pinfield-Wells et al., 2005) and the *ped1/kat2* mutant, which is deficient in the last thiolytic

cleavage step (Hayashi et al., 1998; Germain et al., 2001). Finally, mutations in different enzymatic steps of the peroxisomal glyoxylate cycle, which plays a central role in the conversion of acetyl units derived from  $\beta$ -oxidation to sugars, also gives rise to sugar-dependent seedling establishment phenotypes of varying degrees of severity (Eastmond et al., 2000; Eastmond and Graham, 2001; Cornah et al., 2004). The *icl* and *mls* mutants are devoid of the corresponding isocitrate lyase and malate synthase glyoxylate cycle enzymes, but despite this, they exhibit a relatively weak sugar-dependent seedling establishment phenotype best characterized by impaired hypocotyl elongation in dark-grown seedlings (Eastmond et al., 2000; Cornah et al., 2004). These weak phenotypes are put down due to the fact that in the absence of isocitrate lyase activity, acetyl units from  $\beta$ -oxidation can still be respired (Eastmond et al., 2000), and in the absence of malate synthase, acetyl-CoA can still be used by the seedling for gluconeogenesis, because the glyoxylate from isocitrate lyase can be metabolized by an alternative pathway (Cornah et al., 2004). In contrast to *icl* and *mls* mutant seedlings, the *csy2 csy3* double mutant, which lacks the major peroxisomal citrate synthase isoforms involved in storage reserve mobilization, remains dormant until the seed coat is removed and is then dependent on exogenous sugar for seedling establishment (Pracharoenwattana et al., 2005). This increased dormancy phenotype is also exhibited by the *cts*, *kat2*, and *acx1 acx2* mutants (Pinfield-Wells et al., 2005; Pracharoenwattana et al., 2005). The severity of the *csy2 csy3* double-mutant phenotype is thought to be due to the fact that export of acetyl units from the peroxisome in Arabidopsis is absolutely dependent on their conversion to citrate.

The severity of the *hal3 aaBb* sugar-dependent seedling establishment phenotype (Fig. 3) is much closer to those mutants that are completely blocked in fatty acid breakdown (due either to disruption of a component of the pathway or export of product from the peroxisome) than to the *mls* and *icl* mutants, which are still able to utilize acetyl-CoA derived from fatty acid breakdown. Malate synthase and citrate synthase both use acetyl-CoA derived from the last step of  $\beta$ -oxidation and recycle CoA, thus maintaining the peroxisomal pool. This pool of CoA will almost certainly need to increase to meet demand with the onset of storage lipid mobilization during postgerminative seedling growth. The *hal3 aaBb* mutant appears to be unable to respond to this increased demand resulting in an inability to break down and utilize storage lipid derived fatty acids, which leads to a sugar-dependent seedling establishment phenotype.

The Suc rescue of seedling establishment in the *hal3 aaBb* mutant demonstrates that these seedlings are able to utilize sugars as a respiratory carbon source. In the presence of Suc the mitochondrial CoA pool must therefore be adequate to support the production of acetyl-CoA required for the TCA cycle. It is possible that exogenous sugar feeding, as well as providing a

utilizable carbon source, could also alleviate the limitation in peroxisomal CoA brought about through increased demand, since sugars are known to delay and in some cases inhibit breakdown of storage lipid derived fatty acids (Eastmond et al., 2000; Martin et al., 2002). Furthermore, sugars such as Suc and Glc can also have regulatory roles, acting in many cases to either increase or decrease the activity of various metabolic pathways at the transcriptional and posttranscriptional level (Smeekens, 2000). It is possible that Suc could lead to a sufficient up-regulation of the CoA biosynthetic pathway to alleviate the seedling establishment phenotype of the *hal3 aaBb* mutant. In fact Suc feeding actually increases the total acyl-CoA pool in both wild type and the *hal3a-1* and *hal3b* mutants, which suggests that increased levels of CoA are available in the presence of Suc (Fig. 6).

Increases in the total acyl-CoA pool are also seen in the various mutants disrupted in fatty acid breakdown (Germain et al., 2001; Footitt et al., 2002; Rylott et al., 2003; Fulda et al., 2004; Pinfield-Wells et al., 2005). In contrast, the *aaBb* seedling total acyl-CoA pool measured in this study was lower than that measured for the wild type on a per seedling basis. This suggests that the observed retention of storage lipid specific fatty acids in this line could at least partly be due to a decreased CoA supply to cytosolic and peroxisomal acyl-CoA synthetases, which are required to activate fatty acids to acyl-CoA esters for subsequent  $\beta$ -oxidation. Alternately, a bottleneck in peroxisomal  $\beta$ -oxidation arising as a consequence of a limiting supply of CoA provision for the last thiolytic step in the pathway could result in feedback inhibition of storage lipid breakdown as previously proposed (Graham et al., 2002). Indeed, the accumulation of C4, C6, and C20:x acyl-CoAs (on a mole percentage) in the *aaBb* line suggests a bottleneck operated over the entire range of acyl-CoA chain lengths that are generated during the cyclic 2C cleavage of long-chain fatty acids. That C16:0 and C18:x CoAs do not accumulate despite the fact that these are the predominant fatty acids in storage lipids most likely reflects an impairment in the acylation of fatty acids (because of a reduced supply of CoA) together with compromised de novo fatty acid synthesis.

In conclusion, the data presented in this work provide strong evidence that HAL3B plays the same catalytic role as HAL3A in CoA biosynthesis. Despite the fact that HAL3B is expressed at significantly lower levels than HAL3A (Espinosa-Ruiz et al., 1999; Fig. 1C), it can compensate for the lack of HAL3A during embryo development and the vegetative growth of the plant. HAL3B mRNA is detected (at lower level than HAL3A mRNA) in root, flower, and silique, whereas it is hardly detectable in shoot, leaf, and seed (Espinosa-Ruiz et al., 1999). Therefore, at specific stages (reproductive growth, salt stress, and seedling establishment), presumably due to increased demand for CoA, two rather than one copy of the HAL3B gene are required to compensate for the lack of HAL3A. Thus, *aaBB* seedlings, still containing

65% of wild-type levels of total acyl-CoAs, did not show a Suc-dependent phenotype for seedling establishment. Instead, further reduction (up to 44%) in *aaBb* seedlings correlated with a dependence on exogenous Suc for seedling establishment. As free CoA is only 10% to 15% of total CoAs in tissues with active fatty acid metabolism (Jackowski and Rock, 1986; Tahiliani and Beinlich, 1991), the reduced content of total acyl-CoAs likely reflects impaired CoA synthesis in the *aaBb* mutant. In addition, we clearly show that acetyl-CoA levels are compromised in the *aaBb* line with respect to *hal3a-1*, *hal3b*, or wild-type plants (approximately 80% reduction with respect to wild type), and that this correlates with compromised fatty acid breakdown. The fact that Suc rescues seedling establishment in the *aaBb* line demonstrates that the main cause of this phenotype is a compromised reserve mobilization, rather than other pleiotrophic effects of CoA deficiency. It will be interesting to establish whether the salt hypersensitivity observed in *aaBb* seedlings (Fig. 4C) correlates with similar metabolic defects or whether it is due to other roles of CoA. However, the low fresh weight of these seedlings and their growth arrest in the absence of Suc also suggest that the retention of storage lipid may have been a consequence of detrimental pleiotrophic effects of CoA deficiency (i.e. impaired amino acid biosynthesis) on cell development and expansion.

Finally, the Suc-dependent phenotype of *aaBb* seedlings was complemented upon media supplementation with pantethine. Reduction and phosphorylation of this compound by the cell metabolism must have occurred to generate 4'-phosphopantetheine, which is the CoA precursor generated by HAL3A enzyme. This result suggests that it might be possible to therapeutically deliver pantethine or an alternative intermediary compound to bypass certain enzymatic defects in CoA biosynthesis. In the case of PK-associated neurodegeneration, the only human illness currently associated to a defect in CoA biosynthesis (Zhou et al., 2001), pantethine supplementation might not be effective, as presumably PK activity is required to generate 4'-phosphopantetheine from pantethine. However, for a different illness associated to a defect in other steps of the CoA pathway (CoaB or CoaC), treatment with pantethine might prove to be useful. In general, this result serves to illustrate that a defect in the CoA biosynthesis pathway might be complemented by non-phosphorylated downstream intermediates, provided a functional PK is present.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*Arabidopsis* (*Arabidopsis thaliana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite-soil mixture. For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5% sodium hypochlorite) containing 0.05% Triton X-100 for 10 min, and, finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 4 d. Then (0 DAI), seeds were sowed on Murashige and Skoog plates

(Murashige and Skoog, 1962) composed of Murashige and Skoog basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% agar, and pH adjusted to 5.7 with potassium hydroxide before autoclaving. When stated, 1% Suc was included in the media. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16-h light, 8-h dark photoperiod at 80 to 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ .

### Mutant Isolation by PCR Screening

An *Arabidopsis* insertion mutant collection of 90,000 lines (Columbia background) that carry the T-DNA of vector pPCV6NFHyg was screened as described by Rios et al. (2002). The PCR screen was performed with the T-DNA left border primers HOOK1 and FISH1 (Rios et al., 2002) and the following *HAL3A* primers: 5'-CCAACGGTTTTAGCAGTCTGACTCTTAC and 5'-CAGAGTGGAGCTAGTAGTGCAAATGGTC. Finally, two plants corresponding to donor stock numbers 13332 and 32247 were found to contain independent single T-DNA insertions in the *HAL3A* gene, and they were, respectively, named *hal3a-1* and *hal3a-2*. To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from hygromycin-resistant seedlings and submitted to PCR genotyping using the above-described primers.

A line (Columbia background) containing a single T-DNA insertion in *HAL3B* was identified from the SALK T-DNA collection (<http://signal.salk.edu/cgi-bin/tdnaexpress>), corresponding to donor stock number SALK\_045607. To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant (25  $\mu\text{g}/\text{mL}$ ) seedlings and submitted to PCR genotyping using the following *HAL3B* primers: 5'-TGTGACTGGTCA-TAGTCTTACTGAACAC and 5'-TACTCGAGTCGTTGTGCCACATAAAACC. As T-DNA left border primer of the pROK2 vector, we used the following one: 5'-GCCGATTCGGAACCACCATC.

The T-DNA tagged mutant lines were backcrossed once with the wild type, verified by DNA gel-blot hybridization and also by segregation analysis of the encoded antibiotic resistance gene (for the SALK line, the partially silenced selectable marker was scored under a 25  $\mu\text{g}/\text{mL}$  kanamycin concentration).

### Cytological Techniques

Green and mature siliques were fixed in an ethanol/acetic acid mixture and then cleared using the following solution: chloral hydrate/glycerol/water 8:1:2 (w/v/v) according to the protocol of Weigel and Glazebrook (2002). Seeds, usually cleared for 12 to 16 h, were examined with an Eclipse E600 microscope (Nikon) equipped with Nomarski optics.

### Seedling Establishment, Complementation, and Salt Tolerance Assays

Seedling establishment of the different genetic backgrounds was scored as the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves. Complementation of the Suc-dependent phenotype of *aaBb* individuals was assayed supplementing the medium with 50  $\mu\text{g}/\text{mL}$  of bis[*N*-pantothienylamidoethyl] disulfide (pantethine, Sigma P2125). Salt tolerance assays were performed by transferring 7-d-old seedlings grown in medium containing Suc to a medium supplemented with 125 mM NaCl and lacking or containing 1% Suc. Previous assays for testing salt resistance of 35S:*AtHAL3A* transgenic lines were done by testing plant growth in Murashige and Skoog medium supplemented with 3% Suc and 100 mM NaCl (Espinosa-Ruiz et al., 1999). In tobacco (*Nicotiana tabacum*), salt resistance assays were performed in calli derived of Bright Yellow 2 cells that were transformed with a 35S:*NtHAL3A* construct (Yonamine et al., 2004).

### RNA Analyses

Seedlings were collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy plant mini kit, separated on formaldehyde-agarose gels, and blotted to a nylon membrane. Blots were hybridized with random-priming <sup>32</sup>P-labeled probes. A specific cDNA probe for *HAL3A* was prepared as described previously (Espinosa-Ruiz et al., 1999). RNA samples for RT-PCR analysis were treated with DNase (RNase free) and, after precipitation in ethanol, they were dissolved to a final concentration of 1  $\mu\text{g}/\mu\text{L}$ . One microgram of the RNA solution obtained was reverse transcribed using 0.1  $\mu\text{g}$  oligo(dT)<sub>15</sub> primer and Moloney murine leukemia virus reverse transcriptase

(Roche), to finally obtain a 20- $\mu$ L cDNA solution. PCR reactions were performed on 1- $\mu$ L cDNA template using the following primers: primers *HAL3A*, 5'-ATG GAG AAT GGG AAA AGA GAC and 5'-AAG ATT ATC ACA AAG CCC ACC; primers *HAL3B*, 5'-GATTCAGATAGAGAAGAAGATGA and 5'-CATGCTTACTATACATGTC; primers *TUB*, 5'-CCTGATACTTCGCTT-TGG and 5'-GTGAACTCCATCTCGTCCAT.

## Fatty Acid and Acyl-CoA Profiling

Lipids from 50 dry seeds were extracted and transmethylated to their fatty acid methyl esters (FAMES) together with tripentadecanoin as an internal standard using a one-step procedure (Browse et al., 1986). FAMES were dissolved in hexane, and 2- $\mu$ L aliquots injected for gas chromatography-flame ionization detector analysis using a BPX70 60 m  $\times$  0.25 mm i.d.  $\times$  0.25  $\mu$ m film thickness capillary column (SGE) and a CE instruments GC8000 Top GC (Thermoquest). Injection was made into a hydrogen carrier gas stream at 1.3 mL  $\text{min}^{-1}$  (average linear velocity 35  $\text{cm s}^{-1}$ ) at a 30:1 split ratio. Temperature was ramped as follows: 110°C isothermal 1 min; 7.5°C  $\text{min}^{-1}$  to 260°C; cool down 70°C  $\text{min}^{-1}$  to 110°C; total analysis time 23 min. FAMES were identified by comparison to a 37 FAME mix (Supelco). Ten seedlings (approximately 10 mg) were extracted for quantitative acyl-CoA analysis by HPLC with fluorescence detection of acyl *etheno* CoA derivatives (Larson and Graham, 2001). The lipid portion of the acyl-CoA extracts were used for fatty acid determinations as described above.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF166262 and U80192.

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