Multifunctional acetyl-CoA carboxylase 1 is essential for very long chain fatty acid elongation and embryo development in *Arabidopsis*

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**Summary**

Acetyl-CoA carboxylase (ACCase) catalyses the carboxylation of acetyl-CoA, forming malonyl-CoA, which is used in the plastid for fatty acid synthesis and in the cytosol in various biosynthetic pathways including fatty acid elongation. In *Arabidopsis thaliana*, ACC1 and ACC2, two genes located in a tandem repeat within a 25-kbp genomic region near the centromere of chromosome 1, encode two multifunctional ACCase isoforms. Both genes, ACC1 and ACC2, appear to be ubiquitously expressed, but little is known about their respective function and importance. Here, we report the isolation and characterisation of two allelic mutants disrupted in the ACC1 gene. Both acc1-1 and acc1-2 mutations are recessive and embryo lethal. Embryo morphogenesis is impaired and both alleles lead to cucumber-like structures lacking in cotyledons, while the shortened hypocotyl and root exhibit a normal radial pattern organisation of the body axis. In this abnormal embryo, the maturation process still occurs. Storage proteins accumulate normally, while triacylglycerides (TAG) are synthesised at a lower concentration than in the wild-type seed. However, these TAG are totally devoid of very long chain fatty acids (VLCFA) and consequently enriched in C18:1, like all lipid fractions analysed in the mutant seed. These data demonstrate, *in planta*, the role of ACCase 1 in VLCFA elongation. Furthermore, this multifunctional enzyme also plays an unexpected and central function in embryo morphogenesis, especially in apical meristem development.

**Keywords:** acetyl-CoA carboxylase 1, *Arabidopsis*, embryo development, lethal mutant, pattern formation, very long chain fatty acids.

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**Introduction**

To date, the biochemical pathways producing both constitutive and storage lipid components in plants have been largely described (Voelker and Kinney, 2001). Moreover, many processing enzymes have been characterised and localised in this complex metabolic network and the corresponding genes have been cloned. However, factors regulating fatty acid synthesis and controlling total oil content in oilseed crops are still poorly understood (Thelen and Ohlrogge, 2002). Likewise, the exact contribution of the so far characterised enzymes to the lipid storage process and their regulatory potential are largely unknown. *Arabidopsis thaliana*, which develops embryo accumulating nearly 30% of triacylglycerols (TAG) in cytosolic oil bodies, represents a useful model to investigate storage lipid synthesis and its regulation (Ruuska et al., 2002). To unravel molecular mechanisms involved in seed maturation and its regulation, isolation of *A. thaliana* mutants affected in seed development was carried out. This forward genetic program, based on visual screening of seeds along with their biochemical analyses, led to the isolation of lines producing wrinkled seeds as a result of abnormal embryo development or altered metabolic processes.

Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyses the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate (Konishi and Sasaki, 1994). ACCase contains three functional domains and catalyses a two-step reaction (Ke et al., 2000). First, carboxylation of a biotin cofactor, covalently bound to the central biotin-carboxyl...
carrier (BCC) domain, is catalysed by the biotin carboxylase (BC) domain. Then, transfer of the carboxyl group from carboxy-biotin to acetyl-CoA occurs at the carboxyltransferase (CT) domain, forming malonyl-CoA. ACCases are present in two structurally distinct forms: a multifunctional homodimeric form with subunits >200 kDa, and a dissociable, multisubunit, heteromeric form made of four proteins (Alban et al., 2000). The multisubunit type is present in plastids of dicots and non-Poaceae monocots, whereas the multifunctional form is thought to be a cytosolic form, with the exception of Poaceae that possess a multifunctional form in their plastids (Konishi and Sasaki, 1994; Konishi et al., 1996). In A. thaliana, three of the subunits composing the multisubunit form of ACCase (BCC protein, BC and CT α subunits) are encoded by nuclear genes; the fourth (CT β subunit) is encoded by the chloroplast genome (Ke et al., 2000). Two nuclear genes, contiguously located within a 25-kbp genomic region nearby the centre of chromosome 1, ACC1 and ACC2, encode two multifunctional isoforms (Yanai et al., 1995).

The plastid ACCase activity, which catalyses the first committed step in fatty acid biosynthesis (up to C16:0, C16:1 and C18:1), is believed to be highly regulated and is considered to be a major determinant of the overall rate of fatty acid synthesis (Schulte et al., 1997). This plastid-localised pathway represents the main sink of malonyl-CoA. An independent extra-plastidial malonyl-CoA pool is also required for a wide range of reactions and pathways. This includes the synthesis of secondary metabolites such as flavonoids and the elongation of very long chain fatty acids (VLCFA ≥ C20) incorporated in TAG molecules, cuticle, waxes or sphingolipids (Roesler et al., 1994). Malonylation of some D-amino acids, glycosides and ethylene precursor aminoacyclopropane-1-carboxylate is also dependent on cytosolic malonyl-CoA pool. However, the precise function and the relative contribution of the two multifunctional ACCases in the generation of the cytosolic malonyl-CoA pool remain unclear.

In TAG biosynthesis, ACCase activity also plays a direct and central role. It contributes to the de novo fatty acid synthesis in plastid and so allows fatty acid elongation in ER to form C20:0, C20:1 and C22:1, which represent 25% of A. thaliana TAG fatty acids (Baud et al., 2002). An A. thaliana multifunctional ACCase, targeted to the plastid in Brassica napus, led to a 5% increase of total oil content in the seed (Roesler et al., 1997). Moreover, downregulation of the multifunctional ACCase I in B. napus, using antisense technology, resulted in the production of wrinkled seeds with reduced lipid content (Sellwood et al., 2000). This suggests that multifunctional ACCases can interfere with TAG accumulation in the seed. However, neither the precise mechanism involved nor the relative implication of the two different isoenzymes present in A. thaliana has, so far, been characterised.

Here, we report the isolation of two A. thaliana acc1 mutants and their molecular, cytological and biochemical characterisation. Our results demonstrate the role, in planta, of the ACCase 1 in fatty acid elongation. Furthermore, these imply that this multifunctional enzyme plays a surprising and central role in embryo morphogenesis.

Results

Isolation of two allelic embryo lethal mutants

The acc1-1 mutant line was identified, among T-DNA insertion lines, by visual screening of the T3 seeds of 50 000 T-DNA lines composing the Versailles collection (Bechtold et al., 1993). Heterozygous plants appeared normal except for the production of some severely wrinkled seeds (Figure 1a). The altered seed phenotype was visible, during silique maturation, as white seeds among green ones (Figure 1b). The desiccated wrinkled seeds were unable to germinate after imbibition and this mutant was therefore initially classified as embryo lethal (emb). The emb phenotype segregated as a single Mendelian recessive locus (data not shown). Co-segregation analyses of kanamycin resistance (conferred by the T-DNA) and wrinkled seed phenotype were carried out to check that acc1-1 was a T-DNA tagged mutant. The strict co-segregation pattern suggested that the acc1-1 mutation was genetically linked to a single T-DNA insertion locus. The fact that no homozygous plant was obtained was in agreement with the lethal nature of the mutation at the homozygous state.

When analysing acc1-1/ACC1 plant progeny, only 19% of seeds observed (n = 2817) exhibited a wrinkled phenotype, instead of the 25% expected, in case of a normal recessive Mendelian trait. Statistical analyses (χ²; p < 0.01) confirmed significant differences between the observed and expected ratios. Furthermore, a decreasing ratio of wrinkled seeds was observed from the upper part (adjacent to the stigma) to the lower half of the siliques (data not shown). These results suggested a weak transmission defect on the male side.

A second allele, acc1-2, has been characterised. The acc1-2 mutant was also isolated by visual screening of seeds (T3 progeny) of the Versailles transformatant collection. Acc1-1 and acc1-2 seeds (Figure 1a) displayed similar phenotypes and, after crossing heterozygous plants, no complementation of the phenotype was obtained in F1.

Both mutations affect ACC1 gene

A genomic program is carried out to characterise, molecularly, the Versailles transformant collection, the result of which is available in the FLAGdb/FST database (Samson et al., 2002). As a result, a flanking sequence tag (FST) for
the acc1-1 line was generated for the left T-DNA border (Balzergue et al., 2001) and anchored to the genome sequence of A. thaliana in ACC1 gene (At1g36160). Plant genomic DNA sequence flanking the right and left T-DNA borders of acc1-1 mutant were amplified by PCR and sequenced, confirming the FST found in the FLAGdb/FST database. The T-DNA insertion resulted in a small deletion of genomic DNA (3 bp) at the insertion site, in the 30th exon of ACC1 (Figure 2a). Furthermore, analysing genomic DNA from heterozygous acc1-1 plantlets by DNA gel blot hybridisation using T-DNA probes showed that a single T-DNA copy was present at the insertion locus (data not shown). Several PCR analyses performed on DNA from single excised embryos confirmed the expected polymorphism between homozygous, heterozygous and wild-type embryos (Figure 2b), suggesting that the mutation is tagged at the molecular level.

Southern blot analysis using T-DNA probes showed that no T-DNA copy was present in the genome of acc1-2 progeny tested. However, sequencing of the ACC1 gene revealed a 5-bp deletion in the 30th exon, from nucleotide 9030–9034 (Figure 2a), which leads to a frameshift mutation.
and probable premature termination of the corresponding protein. The molecular characterisation of the two allelic mutants demonstrates that both acc1-1 and acc1-2 mutations are responsible for the wrinkled seed phenotype.

**ACC1 and ACC2 are ubiquitously expressed**

The expression pattern of both ACC1 and ACC2 genes was investigated in various tissues of the wild-type ecotype WS using a quantitative RT-PCR strategy. For both genes, primers were designed at the 3' end of the transcript to amplify a 281- and 140-bp fragment, respectively. The results were standardised to the constitutive EF1A4x gene expression level (Liboz et al., 1990). ACC1 transcripts were present in all tissues analysed at various stages of developing and germinating seeds (Figure 3). The highest expression levels were observed in flower tissues, then in the silique walls at 6 DAF (Figure 3a). During seed development, ACC1 mRNA level did not show significant changes (Figure 3b). ACC2 mRNA was also detected in all
Figure 4. ACCases gene expression and phenotype in the mutants.
(a) Quantitative RT-PCR analysis of ACC1 and ACC2 mRNAs in excised embryos at 15 DAF. Values are the means ± SE of three independent replicates.
(b) Identification of chimerical ACC-T-DNA transcript in acc1-1 embryos. Four sets of primers, upstream (aF/R), overlapping (bF/R and cF/R) or downstream (dF/R) to the T-DNA insertion site were used on cDNA from excised acc1-1 and WS embryos aged 15 DAF and on DNA from excised acc1-1 embryos.
(c) Multifunctional ACCases detection in excised acc1 and WT embryos. Total proteins of excised embryos aged 15 DAF were submitted to SDS-PAGE electrophoresis on a 4–15% polyacrylamide gel. Nitrocellulose blot was probed with peroxidase-conjugated streptavidin. Biotinylated markers (right lane) are indicated in kDa.

AtACC1 is essential for embryo development

Embryos were observed throughout seed development (Figure 1c–j). Mutant embryos exhibited a developmental arrest early in the phase of pattern formation. The morphogenesis of acc1-1 embryos appeared to be blocked at the late globular stage. In contrast to wild-type embryos, the radicle did not elongate, cotyledons did not form a heart stage and no bilateral organisation was acquired. A range of embryo phenotypes was observed, from ball-shaped embryos to cucumber-shaped ones, a minority of which exhibiting an enlargement of the apical region. These abnormal embryos increased in size along seed development, allowing a minority of them to fill a great part of the seed.

It is essential for embryo development

Acc1 mutants lack a biotinylated multifunctional ACCase

Analysis of mRNAs from 15 DAF excised embryos was performed (Figure 4a). Full-length ACC1 transcripts were absent in homozygous acc1-1 embryos, while they were still present, but at significantly lower levels compared to the wild type, in homozygous acc1-2 embryos. A set of different primers then allowed us to detect a chimerical ACC1-T-DNA transcript in homozygous acc1-1 embryos (Figure 4b), in agreement with the structure of the previously characterised mutation.

The presence of a biotinylated multifunctional ACCase in the excised embryos was investigated using a Western blot strategy. The only known plant biotinylated polypeptides, with a molecular mass greater than 200 kDa, are the multifunctional ACCases isoenzymes (Duval et al., 1994). In the wild-type embryos, a biotinylated polypeptide with a molecular weight of 220 kDa was detected, which could correspond to previously characterised multifunctional ACCases

(Roesler et al., 1997). Neither acc1-1 nor acc1-2 homozygous embryos presented this band (Figure 4c). However, a band of about 84 kDa, which could correspond to the constitutively expressed methylcrotonyl-CoA carboxylase (MCC) (Wurtele and Nikolau, 1990), was present in both wild-type and mutant embryos. These results demonstrated the lack of a biotinylated multifunctional ACCase in both acc1-1 and acc1-2 mutant embryos.

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Cytological observations revealed a classical cellular organisation (Jürgens, 1994) of the basal part of acc1-1 and acc1-2 embryos (Figure 1k–m), with a radicle meristem (Figure 1n) under rows of differentiated cells forming a central cylinder and a cortex surrounded by an epidermal layer. In contrast, the apical part of the mutant embryos did not display any typical cellular organisation. All the embryos were lacking in cotyledons and exhibited aberrant organisation of the apical meristematic area. The epidermal layer also showed an irregular pattern in this region. Based on the tetrazolium test applied to excised embryos (see Boisson et al., 2001), the majority of acc1-1 embryos were metabolically active until 17 DAF, while mature desiccated embryos were inactive and therefore dead.

The maturation process still occurs in mutant embryos

Due to the abnormal shape of the acc1-1 embryo, dry matter accumulation in mutant seeds was lower than in wild-type seeds, with only 13.4 (±0.3) μg dry matter (DM) in the acc1-1 mature desiccated seed versus 25.6 (±0.4) μg for the wild type (Figure 5a). Likewise, the water content in acc1-1 seeds was higher during maturation, with a sharp decrease from 60 to 16% during the final desiccation period (Figure 5b). Starch content was measured throughout seed development. The seeds stored high amounts of starch between 6 and 11 DAF that were later degraded (Baud et al., 2002). During maturation, starch content was slightly greater in the mutant seeds compared to the wild type (data not shown). The synthesis of storage compounds was analysed. In the homozygous acc1-1 seeds, the content of total protein showed an evolution pattern similar to that of the heterozygous or wild-type seeds on a dry-weight basis, with an average of 280 (±20) μg mg\(^{-1}\) DM in the mature mutant seed versus 306 (±36) μg mg\(^{-1}\) DM for the wild type. SDS-PAGE analysis revealed the presence of both 2S and 12S storage proteins in mature acc1-2 seeds, suggesting that synthesis of seed storage protein was not prevented. Total fatty acid accumulation was observed in the acc1-1 seeds, but at lower concentrations compared to heterozygous or wild-type seeds (Figure 5c). Mature mutant seeds contained 158 (±8) μg mg\(^{-1}\) DM of total fatty acids versus 238 (±5) for the wild-type mature seeds. Fatty acid analyses were performed on total lipids fractions isolated from mature desiccated seeds to determine fatty acid composition in acc1-1 mutants. The mutant line showed an absence of VLCFA (C20:0, C20:1 and C22:1), an increased proportion of C18:1 and, to a lesser extent, of C16:0 and C18:0 (Table 1). Following purification by thin layer chromatography, analyses of TAG, DAG, phospholipids and free fatty acids confirmed that the TAG were lacking in VLCFA and that these four lipid classes were strongly enriched in C18:1. In acc1-1 seeds, TAG remained the preponderant lipid class, but free fatty acids level was 10-fold higher than that in the wild type. Finally, TEM showed cellular protein and oil bodies with a normal aspect in acc1-1 embryos (data not shown). These biochemical data showed that the maturation process, mainly characterised by storage compound accumulation, still occurred in mutant embryos.

Figure 5. Physiological and biochemical characteristics of seeds produced by ACC1/acc1-1 heterozygotes compared to wild type.

Wild-type seeds (WS), seeds without phenotype (Φ−) and seeds with phenotype (Φ+) in the heterozygous siliques were analysed during their development. Values are the means ± SE of at least three independent measurements.

(a) Evolution of seed dry weight.
(b) Evolution of seed water content.
(c) Evolution of total fatty acid amount.
Table 1 Fatty acid composition of different lipid fractions in mature WS and acc1 seeds

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<th>Type of seed</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
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<th>20:0</th>
<th>20:1</th>
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Total lipid extraction was carried out on batches of 50–100 seeds. Total fatty acid composition (in mol%) of seeds was determined by GC analyses of an aliquot. WS and acc1-1 lipid classes were separated by thin layer chromatography; their FA composition (in mol%) and amount as percentage of the total lipids were determined by GC. Values are the means of three independent replicates. TAG, triacylglycerols; FFA, free fatty acids; DAG, diacylglycerols; PL, phospholipids; TFA, total fatty acids.

Discussion

We have isolated two allelic mutants of ACCase 1: the acc1-1 mutation consists of a T-DNA insertion in the 30th exon of the ACC1 gene, while the acc1-2 mutation corresponds to a 5-bp deletion in the same exon (Figure 2a). Both mutations lead to a premature termination of the corresponding protein deprived of highly conserved amino acid sequences, probably in the carboxyltransferase domain of the enzyme (Roesler et al., 1994). Study of ACCases gene expression in the mutants revealed that a chimerical ACC1-T-DNA mRNA is produced in acc1-1 (Figure 4b), while the level of ACC1 mRNA in acc1-2 is greatly reduced (Figure 4a). More importantly, we observed a total lack of bionylated multifunctional ACCase in both acc1-1 and acc1-2 mutant embryos (Figure 4c). The two mutants exhibit the same biochemical and developmental phenotypes, demonstrating that the multifunctional ACCase 1 is a key enzyme in different processes involved in A. thaliana embryogenesis.

ACC1 is essential for VLCFA elongation

Fatty acid synthesis in the plastids results in C16 and C18 fatty acids, which are elongated in the cytosol to form VLCFA. Waxes derive from C26 to C32 fatty acids, sphingolipids often contain C22 and C24 fatty acids, and TAG, which represent the preponderant lipid class in the mature A. thaliana seed, contain large amounts of C20:1. The absence of VLCFA in both acc1-1 and acc1-2 TAG (Table 1) demonstrates the role, in planta, of ACC1 in VLCFA elongation. Chemical degradation experiments and studies on microsomal fractions (Agrawal et al., 1984) demonstrated that the VLCFAs are formed by cytosolic malonyl-CoA-dependent elongation of preformed chains (Harwood, 1988). Chemical inhibition of the multifunctional ACCases in embryos of B. rapa showed that the elongation of C18:1 to C22:1 was dependent on the cytosolic ACCases to supply malonyl-CoA (Bao et al., 1998). Characterisation of acc1 mutants confirms this hypothesis, in planta, and points out the involvement of ACC1 in malonyl-CoA supply for VLCFA elongation during A. thaliana seed development. The lack of VLCFA elongation led to C18:1 overaccumulation in the mutant seed (Table 1). Indeed, 18:1-CoA is the substrate for C20:1 and C22:1 synthesis by successive condensations of malonyl-CoA. This C18:1 enrichment was observed not only in the TAG fraction, but also in DAG, phospholipids and in the FFA fraction. This illustrates a redistribution process in different lipids of the overabundant C18:1 precursors present in acyl-CoA pools. However, only a part of the remaining C18:1 is re-allocated to pre-existing lipid classes and, consequently, an important FFA pool seems to be formed.

ACC1 is necessary for embryo development

In this study, we have shown that ACC1 is essential for A. thaliana embryo development and plays a key role in apical development. Both acc1-1 and acc1-2 embryos exhibit cucumber-like structures lacking in cotyledons, with a cellular organisation perturbed in the apical region (Figure 1). On the opposite, the shortened hypocotyl and the radicle exhibit a normal radial pattern organisation of the body axis. The radicle also presents a normal meristem. Mutant embryos deviate from wild type during the transition from radial to bilateral symmetry at the triangular or early heart stage when the cotyledon primordia are formed during wild-type embryogenesis (Mayer et al., 1991). Pattern mutations deleting specific embryo regions
such as the apical part have already been described in the emb30/gnom or gurke mutants for example, demonstrating that plant embryos develop from regions that evolve autonomously (Goldberg et al., 1994; for review, see Jürgens, 1994). In the same way, several mutations have been identified (e.g. sim, wus, zll) that affect the differentiation of the shoot apical meristem (SAM) during embryogenesis (Mordhorst et al., 2002 and references therein). These mutations, which disrupt meristem identity and have no other characterised effect on embryonic development (Goldberg et al., 1994), rather emphasise on the importance of gene regulation. ACC1 also appears as a key factor of embryo development, even though it encodes for a common and widespread enzyme of carbon metabolism.

The specific alteration of the apical region in acc1 mutants indeed suggests a signalling process failure rather than a fundamental metabolic or structural defect, such as altered lipid membrane composition. We have listed below known biosynthetic pathways that use cytosolic malonyl-CoA and considered whether they could be required for proper embryo development or not. Flavonoid synthesis can first be ruled out, since all known transparent testa mutants (Winkel-Shirley, 2002) that are impaired in flavonoid biosynthesis exhibit normal embryo morphogenesis. Ethylene biosynthesis is reported to be downregulated through 1-aminocyclopropane-1-carboxylate malonylation (Guo et al., 1993). This pathway does not seem to interfere with embryo development, since ethylene overproducer mutants exhibit normal embryo development (Woeste et al., 1999). Waxes that contain VLCFA are deposited once embryo morphogenesis is achieved and therefore are unlikely to be involved. TAG can be ruled out too, since the development of fae1 mutant embryo is unaltered despite of a lack of VLCFA in TAG (Kunst et al., 1992). The case of sphingolipids is somewhat different. Concentrated in the plasma membrane, they consist of a long-chain amino alcohol (sphinganine) that forms an amide linkage to a fatty acid to constitute a ceramide, the basic component of all sphingolipids. In this structure, the acyl group is often longer than C18 and in fungi, for example, ceramide backbones with very long chains are restricted to the synthesis of the inositol-containing phosphosphingolipids (Leipelt et al., 2001), such as glycosylphosphatidylinositol-anchored proteins (GPI-anchored proteins). These GPI anchors are of particular interest, since they may function in signal transduction pathways in plants, in association with arabino-galactan-proteins which they anchor in the membrane (Schultz et al., 1998). Normal embryo development could involve such actors (Sherrier et al., 1999). Furthermore, in mammals, sphingolipids are also supposed to interact with cholesterol to form clusters of raft-like domains that are implicated in lateral sorting of proteins during cellular trafficking and signal transduction (Carl and Hetherington, 2001). Sphingolipid synthesis impairment or aberrant sphingolipid synthesis due to a lack of VLCFA can therefore constitute the first hypothesis for the abnormal embryo morphogenesis in acc1 mutants. The lack of VLCFA normally included in sphingolipid and GPI-anchor lipid was already suggested to explain lethality of ACC-defective deletion mutants in yeast (Hoj a et al., 1998).

A second hypothesis is based on a more conventional plant signalling molecule. Indeed, B. juncea embryos (Hadfi et al., 1998), treated in vitro with high exogenous indole-3-acetic acid (IAA) concentrations, result in ball-shaped or cucumber-shaped embryos resembling acc1 phenotype. These embryos, immersed in exogenous auxin, were unable to establish the auxin gradients, providing positional information within the apical region of the embryo which is essential during the transition period from globular to heart stage (Souter and Lindsey, 2000). Auxin is also proposed to be a key signal molecule within the developing apical region. A poorly documented use of cytosolic malonyl-CoA, i.e. amino acid malonylation enables us to suggest an hypothetical relation between auxin synthesis regulation and ACC1. N-malonyltransferases catalyse the formation of N-malic conjugates of D-amino acids. The formation of N-malonyl-D-tryptophan has been described and this metabolite is known to occur naturally in a wide range of plants and tissues, including seeds (Klambt, 1960). Experiments on apple trees showed that malonyltryptophan accumulates when IAA metabolism is inhibited (Williams and Stahl, 1970). Similarly, Rekoslawskaya (1986) suggested a role of this compound in the regulation of the endogenous auxin level under various physiological conditions. AIA biosynthesis could be downregulated through tryptophan malonylation. As a consequence, malonylation impairment of this amino acid would lead to an excess of auxin biosynthesis.

**Maturation still occurs in mutant embryos**

Another conclusion of the experiments presented here is that seed maturation processes can take place even when embryo morphogenesis has failed. The acc1 mutants accumulate usual content of storage proteins. TAG, though with an altered fatty acid composition and a reduced concentration (Figure 5c), are stored in lipid bodies. Such a phenomenon has already been described in embryos blocked at the globular stage and still exhibiting cell differentiation process (Yadegari et al., 1994). Some mutants blocked at the heart stage, like cyt1 (Nickle and Meinke, 1998) or gcs1 (Boisson et al., 2001) also exhibited lipid accumulation and formation of cytosolic oil bodies. Embryo morphogenesis and maturation therefore appear as two distinct processes. Concerning TAG storage, the lack of chlorophyll in mutant seeds could contribute to the reduction of oil accumulation, since photosystems and light-driven electron transport provide cofactors for plastid fatty acid synthesis (Bao et al., 1998).
Ubiquitous ACC1 expression copes with multiple uses of malonyl-CoA

Quantitative RT-PCR profile suggested that the ACC1 gene is ubiquitously expressed in Arabidopsis, since transcripts are found in all different tissues analysed (Figure 3). This is consistent with previous RT-PCR (Yanai et al., 1995) and in situ hybridisation (Nikolau and Wurtele, 1998) analyses. During seed development, the ACC1 expression level appears stable, in contrast with some other genes involved in triglyceride biosynthesis, such as fatty acid synthase subunits, that have been shown to increase during the lipid deposition phase (O’Hara et al., 2002). This raises the question of a possible post-translational regulation of the multifunctional ACCase. Some recent data suggest that the pea multisubunit ACCase may be regulated by phosphorylation (Savage and Ohlrogge, 1999).

The highest level of ACC1 mRNA is found in A. thaliana flowers. In situ hybridisation results obtained by Nikolau and Wurtele (1998) revealed that ACC1 mRNA accumulation is high in tissues that synthesise phytochemicals, using a cytosolic pool of malonyl-CoA (e.g. flavonoids), like epidermal cells of carpels or petals. Likewise, various lipids containing VLCFA are massively accumulated in pollen coat (Kim et al., 2002). Some of them control the flow of water to pollen on the stigma and therefore constitute an essential factor allowing pollen tubes to penetrate stigma. As a consequence, the pollen grains of the mutants cer1, cer3, cer6 and pop1, defective in synthesis of VLCFA included in waxes, do not hydrate (Wolters-Arts et al., 1998). This could explain the weak defect of the gametic transmission on the male side observed in the acc1 mutants.

 ACCase 2 function remains unclear

An ACC1 knockout totally blocks VLCFA elongation in the seed. This observation raises the question of ACC2 function. Due to its very low expression level (Figure 3) and to the lack of biotinylated ACCase in acc1-1 and acc1-2 mutant embryos (Figure 4c), the role of ACC2, if any, and its cellular localisation remain unclear. Indeed, ACC1 and ACC2 genes are organised in 31 and 32 exons, respectively, and, with the exception of the initial exon of ACC2, they code for nearly identical proteins (>95% amino acid identity; Nikolau and Wurtele, 1998). The amino acid sequence coded by this ‘additional’ 5′ exon in ACC2 was compared to the one coded by the B. napus ‘additional’ 5′ exon of BnACCaseg8 gene, shown to work as a transit peptide addressing a GFP fusion protein in chloroplasts of tobacco protoplasts (Schulte et al., 1997). A 42% identity score was obtained. In both peptide sequences, there were a high amount of serine and threonine residues, very low amounts of acidic amino acids and a positively charged domain in the centre of the sequence, that are characteristic of a transit peptide (Gavel and von Heijne, 1990). This led us to consider an hypothetical plastidial localisation for ACC2, which would then be irrelevant to generate cytosolic malonyl-CoA pool and could constitute a second example, after the case of BnACCaseg8, of a plastidial localisation of a multifunctional ACCase in dicots.

In conclusion, we have demonstrated, in planta, that the multifunctional ACCase 1 is essential for VLCFA elongation. Interestingly, this enzyme also appears to be necessary for A. thaliana embryo development, since acc1 mutants exhibit an aberrant developmental pattern. Nevertheless, the precise mechanism by which the ACCase 1 interferes with embryo morphogenesis remains to be elucidated.

Experimental procedures

Plant material and growth conditions

A. thaliana of the ecotype Wassilewskija (WS) as well as acc1-1 (DYWS line) and acc1-2 (DCY3 line) mutants (all from the WS ecotype) were obtained from the Station de Génétique et d’Amélioration des Plantes (INRA, Versailles, France). Seeds were surface sterilised and germinated on Murashige and Skoog (MS) medium (Mur2 555, pH 5.6; Duchefa, Haarlem, the Netherlands) solidified with 0.7% (w/v) agar. After a cold treatment of 48 h at 4°C in the dark, the plates were transferred to a growth chamber and incubated at 20°C/15°C day/night, under a 16-h/8-h light/dark regime. Selection of T-DNA-containing seeds was performed by germination on MS supplemented with kanamycin (Sigma, Saint-Quentin Fallavier, France) at 50 mg l−1. After 15 days, the plantlets were transferred to sterilised compost in individual pots, grown under the same conditions as above and irrigated twice a week with a complete mineral nutrient solution (Lesaint and Coic, 1993). Primary shoots only were used and secondary shoots were removed. To harvest siliques of defined developmental stages, individual flowers were tagged using coloured tape on the day of flowering. To analyse the distribution of seeds with phenotype (white and wrinkled seeds), heterozygous acc1-1/ACC1 siliques at 15 DAF were opened and observed without disturbing seed positions. For time course studies, all the developing seeds of one shoot were harvested 3–4 weeks after the onset of flowering: siliques ranging from 3 to 22 DAF were opened and the corresponding seeds removed and subsampled. For acc1-1/ACC1 heterozygous siliques, seeds were partitioned according to their phenotype: normal (green turning to fully yellow seeds) or abnormal (white turning to brown wrinkled seeds). This was only possible after 10 DAF. Pools of 10–20 seeds were weighed (M2P balance; Sartorius, Goettingen, Germany) right after harvest (fresh weight). Dry weight (DW) was measured after drying at 50°C for 48 h. Pools of harvested seeds were freeze-dried and stored at 4°C for biochemical analyses. Material used for RNA or protein extraction was immediately frozen in liquid nitrogen and stored at −80°C prior to extraction. To obtain germinating embryos and young seedlings for RNA analysis, seeds were germinated on 0.8% (w/v) agar in the same conditions as described above.

Microscopy

Developing seeds or excised embryos were placed in a quick-clearing solution of chloral hydrate for 1–24 h (Boisson
et al., 2001), and then observed under differential interference contrast optics (Nomarski) using a light microscope (Axioskop 2; Zeiss, Jena, Germany). Photographs were taken using Kodak Elite 160T film. For bright-field microscopy, developing seeds were fixed in glutaraldehyde-paraformaldehyde (2.5 and 4% w/v, respectively) in 0.1 M phosphate buffer at pH 7.2; Triton (0.1% v/v) was added to facilitate fixation. The fixative solution was vacuum infiltrated and silicues were left overnight at 4°C. Seeds were dehydrated through an ethanol series to 100% ethanol. The ethanol was gradually replaced with the cold-polymerising resin Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Following resin polymerisation, 4-μm sections were cut with a microtome (Leica RM 2055, Rueil-Malmaison, France) using glass knives. Staining was performed with toluidine blue (0.1% w/v) and observation was made as mentioned above. Photographs were scanned using an UMAX Powerbook II (Iconofast, Courbevoie, France) and the figures were prepared with Adobe Photoshop 5.5.

Lipid analyses

For time course studies of total fatty acid accumulation (Figure 5c), pools of 10–20 seeds were ground in a glass reaction tube. For lipid fraction analyses (Table 1), batches of 50–100 mature seeds (approximately 1 mg DW) were used. Extraction, separation of lipid classes by thin layer chromatography plates and analyses of fatty acyl methyl esters by gas chromatography were performed as described previously (Baud et al., 2002). Due to the very low amount of homoyzgous mutant tissues available, analysis of the other lipid classes normally containing VLCFA, e.g. waxes and sphingolipids, was not feasible.

DNA extraction and PCR analyses

Genomic DNA was extracted according to Oard and Dronavalli (1992). When DNA was extracted from single excised embryos, for genotyping, a 45-cycle PCR was performed with half of DNA sample using primers dyw5LB and Ta1A (specific from the left T-DNA border) or dyw5RB2 (5'-gaagatggttggagtgat-3', 5'-attgcttttcttgacc-3', 5'-attgcttttcttgacc-3', 5'-atcatgaaaccagactgc-3', respectively) in a single reaction (Figure 2b). For molecular characterisation of the acc1-1 mutation, genomic regions flanking the T-DNA insertion were amplified using a 35 cycle PCR reaction with primers complementary to the flanking sequence tag available in the FLAgDB/FST database (Samson et al., 2002). For the left border, Ta1A and dyw5LB were used, while Ta11 (5’-ctgataccagactgacctg-3’) and dyw5RB2 allowed amplification of the right border. DNA sequences were then carried out on PCR products purified with Qiagen (Chatsworth, CA) Purification Kit, using the Applied Biosyst. (Foster City, CA) DNA Sequencing Kit (Bigdye Terminator V.3.0) and ABI prism 310 genetic analyser.

RNA analyses

Frozen tissues were ground in liquid nitrogen and total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. The extracts were treated with 30 units of Rnase-free Dnase I (Qiagen) and eluted with 40 μl of diethyl pyrocarbonate-treated water. For reverse transcription (RT)-PCR studies, DNA-free RNA was converted into first-strand cDNA using the SuperScript pre-amplification system for first-strand cDNA synthesis (Gibco BRL, Cergy Pontoise, France) with oligo(dT)22.

Real-time RT-PCR: The reaction was performed on the LightCycler Instrument (Roche, Meylan, France) with the LightCycler-FastStar DNA Master SYBR Green I kit for PCR (Roche) according to the manufacturer’s protocol. Each reaction was performed with 5 μl of 1:10 to 1:100 dilution of the first cDNA strands in a total volume of 20 μl. Specific primer sets were designed in the 5’ region of the ACC1 and ACC2 cDNAs. The F1.c (5’-tggagacggcagaaactact-3’) and R1.b (5’-caacagacgagacacc-3’) primers amplify a 281-bp fragment on ACC1 cDNA, while the F2.b (5’-ggacctgacttctcgc-3’) and R2.a (5’-caagagggagacaaggag-3’) primers amplify a 140-bp fragment on ACC2 cDNA. The reactions were incubated at 95°C for 5 min to activate the hot start recombinant Taq DNA polymerase, followed by 45 cycles of 10 sec at 95°C, 6 sec at 55°C and 20 sec at 72°C (Orsel et al., 2002). The specificity of the PCR amplification was checked with a heat dissociation protocol (from 65 to 95°C) following the final cycle of the PCR; PCR products were then purified and sequenced. The efficiency of the primer sets was calculated by performing real-time PCR on several dilutions of first strands. Efficiencies of the different primer sets used were checked to be almost similar. The results obtained for the different tissues analysed were standardised to the constitutive EF1A2 gene expression level (Liboz et al., 1990), amplified with EF1F (5’-ctgagactttgattctgctgat-3’) and EF1R (5’-caacagagggagacaaggag-3’) primers.

To detect the presence of a chimerical cDNA in acc1-1 embryos, 35 cycles of RT-PCR were performed with different primer sets amplifying fragments upstream, overlapping, or downstream to the T-DNA insertion site (Figure 4b): set a/F is composed of 8.5 (5’-gtgataataagccctggg-3’) and 8.Rb (5’-taagttctctaccacattcact-3’) primers, set b/F is composed of TailA and dyw5LB1 (5’-tttctcaggttagggac-3’), set c/F is composed of Tail1 and dyw5RB2 primers and set d/F is composed of F.1c and R.1b primers.

Western blot

Approximately 20–50 isolated embryos were homogenised in 50 μl of sample buffer according to the Laemmli procedure (Laemmli, 1970). Extracts were incubated for 30 min at 60°C to facilitate total protein extraction. Denatured samples were subjected to SDS-PAGE electrophoresis in 4–15% acrylamide gradient gel (Ready-gel; Biorad, Marnes la Coquette, France). The relative molecular mass of ACCase subunit was determined with biotinylated standards (Sigma) covering an 18- to 180-kDa range. Polyacrylamide gel was blotted to nitrocellulose filter (BiTrace™ NT; Gelman, Pall, Saint-Germain-en-Laye, France). Blots were incubated for 1 h in Tris buffered saline (TBS) containing 3% BSA, then for 2 h with peroxidase-conjugated streptavidin (500 μM ml⁻¹ in TBS, Roche). Biotinylated polypeptides were revealed by adding peroxidase substrate solution (0.8 μl ml⁻¹ of 30% H₂O₂, 0.4 mg ml⁻¹ of luminol and 0.2 mg ml⁻¹ of p-coumaric acid).

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