gurke and *pasticcino3* mutants affected in embryo development are impaired in acetyl-CoA carboxylase

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Normal embryo development is required for correct seedling formation. The *Arabidopsis gurke* and *pasticcino3* mutants were isolated from different developmental screens and the corresponding embryos exhibit severe defects in their apical region, affecting bilateral symmetry. We have recently identified lethal *acc1* mutants affected in acetyl-CoA carboxylase 1 (ACCase 1) that display a similar embryo phenotype. A series of crosses showed that *gk* and *pas3* are allelic to *acc1* mutants, and direct sequencing of the *ACC1* gene revealed point mutations in these new alleles. The isolation of leaky *acc1* alleles demonstrated that ACCase 1 is essential for correct plant development and that mutations in ACCase affect cellular division in plants, as is the case in yeast. Interestingly, significant metabolic complementation of the mutant phenotype was obtained by exogenous supply of malonate, suggesting that the lack of cytosolic malonyl-CoA is likely to be the initial factor leading to abnormal development in the *acc1* mutants.

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INTRODUCTION

Normal embryo development governs correct seedling formation. In *Arabidopsis*, the origin of seedling structures can be traced back to cell groups in the young embryo, due to the invariant pattern of cell division during early embryogenesis (Jürgens, 2001). First, the embryo develops through an apical–basal axis, which is partitioned into three major regions: apical, central and basal. Superimposed on this axis appears a radial pattern of concentric tissue layers established successively from the periphery to the centre (Jürgens, 1995). At the late-globular stage, the radial symmetry of the embryo is transformed into a bilateral symmetry

by the enhanced dividing activity of cells at opposite sites of the apical region, which will determine the future cotyledons. How the apical region is partitioned between the presumptive shoot meristem and the two flanking cotyledon primordia is unknown. However, a number of genes (*WUSCHEL*, *SHOOTMERISTEMLESS*, *CLAVATA1*, *CUP-SHAPED COTYLEDON 1*) were shown to affect the establishment and the development of this region (for reviews, see Souter & Lindsey, 2000; Jürgens, 2001). *gurke* mutants were isolated on the basis of apical defects with the cotyledons missing or reduced to small knob-like structures (Torres-Ruiz *et al*, 1996). Mutations in the *GURKE* gene were mapped to chromosome 1. Likewise, the *PASTICCINO* genes, which are involved in the control of cell division and differentiation, are required for normal organization of the apical region in the embryo (Faure *et al*, 1998). *pasticcino* mutants, which belong to three complementation groups (*pas1*, *pas2*, *pas3*), produce abnormal embryos with altered cotyledon primordia leading to a flat apex. The *PAS1* gene was found to encode an immunophilin-like protein of the FK506 binding protein (FKBP) family (Vittorioso *et al*, 1998), and the *PAS2/PEPINO* gene was shown to encode a tyrosine phosphatase-like protein (Bellec *et al*, 2002; Haberer *et al*, 2002), but their functions remain unknown. *pas3*, which is epistatic to *pas1* and *pas2*, has not yet been identified. However, genetic analyses demonstrated that it is located near the centromere of chromosome 1 (Faure *et al*, 1998).

We recently characterized *acetyl-CoA carboxylase 1* (*acc1*) mutants impaired in embryo morphogenesis (Baud *et al*, 2003). Acetyl-CoA carboxylase (ACCase; EC 6.4.1.2) catalyses the ATP-dependent formation of malonyl-CoA from acetyl-CoA and bicarbonate. In dicots, ACCases are present in two structurally distinct forms, a multifunctional homodimeric form in the cytosol and a dissociable, multisubunit heteromeric form in the plastids (Alban *et al*, 2000). Activity of these ACCases is then able to generate two independent malonyl-CoA pools. The plastidic pool contributes to the *de novo* fatty acid synthesis, and the cytosolic pool is required for a wide range of reactions and pathways including very-long-chain fatty acid (VLCFA $\geq C_{20}$) elongation. These VLCFAs are incorporated into triacylglycerides, cuticle, waxes or sphingolipids. Two genes, *ACC1* and *ACC2*, encode such cytosolic ACCases in *Arabidopsis*, although *ACC2* transcripts

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are barely detectable (Baud *et al*, 2003). *acc1* mutant embryos exhibited cucumber-like structures lacking cotyledons. *acc1-1* and *acc1-2* alleles are devoid of cytosolic ACCase protein and are considered as null alleles. In the search for new alleles exhibiting leaky phenotypes, we identified the *Arabidopsis gurke* and *pas3* mutants as new *acc1* alleles. Like *acc1*, the *pas3* and *gk* alleles also showed a reduced VLCFA accumulation in seeds. Moreover, a significant metabolic complementation of the *acc1* phenotype could be obtained with exogenous malonate, suggesting that the lack of malonyl-CoA is responsible for the developmental defect observed in *acc1/gk/pas3* mutants.

RESULTS AND DISCUSSION

gk and *pas3* mutants are allelic to *acc1* mutants

acc1-1 and *acc1-2* embryos exhibit an early developmental arrest, leading to cucumber-like structures deprived of cotyledons (Baud *et al*, 2003). The pattern defect of these embryos resembles the patterns exhibited by the severe *gurke* and *pas3* alleles (Torres-Ruiz *et al*, 1996; Faure *et al*, 1998). *gk* and *pas3* mutations were both mapped to chromosome 1, near the centromere (Torres-Ruiz *et al*, 1996; Faure *et al*, 1998) and not far from *ACC1*. As the mutants display a similar phenotype and close genetic location, allelism tests were carried out (see Methods). No complementations were obtained, thus demonstrating that *acc1*, *gk* and *pas3* mutants were allelic. The large number of alleles (six *pas3* alleles, ten *gk* alleles and two *acc1* alleles) isolated so far in various screens is consistent with the large size of the *ACC1* gene (about 9 kb).

Molecular characterization of the *gk* and *pas3* alleles

To gain further insights into the molecular defects of the *gk* and *pas3* alleles, *ACC1* was sequenced in *pas3-1*, *pas3-2* and *gk-SC* (Stock Center N8148) alleles. The three mutants were obtained by ethyl methane sulphonate (EMS) mutagenesis of Col0 (*pas3-1*, *pas3-2*) and Ler (*gk-SC*). In all cases, single point mutations were identified in the *ACC1* gene (Fig 1A). In *gk-SC*, a G-to-A transition at position 493 altered a splicing site at the 3' end of the first intron (Fig 1A). Reverse transcription (RT)-PCR experiments using primers designed on both sides of this intron were performed on homozygous embryos, demonstrating a splicing defect in the mutant background (Fig 1B). Two distinct RT-PCR products were obtained and sequenced. The higher and stronger band corresponded to *ACC1* cDNA in which intron 1 was not spliced out. Unspliced intron 1 led to a frameshift and a predicted protein 163 amino acids (aa) shorter than wild-type (WT) protein (2,212 aa). The lower and weaker band corresponded to *ACC1* cDNA in which intron 1 was incorrectly spliced: exon 2 was initiated at position 522 instead of 494 in the WT. Incorrect splicing led to a frameshift and a predicted protein of 131 aa. In *pas3-1*, a G-to-A transition at position 7473 led to the substitution of an E residue to a K residue at amino-acid position 1581. Transition of G to A at position 8070 in *pas3-2* caused a substitution of a G residue to a S residue at amino-acid position 1780. In both cases, a highly conserved amino-acid residue of the carboxyltransferase (CT) domain of *ACC1* was thus mutated (Fig 1C,D).

A western blot strategy using streptavidin was then applied to detect the presence of a biotinylated multifunctional (cytosolic) ACCase in excised embryos (see Baud *et al*, 2003). In WT

Fig 1 | Identification of *gk-SC*, *pas3-1* and *pas3-2* mutations in *ACC1* gene. (A) Structure of *ACC1* gene and position of the point mutations identified in *gk-SC*, *pas3-1* and *pas3-2* mutants; *acc1-1* and *acc1-2* mutations. Closed boxes represent exons, whereas open boxes stand for untranslated regions (UTRs). Nucleotide positions are relative to the translational start site. (B) Characterization of abnormal *ACC1* transcripts in *gk-SC* embryos. Primer sets were used to amplify *EF* (EF1.up and EF1.low; control) and *ACC1* (*ACC1*.up and *ACC1*.low) on cDNA from excised Ler and *gk-SC* embryos aged 15DAA and on Ler DNA. *ACC1*.up was designed in the first exon of *ACC1* gene, and *ACC1*.low in the second exon. (C) Amino-acid sequence alignments of a conserved motif in the CT domain of ACCases. Bold residues indicate positions where amino acids are highly conserved. The asterisk indicates the conserved amino-acid residue mutated in *pas3-1*. (D) Amino-acid sequence alignments of a conserved motif in the CT domain of ACCases. Bold residues indicate positions where amino acids are highly conserved. The asterisk indicates the conserved amino-acid residue mutated in *pas3-2*. AtACC1, *A. thaliana* ACCase 1 (At1g36160); ZmACC, *Zea mays* ACCase (U19183); TaACC, *Triticum aestivum* ACCase (U10187); GgACC, *Gallus gallus* ACCase (J03541); HsACC, *Homo sapiens* ACCase 1 (U19822); ScFAS3, *S. cerevisiae* FAS3 (M92156).

Fig 2 | Multifunctional ACCase detection in excised *pas3* and *gk-SC* embryos. Fresh seeds were removed from heterozygous siliques and mutant embryos excised from severely wrinkled seeds were sampled. Total proteins of excised embryos aged 15 DAA were submitted to SDS–polyacrylamide gel electrophoresis on a 4–15% polyacrylamide gel. A nitrocellulose blot was probed with peroxidase-conjugated streptavidin, which binds biotin cofactor. Biotinylated polypeptides such as methylcrotonoyl-CoA carboxylase (MCCase) or ACCase were thus revealed.

embryos, a biotinylated polypeptide with a molecular mass of 220 kDa was detected, which corresponds to the multifunctional ACCase. The *gk-SC* mutation resulted in a lack of biotinylated multifunctional ACCase in isolated embryos (Fig 2). The truncated peptides corresponding to the unspliced mRNA or to the incorrectly spliced mRNA are indeed deprived of the central biotin-carboxyl carrier (BCC) domain that normally binds biotin. By contrast, the protein was detected in both *pas3-1* and *pas3-2* (Fig 2 and data not shown). This is consistent with point mutations identified in *pas3* alleles that are likely to alter the protein activity because they affect highly conserved motifs in the CT domain of the enzyme (Fig 1).

Modification of fatty acid composition and consequences

To measure the impact of the mutations on plant metabolism, the fatty acid composition of mature mutant seeds was analysed. *acc1-1* and *acc1-2* mutations resulted in a lack of VLCFAs, the elongation of which requires cytosolic malonyl-CoA (Bao *et al*, 1998). The synthesis of shorter acyl chains, requiring solely plastid malonyl-CoA, was not prevented in the mutant. The level of VLCFAs was significantly lower in *pas3*, whereas only traces of VLCFAs were found in *gk-SC* (Fig 3). Compared with WT, $C_{22:1}$ was missing in *pas3* and *gk-SC* as in *acc1*; $C_{20:1}$ was almost absent in *gk-SC* and strongly reduced in *pas3*. Conversely, $C_{16:0}$ and $C_{18:1}$ were increased compared with WT in *pas3* and *gk-SC* mutant as in *acc1*. In all cases, the biochemical analyses performed confirmed that cytosolic ACCase activity was impaired in the *pas3* and *gk* mutants. Consistently, the decrease in VLCFA level correlated with the strength of each allele. *acc1-1* and *gk-SC* alleles, which are embryo lethal, had the lowest VLCFA level, whereas *pas3* embryos, which are viable and able to germinate, exhibited an intermediate VLCFA level (Fig 4B).

Although VLCFAs (mainly $C_{20:1}$) are mainly incorporated into triacylglycerol molecules stored during seed maturation, some VLCFAs (from C_{26} to C_{32}) are also required for cuticle and wax synthesis (Kolattukudy, 1980). Mutation in *ALE1*, a subtilisin-like serine protease required for epidermal surface formation in

Fig 3 | Fatty acid composition of mature mutant seeds. Total lipid extraction and fatty acid transmethylation were carried out on batches of 20 seeds. Total fatty acid composition (in mol%) of seeds was determined by GC analyses. Results are the mean of three independent measurements; s.d. was always less than 1% of the mean. Fatty acid composition of WT Ws (*acc1-1* background), Ler (*gk* background) and Col0 (*pas3* background) seeds was analysed; the results presented for the Ws seeds are representative of the three ecotypes.

Arabidopsis embryos, demonstrated that a normal cuticle is required for separation of the endosperm from the embryo (Tanaka *et al*, 2001). Abnormal adherence observed between endosperm and embryo in *acc1* alleles (data not shown) seems to indicate that cuticle formation was altered in these mutant embryos. Moreover, organ fusion was reported in leaky *gk* and *pas3* alleles (Torres-Ruiz *et al*, 1996; Faure *et al*, 1998), and this phenotype could be mimicked (data not shown) in WT plants sprayed with quizalofop, a specific inhibitor of multifunctional ACCase (Dehaye *et al*, 1994). Interestingly, mutations in *ALE1* or *FIDDLEHEAD*, a gene encoding a fatty acid elongase involved in cuticle biosynthesis, resulted in a similar phenotype (Yephremov *et al*, 1999). Taken together, these results suggested that cuticle formation is severely altered in *acc1*, *gk* and *pas3* alleles. However, the phenotype of mutants with altered cuticle formation, which have been previously described, suggests that this specific defect is not responsible for the developmental phenotype observed in *acc1* embryos.

Partial complementation of the phenotype by malonate

As cytosolic ACCase is involved in malonyl-CoA synthesis, we investigated whether the developmental defect observed in *acc1* mutants could be compensated for by exogenous malonyl-CoA. Owing to membrane impermeability to CoA-bound compounds, malonate was supplied to immature embryos (Fig 4A). Malonyl-CoA synthetase (MCS) was shown to catalyse the formation of malonyl-CoA directly from malonate and CoA in microorganisms such as *Bradyrhizobium japonicum* (Koo & Kim, 2000). We expected that an *A. thaliana* MCS homologue (encoded by At3g16170) could use malonate to generate the malonyl-CoA lacking in the mutant embryos. When WT embryos excised at 15 days after anthesis (DAA) were cultured *in vitro* on a basal medium, they quickly generated green plantlets with small leaves

Fig 4 | Complementation of *acc1* mutations by exogenous supply of malonate. (A) Response of immature *acc1-1* mutant embryos. Arrested embryos were removed from immature siliques of heterozygous plants at 15 DAA, plated on basal medium, treated every 2 days with malonate and observed after several weeks of culture. Pictures on the bottom row are close-ups of embryos or plantlets presented in pictures on the top row. The development of WT Ws embryos was unaffected by malonate supply (not shown). (B) Response of *pas3-1* embryos during germination. Col0 and *pas3-1* seeds were germinated on basal and enriched (1 mM malonate) medium and observed after several weeks of culture. Scale bars, 5 mm.

and a branched root system. However, *acc1-1* embryos exhibited a slight elongation of the radicle and then died. When treated every 2 days with 3 mM malonate, mutant embryos exhibited a significant increase in root elongation, whereas callus-like structures appeared in the apical region of some of these embryos (Table 1). When treated with 10 mM malonate, branched root systems were formed, and mutant embryos occasionally developed into leafy plantlets (Fig 4A). As *pas3-1* mutation was not embryo lethal, germination experiments were conducted to test the effect of malonate. Seedlings were germinated on 1 mM malonate and, after 10 days of culture, the root growth was completely restored, leaf fusion was prevented and the apical part of the seedling was partly restored (Fig 4B).

In both experiments, exogenously supplied malonate compensates for at least part of the mutant developmental defect. These data suggest that the lack of cytosolic malonyl-CoA is likely to be the main factor leading to this strong phenotype. *acc1/pas3/gk*

mutations are the first mutations affecting ACCase described in a multicellular eukaryotic organism. If *acc1-1* and *acc1-2* lethal mutations allowed the key role of ACC1 during embryo morphogenesis to be demonstrated, identification of leaky alleles now emphasizes the role of ACC1 in the development of the plant.

SPECULATION

Further biochemical and transcriptional analyses are needed to elucidate which specific biosynthetic pathway(s) requiring cytosolic malonyl-CoA is (are) involved in this developmental process (Baud et al, 2003). Recently, a *Saccharomyces cerevisiae* mutant strain defective in ACCase was shown to be blocked at the G2/M phase of the cell cycle (Al-Feel et al, 2003). The authors pointed to the role in the cell cycle of ceramides, a basic component of all sphingolipids the biosynthesis of which requires VLCFA. Interestingly, in developing *gk* seeds exhibiting strong phenotypes, the number of cell divisions in rudimentary cotyledon primordia was

Table 1 | Response of *acc1-1* embryos cultured *in vitro* to exogenously supplied malonate

Concentration of malonate (mM)	Embryos cultured	Embryo response after 2 weeks in culture (%)			
		Dead embryos	Yellow calli	Green calli	Leafy plantlets
0	65	100	0	0	0
3	62	10	66	24	0
10	110	6	41	45	8

Mutant embryos excised at 15 DAA were cultured on basal medium and sprayed every 2 days with malonate.

severely reduced (Torres-Ruiz *et al*, 1996), whereas *pasticcino* mutants were shown to display uncoordinated cell divisions enhanced by cytokinin (Faure *et al*, 1998). Studying sphingolipid content in mutant backgrounds and considering their putative role in the control of cell division could thus shed new light on the *acc1* phenotype. Sphingolipids have been reported to interact with cholesterol to form clusters of raft-like domains in mammalian membranes (Carl & Hetherington, 2001). *FAKEL*, a gene involved in sterol biosynthesis in *Arabidopsis*, was shown to be required for proper cell division and expansion in the embryo (Schrack *et al*, 2000). Some membrane sphingolipids and phytosterols may be important for normal organization of the embryo through the regulation and coordination of cell division during its morphogenesis, thus pointing out a novel role for lipids in the embryogenesis of plants.

METHODS

Plant material and growth conditions. *Arabidopsis thaliana* of ecotypes Wassilewskija (Ws), Landsberg erecta (Ler) and Columbia (Col0) were obtained from the Station de Génétique et d'Amélioration des Plantes (INRA Versailles, France). *acc1-1* and *acc1-2* mutants (from the Ws ecotype) have been described previously (Baud *et al*, 2003). *pas3-1* and *pas3-2* were isolated from an EMS-mutagenized seed stock from the Col0 ecotype (Faure *et al*, 1998). The *gk-SC* allele, isolated from an EMS-mutagenized seed stock from the Ler ecotype, was obtained from the Nottingham *Arabidopsis* Stock Center (N8148). Seeds were surface sterilized and germinated on Murashige and Skoog (MS) medium (M02 555, pH 5.6; Duchefa, Haarlem, The Netherlands) solidified with 0.7% (w/v) agar. Culture conditions were the same as described previously (Baud *et al*, 2003). For allelism tests, reciprocal crosses between *gk-SC* and both *acc1-1* and *pas3-1* lines were performed. In reciprocal crosses between *gk-SC* and *acc1-1*, a segregation ratio of 22.9% was observed for wrinkled seeds, which was not different from the theoretical segregation ratio of 25% if no complementation occurs ($\chi^2 = 0.507$, two-sided *P*-value 0.476). In the reciprocal crosses between *gk-SC* and *pas3-1*, we observed a segregation ratio of 24.5% for wrinkled seeds, and again no complementation occurred ($\chi^2 = 0.057$, two-sided *P*-value 0.812). For complementation assays, the whole siliques were surface sterilized. Wrinkled mutant seeds were then removed from the silique under sterile conditions. Embryos were excised from the seeds and cultured as described above on MS medium comple-

mented with Gln at 2.5 mM. Every 2 days, embryos were treated with water (control plants), or 3 mM or 10 mM malonate (pH 5.3).

DNA extraction and sequencing. Genomic DNA was extracted according to Oard & Dronavalli (1992). For molecular characterization of the *gk-SC* and *pas3* mutation, DNA sequences were then carried out on PCR products purified with a Qiagen (Chatsworth, CA) Purification Kit, using the Applied Biosystems (Foster City, CA) DNA Sequencing Kit (Bigdye Terminator V.3.0) and ABI prism 310 genetic analyser.

RNA analyses. Frozen embryos harvested at 15 DAA were ground under 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 U of RNase-free DNase I (Qiagen) and eluted with 40 µl of diethyl pyrocarbonate-treated water. For RT-PCR studies, DNA-free RNA was converted into first-strand cDNA using the SuperScript preamplification system for first-strand cDNA synthesis (Gibco BRL, Cergy Pontoise, France) with oligo(dT)₂₂. Then, 35 cycles of RT-PCR were performed with different primer sets amplifying fragments in the *EF1-ALPHA-A4* cDNA (EF1.Up, 5'-atgccccaggacatcgtgattcat-3'; EF1.Low, 5'-ttggcggcacccttagctggatca-3') or in *ACC1* cDNA, on both sides of the first intron (ACC1.Up, 5'-atggctgctcggttaacgg-3'; ACC1.Low, 5'-accaatcttatctcccagtcg-3').

Western blot. Approximately 20–50 isolated embryos were homogenized in 50 µl of sample buffer according to the Laemmli procedure (Laemmli, 1970). Extraction, electrophoresis, transfer and visualization were performed as described previously (Baud *et al*, 2003).

Lipid analyses. Pools of 20 mature seeds were ground in a glass reaction tube. Extraction and analyses of fatty acid methyl esters by gas chromatography were performed as described previously (Baud *et al*, 2002).

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