The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in Arabidopsis

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Summary

The sucrose transporter gene AtSUC5 was studied as part of a programme aimed at identifying and studying the genes involved in seed maturation in Arabidopsis. Expression profiling of AtSUC5 using the technique of real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) showed that the gene was specifically and highly induced during seed development between 4 and 9 days after flowering (DAF). Analysis of the activity of the AtSUC5 promoter in planta was consistent with this timing, and suggested that AtSUC5 expression is endosperm specific, spreading from the micropylar to the chalazal pole of the filial tissue. To demonstrate the function of AtSUC5, the corresponding cDNA was used to complement a sucrose uptake-deficient yeast mutant, thus confirming its sucrose transport capacity. To investigate the function in planta, three allelic mutants disrupted in the AtSUC5 gene were isolated and characterized. A strong but transient reduction in fatty acid concentration was observed in mutant seeds 8 DAF. This biochemical phenotype was associated with a slight delay in embryo development. Taken together, these data demonstrated the role of the AtSUC5 carrier in the nutrition of the filial tissues during early seed development. However, additional sugar uptake systems, which remain to be characterized, must be functional in developing seeds, especially during maturation of the embryo.

Keywords: Arabidopsis, seed, endosperm, embryogenesis, sucrose transporter, fatty acids.

Introduction

In higher plants, seed development is a key process linking two distinct sporophytic generations. This reproductive strategy requires the coordinated growth of three tissues: the maternal seed coat, the endosperm and the embryo. The double fertilization of the egg cell and two polar nuclei in the embryo sac initiates the development of the diploid embryo and of the triplid endosperm, respectively. These filial organs are surrounded by the seed coat derived from ovular integuments. During seed maturation, storage compounds are synthesized and accumulated in the zygotic tissue (Baud et al., 2002). Carbon and nitrogen resources thus stored in the form of lipid, starch and protein fuel the establishment of the seedling after germination (Bewley and Black, 1994).

Sucrose represents the major form in which photosynthetically assimilated carbon is transported in plants. Sucrose is synthesized in green leaves, and is then transported through the phloem to supply sink organs like seeds. Because of the absence of symplastic linkage between maternal and filial tissues, partitioning of post-phloem assimilate in seeds requires membrane-located transport mechanisms (for review, see Patrick and Offler, 2001). These transfer events and the transporters involved have been widely investigated in cereals and legume grains (for review, see Weber et al., 1997b). The size of the seeds in these plants has allowed physiological and biochemical approaches to be combined with an analysis of the underlying developmental processes. Sieve elements in the seeds of grain legumes and cereals terminate in the seed coat and are symplastically interconnected with the surrounding ground tissues. These symplastic routes allow sieve elements to
unload water, sugars and other compounds like amino acids or ions. These solutes are then released from maternal integument cells and accumulate in the apoplast of the seed. Finally, they are retrieved from the apoplast by specialized cells located at the maternal/filial interface and moved to the cellular site of storage (Patrick and Offler, 2001). Transporters involved in retrieval of sucrose from the apoplast by the embryo or the endosperm have been isolated and characterized in *Vicia faba* (Harrington et al., 1997a; Weber et al., 1997a), pea (Tegeder et al., 1999), rice (Hirose et al., 1997), maize (Aoki et al., 1999) and barley (Weschke et al., 2000). In *V. faba*, the key role of sucrose/H⁺ symport in the accumulation of storage product has been suggested by the strong correlation observed between rates of gain of dry matter during seed development and transport activity (Harrington et al., 1997b). Since the compounds stored in seeds are of agronomic significance, the understanding of sucrose transport activities involved in post-phloem assimilate partitioning appears to be of major economic importance.

In small oleaginous seeds, post-phloem transfer processes remain unknown, contrasting with the increase in studies concerning biosynthetic pathways for storage lipid in the filial tissues (Voelker and Kinney, 2001). *Arabidopsis thaliana* seed, the embryo of which accumulates nearly 30% of triacylglycerols in cytosolic oil bodies, has been widely used as a model system for investigating the synthesis and regulation of storage lipid (Ruuska et al., 2002). However, the small size of the seeds limits fine anatomical investigations. Consequently, no structural framework is available which evaluates the cellular pathways of post-sieve-element transport of photoassimilates and the cellular locations of potential sucrose transporters facilitating exchange between maternal and filial tissues. The complete sequencing of the *A. thaliana* genome revealed nine putative sucrose transporters (Figure 1). The AtSUC family is the largest SUC family identified to date. But, so far, no seed-specific member has been characterized. AtSUC1 (At1g71880) is thought to control the osmotic driving force for anther dehiscence and pollen tube growth (Stadler et al., 1999). AtSUC2 (At1g22710) functions in loading sugars from the apoplast into the conductive sieve elements and the corresponding suc2 mutant exhibits stunted growth (Gottwald et al., 2000). This companion cell-specific carrier (Stadler and Sauer, 1996) has also been shown to be in nematode-induced syncytia (Juergensen et al., 2003). AtSUC3 (SUT2; At2g02860) is expressed in sieve elements and in numerous sink tissues; its expression is enhanced by wounding (Meyer et al., 2000, 2004). The putative sucrose sensor function of this carrier (Barker et al., 2000) is still debated. AtSUC4 (SUT4; At1g09960) is a low-affinity sucrose carrier localized in enucleate sieve elements (Weise et al., 2000). The AtSUC5 (At1g71890) sucrose and biotin transporter has only been characterized in heterologous systems (Ludwig et al., 2000). AtSUC8 (At1g66570) and AtSUC9 (At5g06170) encode functional sucrose transporters detected in floral tissues and AtSUC6 (At5g43610) and AtSUC7 (At1g66570) seem to encode aberrant protein in different *A. thaliana* ecotypes (Sauer et al., 2004).

Here we report on the genetic and functional characterization of AtSUC5 in planta. Quantitative RT-PCR, promoter-
results

isolation and expression analysis of the AtSUC5 gene

A reverse genetic approach was used to unravel the molecular mechanisms involved in seed maturation in A. thaliana. The first step consisted of a non-quantitative RT-PCR study aimed at isolating the genes for carbon metabolism specifically during seed development. Among the candidates thus isolated, a putative sucrose transporter gene was identified which proved to be AtSUC5 (Figure 1). AtSUC5 transcripts were detected in early developing seeds (data not shown). This was consistent with the report on expressed sequence tags (EST) from green siliques previously described [available at www.arabidopsis.org].

To complete these preliminary data, the expression pattern of AtSUC5 was then further investigated in various tissues of the wild-type ecotype Wassilewskija (Ws) using a quantitative RT-PCR strategy. Specific primers were designed at the 5′ end of the AtSUC5 transcript to amplify a 189 bp fragment (see Experimental procedures). The results obtained were standardized to the expression level of the EF1αA4 (EF) gene (Liboz et al., 1990; Nesi et al., 2000). In vegetative organs, in flowers and in young siliques aged 3 DAF, AtSUC5 mRNA was detected at very low levels (Figure 2a). Likewise, AtSUC5 transcripts were hardly detectable in siliques walls after 4 DAF (data not shown). The relative expression profile of the gene was finally investigated in developing seeds. A six-point kinetic analysis was performed (Figure 2b) that was representative of the three stages of A. thaliana seed development (Baud et al., 2002): early embryonic morphogenesis at 4 and 6 DAF, maturation at 9, 12 and 15 DAF, and late maturation at 18 DAF. At 4 DAF, an EF expression level of 4% was measured. The AtSUC5 transcript level then increased tenfold between 4 and 6 DAF, peaking at 40% EF, before falling sharply to 8% EF at 9 DAF. The AtSUC5 transcript was not detected further during late maturation.

pattern of AtSUC5 promoter activity

To gain further insights in AtSUC5 expression, the spatiotemporal activity of the AtSUC5 promoter was investigated. A 1.5 kb fragment (referred as to ProSUC5) was fused translationally to both the uidA reporter gene (which encodes GUS), and the mGFP5-ER reporter gene [which encodes GFP fused to an endoplasmic reticulum (ER) retention sequence]. GUS activity was investigated using fluorometric assays. No significant activity could be found in vegetative organs (data not shown). On the contrary, a strong peak of GUS activity was measured in early developing seeds (Figure 3a), and was localized in the endosperm (data not shown). The slight shift observed when GUS activity measurements are compared with quantitative RT-PCR data might reflect both the delay due to translation and the stability of GUS protein. The ProSUC5:mGFP5-ER construct was used to monitor AtSUC5 promoter activity at various stages of seed development. A strong GFP signal was first detected 3–4 DAF in the micropylar region of the endosperm (Figure 3b,c). At 6 DAF, ProSUC5:mGFP5-ER expression progressed at the micropylar pole (Figure 3d). Observation of confocal sections at the globular stage of embryo development showed that the GFP signal was endosperm specific and confirmed that the signal was absent in the embryo and in its suspensor (Figure 3d). At the torpedo stage, expression extended to the chalazal pole of the endosperm. A strong signal was observed around the chalazal endosperm cyst, at the interface with the maternal tissues (Figure 3e). Finally, expression decayed at the upturned-U stage, 9 DAF, as the endosperm became limited to a few cell layers embedding the maturing embryo (Figure 3f).

Study of AtSUC5 expression by in situ hybridization

An in situ hybridization experiment was carried out to link the activity of the AtSUC5 promoter to mRNA accumulation...
The AtSUC5 sucrose carrier is endosperm specific.

and to precise expression of AtSUC5 in maturing seeds, where the detection of GFP signal is severely compromised by the integuments. Due to the high level of similarity between the sequences of AtSUC1 and AtSUC5 in particular, an oligonucleotide hybridization approach was chosen. The specificity of the oligonucleotide used was demonstrated using null suc5 mutants as negative controls (see below). In the wild-type seeds, AtSUC5 transcripts were specifically detected in the endosperm. At the globular-to-heart transition stage, a strong staining was observed in the region of the endosperm surrounding the embryo, at the micropylar pole (Figure 3l). At the torpedo stage (Figure 3m) and at the bent-cotyledon stage (data not shown), the transcripts had spread to the whole endosperm, and a strong staining could be observed in the cell layer directly facing the embryo. The results obtained were consistent with the GUS and GFP expression profiles previously described (Figure 3).

Isolation and molecular characterization of three suc5 mutants

The AtSUC5 cDNA sequence corresponding to the Ws ecotype was cloned, sequenced and compared with the Ler accession characterized by Ludwig et al. (2000). Eight nucleotide substitutions were detected in the Ws background compared with Ler, resulting in an S to G substitution at position 26 of the AtSUC5 peptide and the addition of three amino acids (GFH) at the C-terminal position of the peptide. To analyse the function of the AtSUC5 protein, the cloned cDNA was used to complement a sucrose uptake-deficient yeast mutant (data not shown), thus confirming its sucrose transport capacity. To investigate the role of the AtSUC5 transporter in planta, suc5 alleles were then isolated among the 50 000 transferred (T)-DNA lines of the Versailles collection (Bechtold et al., 1993). Three flanking sequence tags (FSTs) corresponding to the left T-DNA borders were generated (Balzergue et al., 2001), which were anchored to the genome sequence of A. thaliana in the AtSUC5 gene. Plant genomic DNA flanking the right and left T-DNA borders of the three corresponding suc5 mutants were amplified by PCR and sequenced, confirming the FSTs found in the FLAGdb/FST database. In the suc5.1 mutant (line EEU50), the T-DNA insertion resulted in a 2.7 kb deletion of genomic DNA at the insertion site (Figure 4a). In the suc5.2 mutant (line DYO5), the T-DNA insertion led to a 556 bp deletion beginning in the 5'-untranslated region (UTR) of the AtSUC5 gene and ending in its first exon. In the suc5.3 mutant (line T990), the insertion occurred in the 3'-UTR of the gene and resulted in a 32 bp deletion of genomic DNA.

Analysis of mRNAs from seeds aged 5–6 DAF was performed by quantitative RT-PCR on homoyzogous mutant lines (Figure 4b). AtSUC5 transcripts were not detected in suc5.1 or in suc5.2, but they were still present in the suc5.3 line. A set of different primers then allowed us to establish that the transcripts detected in the suc5.3 mutant were chimeric AtSUC5-T-DNA transcripts (Figure 4c), in agreement with the structure of the previously characterized mutation (Figure 4a). In situ hybridization experiments were carried out using the two putative null mutants, which confirmed both the lack of AtSUC5 transcripts in these mutants (Figure 3n) and the specificity of the probe used.

Study of suc5 seed development and maturation

The three mutant lines developed normally and no abnormal phenotype could be detected by visual observation of their progenies. The shape and colour of the mature and dry mutant seeds were similar to the wild type. Several cultures were conducted in the greenhouse to characterize the suc5 mutant phenotype. In each experiment the dry weight (DW) of the seed of suc5 mutants was reduced compared with the wild-type. Depending on the culture, the reduction in seed DW for the putative null alleles ranged from 5% to 29% of the wild-type. Although variations in the severity of this phenotype could be observed among the cultures conducted, the reduction in seed DW observed in the mutants was always statistically significant. Considering the very specific spatiotemporal expression pattern of AtSUC5, the whole process of seed development was then carefully investigated in the suc5 mutants.

First, microscopic observations of cleared seeds were carried out during early embryonic morphogenesis and early maturation. The structure of the three types of tissues comprising the seed, i.e. integuments, endosperm and embryo, appeared normal. However, a slight delay in embryo development was reproducibly observed in the mutant lines (Figure 5a). At 8 DAF, the wild-type embryo encountered a phase of rapid elongation, progressing from the torpedo stage (55% of embryos) to bent-cotyledon stages (walking stick and upturned-U stages; 44% of

embryos). In the suc5.3 mutant line, fewer than 30% of the embryos exhibited cotyledon curvature, while in suc5.1 and suc5.2 (the putative null alleles), more than 80% of the embryos were still torpedo shaped. This observation was repeated twice on distinct embryo populations, and each time a statistically significant difference in embryo distribution between the wild-type and suc5 mutants was observed (chi-squared; $p < 0.01$). To confirm the link existing between this phenotype and the suc5 mutations, the mutant lines were crossed. For each of the three crosses, the progeny obtained was cultured and genotyped. No complementation of the phenotype could be observed in F$_2$ (data not shown).

Sugar content exhibited a similar evolution in mutant and wild-type seeds (data not shown). To investigate the putative effect of the suc5 mutations on the accumulation of storage compounds, time-course analyses of fatty acid content were then made. A classical sigmoid pattern of oil accumulation which closely paralleled the increase in seed DW was observed in the wild-type (see Figure 5 in Baud et al., 2003). Until 6 DAF, the fatty acid concentration in mutant seeds was fairly similar to the wild-type (Figure 5b; Table 1). Then at 8 DAF, the suc5.1 and suc5.2 seeds exhibited an important reduction (~45%) in fatty acid concentration compared with the wild-type. A smaller reduction of 20% was measured in the seed of the leaky suc5.3 allele. This marked delay in lipid accumulation in

Figure 4. Characterization of three suc5 mutants.
(a) Structure of the AtSUC5 gene showing the position of the T-DNA insertion in suc5.1, suc5.2 and suc5.3. Closed boxes represent exons while open boxes stand for untranslated regions (UTR). Nucleotide positions are relative to the translational start site and primer positions are shown as arrows. LB, left border; RB, right border.
(b) Quantitative RT-PCR analysis of AtSUC5 mRNAs in seeds aged 5–6 DAF. Values are the means ± SE of three replicates carried out on cDNA dilutions obtained from three distinct mRNA extractions. The asterisks indicate that transcripts were not detectable.
(c) Characterization of chimeric AtSUC5-T-DNA transcripts in suc5.3 seeds. Three sets of primers were used for a PCR experiment on cDNA from suc5.3 and wild-type seeds aged 6 DAF, and on DNA from a hemizygous SUC5/suc5.3 plant. The position of the primers used is indicated in (a).
Figure 5. Effect of the **suc5** mutation on seed development.

(a) Delayed elongation of the embryo in **suc5** seeds. Microscopic observations of developing seeds aged 8 DAF produced by mutant and wild-type plants were made. The quick-clearing solution of chloral hydrate in which the seeds were mounted allowed us to distinguish the developmental stage of the embryo. The relative distribution of the embryos amongst four developmental categories, e.g. small torpedo, torpedo, walking stick and upturned-U stages, is presented. For each genotype, the number of embryos observed is indicated.

(b) Altered accumulation of fatty acid in **suc5** seeds. A time course analysis of total fatty acid concentration was made for **suc5.1**, **suc5.2**, **suc5.3** and wild-type seeds. For each point of the kinetic and for each genotype, three independent measurements on batches of 20 seeds were performed. The three values obtained were averaged and the difference in fatty acid concentration compared with the wild-type, in %, was calculated for each mutant. The vertical arrow represents the peak in **AtSUC5** expression.

(c) Decreased content of fatty acids in mature **suc5** embryos and endosperms. Measurements were first carried out on batches of 15 mature dry seeds. To investigate the fatty acid content of separate embryos and endosperms, dry seeds were imbibed for 2 h at 4°C and then dissected under a binocular microscope. Batches of 15 embryos or endosperms were prepared and analysed. Each value is the mean ± SE of five replicates. Statistical analysis of the results was performed using the Dunnet multiple comparisons test, the control corresponding to the wild-type values. One asterisk indicates a significant difference at the 95% level and two asterisks a very significant difference at the 99% level.
the mutant backgrounds was transient. After 8 DAF, the differences observed between the wild-type and the suc5 lines steadily decreased, so that the concentration of fatty acid in dry mutant seeds was only 2–13% lower than in the wild-type, depending on the culture. The composition of the fatty acid was then considered (Table 1). At 4 DAF, both wild-type and mutant seeds contained high amounts of 16:0 (palmitic acid), 18:0 (stearic acid), 18:2 (linoleic acid) and 18:3 (alpha-linolenic acid), plus minor amounts of 18:1 (oleic acid) and 20:0 (behenic acid). With the onset of active synthesis of fatty acids at 8 DAF, this composition evolved; the proportion of 16:0 and 18:0 dropped markedly, while relative levels of 18:1 and 20:1 (eicosanoic acid) increased. Yet the fatty acid composition of the mutants shifted from the wild-type one at this stage. Compared with the wild-type, suc5 seeds aged 8 DAF exhibited a significant enrichment in 16:0, 18:2, 18:3 and a decrease in 18:1 and 20:1 (Table 1). These differences then gradually disappeared during seed maturation, so that the fatty acid compositions of wild-type and mutant dry seeds aged 22 DAF were very similar, with very long chains accounting for more than 50 mol.%.

If the small size of A. thaliana seed impaired the dissection and analysis of its constitutive tissues during their development (Hill et al., 2003), fatty acid measurements could be carried out separately on the embryo and the surrounding cellular endosperm of mature imbibed seeds (Penfield et al., 2004). In the suc5 mutant background, the two tissues exhibited a reduction in fatty acid content compared with the wild-type (Figure 5c). A 15% loss was measured in embryos of the suc5.1 and suc5.2 lines, whereas the corresponding mature mutant endosperm exhibited a 35% loss of fatty acids. Due to the small size of the material considered, it was not possible to weigh the samples and thus to determine whether these variations in total fatty acid content corresponded to significant differences in terms of fatty acid concentrations or merely reflected a reduction in the size of the tissues analysed, as suggested by whole seed analyses (Table 1). Penfield et al. (2004) demonstrated that wild-type endosperm contained proportionally higher levels of 18:1n7 and 20:1n7 long-chain fatty acids compared with embryos. In the suc5 lines, the relative proportion of n7 monounsaturated fatty acids exhibited a 20% increase compared with the wild-type (data not shown). Correspondingly, levels of n9 monounsaturated fatty acids were proportionally decreased. In spite of this altered lipid content, we were unable to detect any germination phenotype, either in the light or in the dark.

Discussion

During the development of seed in A. thaliana, the endosperm is assumed to serve as a transient medium supporting embryogenesis, which is almost completely consumed during maturation of the embryo (Lopes and Larkins, 1993). To fulfil its biosynthetic functions, this tissue has to import nutrients like sucrose. In this paper we report the isolation and characterization of the endosperm-specific sucrose translocator AtSUC5 and provide evidence that this carrier plays an important but transient role during early seed development.

Sucrose import in developing seeds of A. thaliana

The AtSUC5 gene, which is localized next to AtSUC1 on chromosome 1, exhibits a high sequence homology to previously characterized A. thaliana sucrose transporter genes. Its sucrose transport properties were clearly demonstrated in a heterologous system by Ludwig et al. (2000) and confirmed in this study. AtSUC5 behaves like a typical sucrose carrier and its Biotin-H+ symport capacity might represent a

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**Table 1** Fatty acid composition of WS and suc5 seeds. Total lipid extraction was carried out on batches of 20 seeds. The total fatty acid composition (in mol.%) of the seeds was determined by gas chromatographic analysis of an aliquot. Values are the means ± SE of three independent replicates. TFA, total fatty acids. Values in bold are significantly different from the WT (unpaired t-test)

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<th>18:0</th>
<th>18:1</th>
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<th>18:3</th>
<th>20:0</th>
<th>20:1</th>
<th>22:1</th>
<th>TFA (μg mg⁻¹ DW)</th>
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The general feature of sucrose transporters in all higher plants (Ludwig et al., 2000). The A. thaliana genome contains nine putative sucrose transporter genes, raising the question of why so many carriers are present in this plant. Apart from substrate specificity, an explanation might be that the genes are regulated differentially in specific cell types. Data obtained to date seem to confirm this hypothesis. Seven members of the AtSUC family have been functionally characterized and proved to be energy-dependent sucrose transporters (Figure 1). Six of the AtSUC genes have been studied in planta and exhibited distinct expression patterns, together with distinct protein localization. Accordingly, different roles for these transporters have been suggested, ranging from control of the osmotic driving force involved in anther dehiscence and pollen tube growth (Stadler et al., 1999), response to wounding (Meyer et al., 2004), loading of sugar into sieve elements (Stadler and Sauer, 1996) to sugar sensing (Barker et al., 2000). So far no carriers specifically involved in sugar uptake in A. thaliana seeds have been isolated. However, studies on legumes and cereals suggest that retrieval of sucrose from the apoplastic space by filial tissues, namely the embryo and the endosperm, plays a key role in gain of dry matter and seed filling (Harrington et al., 1997b; Weber et al., 1997a). When the GFP gene was expressed in A. thaliana under the control of the AtSUC2 promoter, a GFP signal was observed in the seed coat. But this signal simply reveals a symplastic unloading of the fluorescent protein from the adjacent phloem cells (Imlau et al., 1999). As for AtSUC3, although its complex and large expression pattern includes the embryo suspensor and the external layer of the embryo root tip, its implication in uptake of sucrose by the seed has not been demonstrated to date (Meyer et al., 2004). AtSUC5 is the first member of the AtSUC gene family to exhibit a marked seed-specific expression pattern (Figure 2). Thus, it has the potential to take part in post-phloem sucrose transport in the developing seed. This is discussed further below.

**AtSUC5 expression is endosperm specific**

Quantitative RT-PCR studies, promoter–GUS and promoter–GFP analyses and in situ hybridization experiments have demonstrated that AtSUC5 is exclusively expressed in the endosperm. The GFP signal is first seen between 4 and 6 DAF at the micropylar pole of the endosperm, in the region embedding the embryo and its suspensor (Figure 3). At the torpedo stage, the expression pattern of the gene reaches the chalazal pole, before gradually disappearing from the upturned-U stage onwards. In A. thaliana, the endosperm develops in two phases: first syncytial and then cellular (for review, see Berger, 1999). The syncytial A. thaliana endosperm contains three distinct domains arranged along the anteroposterior axis defined by the site of delivery of the sperm: the micropylar endosperm surrounding the embryo at the anterior pole (MCE), the peripheral endosperm (PEN) and the chalazal endosperm (CZE) at the posterior pole (Brown et al., 1999). Cellularization is initiated at the globular stage in the MCE. This process then spreads in a wave-like manner across the PEN toward the CZE (Mansfield and Briarty, 1990; Sorensen et al., 2002). Several genes that are involved in endosperm differentiation and that have domains of expression arranged along this anteroposterior axis have been isolated in A. thaliana (for review, see Berger, 2003). The expression pattern of AtSUC5 exhibits a similar trend and might be correlated to the de novo synthesis of membranes limiting the newly cellularized endosperm cells. Recent studies have allowed the identification of another A. thaliana transporter gene expressed in the endosperm of developing seeds (Hirner et al., 1998). The AAP1 gene encodes a seed-specific amino acid permease. The spatiotemporal analysis of the GUS staining in AAP1:GUS transformants revealed that the gene expression started as a peripheral ring up to the globular stage, then strongly increased at the heart stage, and finally retracted to the chalazal region before decaying during desiccation. If AtSUC5 constitutes the first endosperm-specific sugar carrier isolated in dicots, another sucrose transporter, namely HvSUT1, has been isolated in barley which is preferentially expressed in the endospermal transfer-cells of developing caryopses (Weschke et al., 2000). However, endosperm structure and function in dicots and monocots are quite distinct, and the role of AtSUC5 cannot be easily deduced from the barley model.

**Importance and function of the AtSUC5 transporter**

In monocots like barley the endosperm constitutes the main site of accumulation of storage compound. Persistent in the mature dry seed, this storage organ provides the nutrients and hormones required during germination. In contrast, in many dicots the endosperm is non-persistent. In such species, reserves are stored in the embryo and the endosperm is assumed to serve as a transient medium supporting embryonic morphogenesis and early maturation (Lopes and Larkins, 1993). If the A. thaliana endosperm represents a transient storage buffer that is mainly consumed during embryogenesis (Mansfield and Briarty, 1990), a limited number of compact endosperm layers are persistent in the dry seed, the lipidic reserve of which partially fuels skotomorphogenesis when seeds are germinated in the dark (Penfield et al., 2004). Since the storage compounds accumulated in this tissue may be used to nurture the embryo during both early embryogenesis and germination, their synthesis appears to be a key determinant of an efficient reproductive strategy. The expression pattern of AtSUC5 first suggests that this sucrose translocator provides the endospermic tissue with carbon precursors and/or biotin for its syntheses. This is characterized by a strong GFP signal in

The AtSUC5 sucrose carrier is endosperm specific

endospermic regions located at the interface with areas of nutrient delivery in the maternal tissues, both at the micropylar (from 4 DAF) and chalazal (from 7 DAF) pole of the endosperm (Figure 3). The AtSUC5 gene is also highly expressed in the region of the micropylar endosperm surrounding the young embryo, from globular to bent cotyledon stage. It is tempting to speculate that these filial tissues interact (Hill et al., 2003) and that AtSUC5 thus contributes to the nutrition of the embryo.

To further evaluate the importance of this carrier in planta, three suc5 mutants have been isolated and characterized. Due to the high rates of sucrose consumption in the maturing seed, the disaccharide is hardly detectable in the wild-type during early maturation (see Figure 3a in Baud et al., 2002). As a consequence, no reduction in sucrose content can be directly measured in the suc5 lines. However, these mutants exhibit a transient but strong reduction in accumulation of fatty acids concomitant with the peak of AtSUC5 expression (Figure 5b). This indirectly demonstrates that the nutrition of the filial tissues is altered in the mutant background. The small size of developing A. thaliana seeds prevents independent analyses of the endosperm and of the embryo during development of the seed. However, separate measurements of fatty acids are possible in mature imbibed seeds. The results obtained demonstrate that both the embryo and the endosperm are affected by the mutation (Figure 5c).

The characterization of suc5 mutants clearly demonstrates the importance of AtSUC5 in early synthesis of storage compounds in the two filial tissues of A. thaliana seeds. The suc5 mutation not only affects the fatty acid content in seeds aged 8 DAF but also results in a modification of the relative fatty acid composition of these seeds (Table 1). A clear alteration observed consists of a relative enrichment in 18:2 and 18:3 associated with a drop in the proportion of 18:1. This relative increase in highly desaturated chains suggests that the activation of desaturases in early maturing seeds is not tightly controlled in response to perturbations affecting carbon fluxes through the fatty acid biosynthetic pathway. Apart from affecting storage product synthesis, the suc5 mutation also slightly delays elongation of the embryo (Figure 5a). A possible reduction in availability of nutrients to the embryo due to the lack of AtSUC5 might explain this phenotype. Apart from this nutritional hypothesis, sucrose is also considered to be a signalling molecule, and its decreased delivery in the suc5 background could affect the induction of cell elongation in the embryo (Weber et al., 1997b).

The phenotype observed in the mutant seeds is transient and gradually disappears during maturation of the seed. Since the suc5 mutation is not lethal and since endosperm development in suc5 seeds is not compromised, other transport systems probably operate in the endosperm during early seed development, so that the nutrient deprivation detected in the suc5 background is only partial. Yet uncharacterized sucrose and/or hexose transporters may be involved that are either normally activated in the wild-type background during early seed development or are specifically induced in the mutant background in response to the mutation. As for the progressive disappearance of the phenotype after 8 DAF, this certainly reflects the activation of embryo-specific transport systems once early embryonic morphogenesis is completed. Sucrose transporters in the epidermis of the embryo could be involved in nutrient uptake during the maturation process, as described in the fava bean (Weber et al., 1997a). However, these putative carriers remain to be isolated and characterized in A. thaliana.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana of the ecotype Wassilewskija (Ws) as well as suc5.1 (DYO5 line), suc5.2 (EEU50 line) and suc5.3 (T990 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 sterilized and germinated on Murashige and Skoog (MS) medium (M20 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 20/15°C day/night, under a 16/8-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 sterilized 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, 1983). Only primary shoots were used for the experiments and secondary shoots were removed. To harvest siliques of defined developmental stages, individual flowers were tagged using coloured tape on the day of flowering. Pools of harvested seeds were freeze-dried and stored at 4°C for biochemical analyses. Material used for RNA extractions was immediately frozen in liquid nitrogen and stored at −80°C prior to extraction.

Construction of ProSUC5:uidA and ProSUC5:mGFP5-ER transgenes and plant transformation

The AtSUC5 promoter used in this study corresponds to region −1503 to −1 bp relative to the AtSUC5 translational start site and was amplified from Ws genomic DNA with 5′-attB1-TAGTAA-GAACAGTGATCC-3′ and 5′-attB2-TATGAAAGAAAAACGAG-CAG-3′, attB1 and attB2 referring to the corresponding Gateway recombination sequences. The PCR product was introduced by a BP recombination reaction into pDONR207 entry vector (Invitrogen, Carlsbad, CA, USA) and transferred to the binary vector pBI101-R1R2-GUS or pBI101-R1R2-GFP (F. Divol, J.-C. Palauqui and B. Dubreucq, Institute Jean-Pierre Bourgin, INRA, Versailles, France, unpublished data) by a LR recombination reaction, to obtain a transscriptional fusion between the AtSUC5 promoter and uidA or mGFP5-ER reporter genes. The resulting binary vectors were electroporated into Agrobacterium tumefaciens strain C58C1mpMP90 (Koncz and Schell, 1986) and used for agroinfiltration of A. thaliana inflorescences (Betchold et al., 1993). For each construct, six kanamycin-resistant transformants were selected on MS medium and

then transferred to the soil for further characterization. The six lines exhibited similar staining patterns. To conduct measurements of GUS activity during seed development, two representative Pro\textsubscript{SUC5}:uidA lines were chosen.

**In situ hybridization**

Developing siliques were fixed in 4% formaldehyde (fresh from paraformaldehyde) in PBS (Eurobio, Les Ulis, France) under vacuum for 1 h and left in fixative overnight. After fixation, siliques were washed, dehydrated slowly and embedded in paraﬃn (Paraplast Plus, Labonord Templemars, France). Paraﬃn sections (8 µm) were cut with a disposable metal knife and attached to pre-coated glass slides (DAKO Cytomation, Trappes, France). A 19 bp oligonucleotide probe speciﬁc for AtSUC\textsubscript{5} (5'-CATTTGGGCGGCTCTTTC-3') was designed after alignment of all the AtSUC sequences. The probe was synthesized using digoxigenin (DIG 11d-UTP; DIG oligonucleotide tailing kit; Roche, Meylan, France) according to the manufacturer’s instructions. In situ hybridization and immunodetection of the DIG-labelled probes were performed using an anti-DIG antibody coupled with alkaline phosphatase as described by the manufacturer (Roche). Control experiments were designed using the two null mutants, namely suc\textsubscript{5-1} and suc\textsubscript{5-2}, to check the speciﬁcity of the probe used.

**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 (Axioplan 2; Zeiss, Jena, Germany). Photographs were taken using Kodak Elite 160T ﬁlm.

Imaging of living tissues expressing the mGFP5-ER reporter gene was performed with a ﬂuorescence microscope (Axioplan 2; Zeiss) equipped with epifluorescence optics after mounting in water. Observations were also made with an inverted Leica TCS-NT confocal laser scanner equipped with an argon/krypton laser (Omnichrome, Chino, CA, USA) and an acousto-optic tunable ﬁlter for excitation after mounting in the lipophilic dye FM 4-64 (Molecular Probes, Eugene, OR, USA). Photographs were digitized using an ARCUS 1200 scanner (Agfa-Gevaert, Mortsel, Belgium).

**Assays for GUS activity**

The ﬂuorometric assay was performed as described by Jefferson (1987) on extracts made from diﬀerent parts of transgenic and control plants. The reaction product of the assay, namely 4-methylumbelliferone, was measured kinetically, in duplicate, using a ﬂuorometer (Fluoroskan II; Labsystems, Helsinki, Finland). Protein concentration was estimated for each extract using the method of Bradford (1976).

**Lipid analyses**

For time-course studies of total fatty acid accumulation, pools of 20 seeds were ground in a glass reaction tube. For analyses of dissected embryos and endosperms, pools of 15 seeds were imbibed for 2 h at 4°C then dissected under a binocular; the tissues thus isolated were frozen in liquid nitrogen and then ground in a glass reaction tube. Extraction and analyses of fatty acyl methyl esters were performed using gas chromatography as described previously (Baud et al., 2002).

**DNA extraction and PCR analyses**

Genomic DNA was extracted according to Oard and Dranavalli (1992). For molecular characterization of the suc\textsubscript{5} mutations, 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). To amplify the left border of suc\textsubscript{5-1}, EEU.LB (5'-ATGAAATGGAAACCTATGG-3') and TailA (5'-ATTGCTTTTTCTCTACGACC-3') were used; for the right border, EEU.RB (5'-CCGGCTGTGACCTACAC-3') and Tail1 (5'-CTGATACCA-GACGTTGCC-3') were used. To amplify the left border of suc\textsubscript{5-2}, DY05.LB2 (5'-CCCTTTTGAAGCTTTTAC-3') and TailA were used; for the right border, DY05.RB (5'-CATAAAGAGAAGAAACACG-3') and Tail1 were used. To amplify the left border of suc\textsubscript{5-3}, T990.LB (5'-GCGGTGATGGAGCTTTAG-3') and TailA were used; for the right border, T990.RB1 (5'-TTGGGGTGTTCCTTTGCG-3') and Tail1 were used. DNA sequencing was then carried out on PCR products puriﬁed with a Qiagen (Chatsworth, CA, USA) puriﬁcation kit, using the Applied Biosystems (Foster City, CA, USA) DNA sequencing kit (Bigdye Terminator V3.0) and an 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 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)\textsubscript{12-18}. For real-time quantitative RT-PCR: the reaction was performed on the LightCycler Instrument (Roche) 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. The speciﬁc primers used, namely T990.up (5'-AGA-AAGGCGGCACAAA-3') and Trev.1 (5'-GACCATTGTGGAGGATC-3'), were designed in the 5' region of the AtSUC\textsubscript{5} cDNA. The reactions were incubated at 95°C for 8 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. 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 results obtained were standardized to the constitutive EF\textsubscript{1α}A4 gene expression level (Liboz et al., 1990), ampliﬁed with the EF1\textsubscript{F} and EF1\textsubscript{R} primers (Baud et al., 2003). The efﬁciencies of the diﬀerent primer sets used were checked to be almost similar.

To detect the presence of a chimerical cDNA in suc\textsubscript{5-3}mutants, 35 cycles of RT-PCR were performed with primers located upstream (T990.LB), downstream (T990.RB1) or in (TailA and Tail1) the T-DNA element (Figure 4a).

**Complementation of a sucrose uptake-deﬁcient yeast mutant**

The AtSUC\textsubscript{5} cDNA was ampliﬁed with a proofreading DNA polymerase Pfu (Pfu-turbo; Stratagene, La Jolla, CA, USA) using TSUC-Catg1 (5'-ACATCCAAAAACATTTAATCCCC-3') and TSUCstop2 (5'-TAAAGAGACAGCAGCAAGG-3') primers, and a cDNA
preparation from seeds aged 6 DAF as a template. The PCR product was cloned in pBluescript SK+ (pBS; Stratagene) digested by EcoR1, sequenced, transferred in YEP112 plasmid, and introduced into the yeast strain SUSY7 (Riesmeier et al., 1992). Yeasts complemented with AtSUC5 were able to grow on minimal medium with sucrose as the sole carbon source, whereas yeast complemented with plasmid YEP112 did not (data not shown). This confirmed that the cloned cDNA encoded a functional sucrose transporter.

Construction of the distance tree

The multiAlign program (Corpet, 1988) was used to create an alignment of the sequences that was then submitted to a neighbour-joining analysis to generate a branching pattern using the CLUSTALX package (Thompson et al., 1997). Bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology. The consensus tree was drawn using the TREEVIEW program (version 1.5.3, Roderic DM Page, University of Glasgow, UK).

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