SUCROSE TRANSPORTER 5 supplies Arabidopsis embryos with biotin and affects triacylglycerol accumulation

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SUMMARY

The Arabidopsis SUC5 protein represents a classical sucrose/H+ symporter. Functional analyses previously revealed that SUC5 also transports biotin, an essential co-factor for fatty acid synthesis. However, evidence for a dual role in transport of the structurally unrelated compounds sucrose and biotin in plants was lacking. Here we show that SUC5 localizes to the plasma membrane, and that the SUC5 gene is expressed in developing embryos, confirming the role of the SUC5 protein as substrate carrier across apoplastic barriers in seeds. We show that transport of biotin but not of sucrose across these barriers is impaired in suc5 mutant embryos. In addition, we show that SUC5 is essential for the delivery of biotin into the embryo of biotin biosynthesis-defective mutants (bio1 and bio2). We compared embryo and seedling development as well as triacylglycerol accumulation and fatty acid composition in seeds of single mutants (suc5, bio1 or bio2), double mutants (suc5 bio1 and suc5 bio2) and wild-type plants. Although suc5 mutants were like the wild-type, bio1 and bio2 mutants showed developmental defects and reduced triacylglycerol contents. In suc5 bio1 and suc5 bio2 double mutants, developmental defects were severely increased and the triacylglycerol content was reduced to a greater extent in comparison to the single mutants. Supplementation with externally applied biotin helped to reduce symptoms in both single and double mutants, but the efficacy of supplementation was significantly lower in double than in single mutants, showing that transport of biotin into the embryo is lower in the absence of SUC5.

Keywords: biotin transport, embryo, fatty acid content, endosperm, sucrose transport, AtSUC5.

INTRODUCTION

Biotin (vitamin B7 or vitamin H) is a prosthetic group in a small number of enzymes that catalyse essential carboxylation, decarboxylation and transcarboxylation reactions (Knowles, 1989; Nikolau et al., 2003; Smith et al., 2007; Ding et al., 2012). Its most prominent role is that of an essential co-factor for both the cytosolic and plastidic isoforms of acetyl CoA carboxylase, which both catalyse the first and rate-limiting step in fatty acid biosynthesis (Nikolau et al., 2003). Bacteria, plants, some fungi and a few animals are capable of synthesizing the biotin required for these reactions. All other organisms, including humans and baker’s yeast (Saccharomyces cerevisiae), depend on uptake of biotin from their environment, and cDNAs of plasma membrane-localized biotin transporters have been cloned [SODIUM-DEPENDENT MULTIVITAMIN TRANSPORTER in human (SDMT; Prasad et al., 1998) and VITAMIN H TRANSPORTER1 in yeast (VHT1; Stolz et al., 1999)].

For identification of possible plant biotin transporters, a yeast mutant lacking the VHT1 gene (∆vht1) was complemented with an Arabidopsis cDNA library and screened for growth on medium containing low biotin concentrations (Ludwig et al., 2000). Surprisingly, this screening identified a sequence with high similarity to sucrose transporter cDNAs (e.g. AtSUC1 and AtSUC2 from Arabidopsis). Functional analyses of the encoded protein demonstrated that it was in fact a member of the Arabidopsis sucrose transporter family (named SUC5; At1g71890), with transport characteristics similar to those of previously published...
sucrose transporters (Ludwig et al., 2000). However, analyses with radiolabelled biotin confirmed that SUC5 also transports biotin (Ludwig et al., 2000), a molecule that has no structural similarity to sucrose. Baud et al. (2005) showed SUC5 expression mainly in the seed endosperm, in agreement with SUC5’s potential role in biotin delivery into the seed, where large amounts of fatty acids are synthesized and stored as triacylglycerols (TAG). However, evidence for a role of SUC5 in catalysis of biotin transport in planta has so far not been provided. Analyses of three suc5 mutants (suc5.1, suc5.2 and suc5.3) studied from 4 to 22 days after fertilization (DAF) revealed that the fatty acid contents of mutant and wild-type were not or were hardly affected in 22 DAF seeds. However, 8 DAF suc5 seeds showed a 20–45% transient decrease in their total fatty acid content. This time point (8 DAF) coincides with the onset of active fatty acid biosynthesis in developing Arabidopsis wild-type seeds (Baud et al., 2002). Obviously, this transient phenotype may result from a reduction in SUC5-driven sucrose import (i.e. reduced availability of organic carbon for fatty acid biosynthesis), a reduction in SUC5-driven biotin supply (i.e. reduced availability of a co-factor required for fatty acid elongation), or a combination of both. Here we describe use of physiological, genetic and biochemical approaches to elucidate the role of the SUC5 protein as a biotin transporter in planta, making use of T-DNA insertion mutants of SUC5. We isolated two allelic mutants disrupted in the SUC5 gene (suc5.4 and suc5.5), and used one (suc5.4) to measure uptake of the two putative substrates, biotin and sucrose, into isolated embryos. Analysis of substrate uptake into intact plants or embryos has been shown to be a suitable method to demonstrate in planta transport activity. Sherson et al. (2000) successfully analysed STP1-mediated transport of glucose by comparing uptake of [14C]-D-glucose into wild-type and stp1 seedlings for up to 60 min. In another study, Matsuura et al. (2000) incubated rice embryos for between 24 and 120 h with radiolabelled glucose, fructose and sucrose, and showed that uptake of [14C]-sucrose into embryos is driven by the action of the rice sucrose transporter OsSUT1. Our data also demonstrates that isolated Arabidopsis embryos are a suitable system for measuring active transport processes. In addition, we studied the physiological role of SUC5 by analysing developmental and biochemical properties of suc5 transporter mutants in the background of Arabidopsis lines that are defective in biosynthesis of biotin, one of the putative substrates. Arabidopsis plants with defects in biotin biosynthesis were first identified in analyses of embryo-lethal mutants. One mutant, bio1.1, was shown to be defective in synthesis of the biotin precursor 7,8-diaminopelargonic acid (Schneider et al., 1989; Muralla et al., 2008). The other mutant, bio2.1, is defective in conversion of dethiobiocin to biotin (Baldet and Ruffet, 1996; Patton et al., 1996, 1998; Weaver et al., 1996). The developmental arrest observed in homozygous (bio1.1/bio1.1 or bio2.1/bio2.1) mutant embryos in the siliques of heterozygous (BIO1/bio1.1 or BIO2/bio2.1) mother plants was rescued by watering the soil-grown heterozygotes with 0.5 mM biotin. This shows that the supplied biotin is taken up by the roots, translocated to the developing seeds, and imported into the endosperm and the developing embryo. This process requires transfer of biotin from the xylem into the sieve element/companion cell complexes of the phloem, which includes transport of biotin across several membranes. Exclusively passive diffusion of biotin across these various membranes seems unlikely. We compared the development of embryos, seedlings and seeds in the various single mutants and bio suc5 double mutants, and quantified the TAG content and the fatty acid composition in dry seeds of the various plants. Both suc5 single mutants resembled wild-type plants, but the absence of SUC5 strongly enhanced all developmental and biochemical phenotypes observed in bio1.1 and bio2.1 mutant plants. In summary, our data show that SUC5 transfers biotin from the maternal tissue into the endosperm and embryos of developing Arabidopsis seeds, and that this activity is essential under conditions of biotin limitation.

RESULTS

Analyses of pSUC5/sGFP and pSUC5/tmGFP9 plants

We generated two pSUC5/reporter lines under the control of a 2030 bp SUC5 promoter. These lines expressed the open reading frames of soluble and freely mobile green fluorescent protein (sGFP) or a non-mobile version of GFP (tmGFP9) that is membrane-associated via N-terminal transmembrane helices (Stadler et al., 2005a). Analyses of these plants by confocal microscopy confirmed the previously reported SUC5 expression in the endosperm (Figure 1a,b) (Baud et al., 2005), and accumulation of GFP at the chalazal end of the endosperm (Figure 1b). No expression of GFP was observed in globular (Figure 1c) or heart-stage embryos (Figure 1d). Unexpectedly, we observed SUC5 activity for both constructs during the later stages of embryo development (Figure 1e–i). This SUC5 expression in the embryo was not observed in earlier analyses performed using standard fluorescence microscopy and a shorter 1500 bp promoter fragment (Baud et al., 2005).

Optical sections through pSUC5/tmGFP9 embryos confined pSUC5 activity specifically on the epidermis of the outer surface of the cotyledons (Figure 1g,h). In previous analyses (Stadler et al., 2005a), it was shown that sGFP synthesized in the embryo epidermis from Arabidopsis GLABRA2 promoter (gGL2/sGFP constructs moves symptomatically into all other cells of the developing embryo.
due to the presence of large plasmodesmata. This explains the homogenous fluorescence seen in early torpedo-stage embryos expressing sGFP from pSUC5 (Figure 1e). Embryos from wild-type seeds showed no fluorescence at any developmental stage (Figure 1i).

In addition, we analysed the subcellular localization of the SUC5 protein by transiently transforming Arabidopsis protoplasts with N- and C-terminal GFP fusion constructs of SUC5. Both fusion proteins localized to the plasma membrane (Figure 1j,k). We verified this localization in particle-bombed leek (Allium ampeloprasum) epidermis cells (Figure 1l,m).

**Identification of two allelic mutant lines defective in SUC5**

The suc5.1, suc5.2 and suc5.3 mutants described by Baud et al. (2005) were generated in the Wassilewskija background. As the bio1.1 and bio2.1 mutants were in ecotype Columbia (Col-0), we characterized two suc5 mutants (SAIL_365_D07, suc5.4; SALK_092412, suc5.5; http://signal.salk.edu/cgi-bin/tdnaexpress) in the same ecotype. These mutants carry insertions after nucleotide 250 of the 2nd intron (suc5.4) or between nucleotides 98 and 105 of the 3rd exon (suc5.5, six nucleotides deleted), with suc5.5 having a tandem insertion with the two right borders facing each other (Figure 2a). For the suc5.4 allele, only the orientation of the left border was determined. Comparative RT-PCR using RNA isolated from flower tissue (Figure 2c) demonstrated that both lines produced mRNA from the first exon, but failed to produce the complete SUC5 mRNA. Therefore, both mutants produce truncated SUC5 proteins, with the T-DNA insertion in suc5.4 resulting in a truncated protein lacking the last two transmembrane helices, and the T-DNA insertion in suc5.5 resulting in a truncated protein lacking the last transmembrane helix. Studies on the structurally related HUP1 hexose transporter of Chlorella demonstrated that the C-terminal region and the 11th
transmembrane domain in particular are essential for transport function, and it is therefore unlikely that the truncated SUC5 proteins analysed here retain any transport activity (Caspari et al., 1994; Will et al., 1998).

**Uptake of biotin and sucrose by wild-type and suC5 embryos**

SUC5-mediated transport of biotin was demonstrated by heterologous expression of SUC5 in a yeast strain lacking VHT1, the endogenous transporter for biotin (Ludwig et al., 2000). To confirm the biotin transport activity of SUC5 in planta, we compared the uptake of radiolabelled biotin and sucrose into isolated embryos (8 DAF) from suC5.4 single mutants and wild-type (Figure 3). The embryo is separated from the maternal tissue by the seed apoplasmic space, and therefore import into the embryo may only be driven by active transport processes. These uptake measurements revealed that the uptake rate of biotin into isolated wild-type embryos was concentration-dependent, and that the uptake of biotin into suC5.4 embryos was significantly lower (for 10 and 25 μM biotin; P < 0.05, Student’s t-test) than that into wild-type embryos (Figure 3a). In contrast to biotin, uptake of sucrose was unaltered in wild-type and suC5.4 (Figure 3b). No phenotypic differences were observed between the suC5.4 and wild-type embryos (Figure 3c).

**Comparative analyses of embryo and seed development**

The originally described bio1.1 and bio2.1 mutations (Schneider et al., 1989; Patton et al., 1998) were induced by chemical mutagenesis. Homozygous suC5.4 or suC5.5 plants were crossed with these bio1.1 or bio2.1 mutants, and double homozygosity was determined by PCR (for the T-DNA insertions in suC5.4 and suC5.5) and by confirming the growth defect on biotin-free medium (for bio1.1 and bio2.1) in the subsequent generations.

On soil, these double homozygotes developed normal rosettes, flowered and produced fertile seeds when watered with 1 mM biotin. Without supplemented biotin, the homozygous bio1.1 and bio2.1 plants and all double homozygotes germinated poorly, and developed into tiny plants that turned pale and eventually died (Figure S1). However, when the seeds were first germinated on biotin-
containing (1 mM) agar medium for approximately 10 days and then transferred to soil, normal-looking plants developed that flowered even without further biotin supplementation (Figure S2).

To compare the development of seeds and embryos in wild-type plants, suc5.4, suc5.5, bio1.1 and bio2.1 single mutants and bio suc5 double mutants, seeds of all lines were germinated on agar medium containing 1 mM biotin. After 12 days, all seedlings were an identical size and were transferred to soil (Figure S2). These seedlings were then watered either without additional biotin, with 0.1 mM supplemental biotin, or with 1 mM supplemental biotin.

We harvested siliques at the same developmental stage from these plants, and analysed seed and embryo development (Figure 4a,b). Whereas developing wild-type and suc5 seeds and embryos looked normal under all growth conditions, bio2.1 seeds and embryos showed a biotin-dependent phenotype. Without biotin supplementation, the bio2.1 seeds were yellowish and pale, and embryos isolated from these seeds showed strongly retarded development (0 mM biotin, Figure 4a,b). This defect was partly rescued in plants supplemented with 0.1 mM biotin. Embryos from these plants were the same size as wild-type embryos; however, they were unable to synthesize chlorophyll and had a faint yellowish colour. Embryos from bio2.1 plants supplemented with 1 mM biotin were able to synthesize chlorophyll and looked essentially like wild-type embryos.

A significantly stronger phenotype was observed in developing seeds and embryos of bio2.1 suc5.5 double mutants that were not supplemented with biotin (0 mM biotin, Figure 4a,b). Seeds from these plants were white and smaller than seeds of bio2.1 single mutants, indicating a stronger biotin deficiency. Without biotin supplementation, no embryos were detected in these seeds, and even embryos from plants supplemented with 0.1 mM biotin showed a strong developmental phenotype. Only in seeds from...
plants that were supplemented with 1 mM biotin were wild-type-like embryos formed.

We finally compared the morphology (Figure 5a) and the weight (Figure 5b) of dry seeds from homozygous single and double mutants, and from wild-type plants. As expected, wild-type and suc5.5 mutant seeds looked normal under all growth conditions. In contrast, the seeds of bio2.1 and bio2.1 suc5.5 plants had a slightly (bio2.1) or strongly wrinkled appearance (bio2.1 suc5.5) when the plants had not been supplied with biotin. Supplementation of 0.1 mM biotin complemented these phenotypes to various extents. Whereas the bio2.1 seeds looked almost normal, all of the bio2.1 suc5.5 seeds still had a wrinkled appearance. Watering of the parent plants with 1 mM biotin completely (bio2.1) or almost completely (bio2.1 suc5.5) reversed this phenotype. Similar results were obtained for developing seeds and embryos and for dry seeds from bio1.1 and bio1.1 suc5.4 plants.

Because only very few seeds were obtained from bio suc5 double mutants that were watered without any biotin supplementation, we compared the seed weight only from plants supplemented with 0.1 and 1 mM biotin. The 100-seed weight from wild-type, suc5.4 and suc5.5 mutants was unaltered under both supplementation conditions (0.1 and 1 mM biotin, Figure 5b), but that of bio1.1, bio2.1, bio1.1 suc5.4 and bio2.1 suc5.5 mutants was significantly lower ($P < 0.005$, Student’s t-test) than in wild-type or suc5 single mutants under both conditions. The seed weight of bio1.1 suc5.4 and bio2.1 suc5.5 was also lower than that of the bio1.1 and bio2.1 single mutants, but this was not significant for 0.1 mM biotin. For 1 mM biotin, a significant difference was observed for bio1.1 versus bio1.1 suc5.4 (indicated by an asterisk, $P < 0.05$, Student’s t-test). The differences in seed weight reflect the observed morphological differences in these seeds.

Figure 5. Phenotypes and 100-seed weight of dry seeds from wild-type plants, and single and double mutants supplemented with various biotin concentrations. (a) Dry seeds from the indicated plant lines. Seeds of wild-type and suc5.5 plants are shaped normally under all growth conditions. Seeds of bio2.1 plants are wrinkled and seeds of bio2.1 suc5.5 double mutants have the appearance of ‘empty bags’ when their parent plants are not supplemented with biotin. Supplementation with 0.1 mM biotin complemented this defect partly (bio2.1 seeds are almost normal looking; bio2.1 suc5.5 seeds are still wrinkled). Seeds looked normal when the parent plants were watered with 1 mM biotin. Scale bars = 500 μm. (b) Weight of dry seeds in mg per 100 seeds for wild-type and the indicated genotypes supplemented with either 0.1 or 1.0 mM biotin. Bars labelled with different letters represent 100-seed weights that differ significantly from each other ($P < 0.005$, Student’s t-test). The difference between bio1 and bio1.1 suc5.4 at 1 mM (white asterisks) is slightly significant ($P < 0.05$, Student’s t-test, black asterisk above bar).

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Phenotypic comparison of seedlings

Homozygous single mutant seedlings (\textit{bio}1.1, \textit{bio}2.1, \textit{suc}5.4 and \textit{suc}5.5) developed normally on high-biotin medium and were indistinguishable from wild-type plants (Figure 6, MS + biotin). However, similar analyses on biotin-free medium (Figure 6, MS) confirmed the previously described defects for \textit{bio}1.1 and \textit{bio}2.1 seedlings (Schneider et al., 1989; Patton et al., 1998). Germinable homozygous \textit{bio}1.1 and \textit{bio}2.1 seeds were obtained exclusively from biotin-watered plants. After germination, the seedlings started to form normally sized, green cotyledons. However, the first pair of rosette leaves developed poorly, and was tiny and yellowish (Figure 6), and eventually these plants died before developing inflorescences. In contrast, \textit{suc}5.4 and \textit{suc}5.5 (parent plants not watered with biotin) showed no phenotypic difference from wild-type seedlings on biotin-free medium.

When the same analyses were performed using seeds of \textit{bio}1.1 \textit{suc}5.4, \textit{bio}1.1 \textit{suc}5.5, \textit{bio}2.1 \textit{suc}5.4 and \textit{bio}2.1 \textit{suc}5.5 double homozygotes (all parent plants watered with biotin; Figure 6, right panel), we observed a high percentage of seedlings with severely aberrant cotyledon phenotypes on biotin-free medium (Figure 6, MS). The cotyledons of almost all seedlings were smaller than those of wild-type seedlings, and these cotyledons were partially or completely white in a high percentage of seedlings. Interestingly, even these strong phenotypes were rescued by 1 mM biotin in the growth medium (Figure 6, MS + biotin). However, the development of these rescued double mutant seedlings was delayed compared to rescued \textit{bio}1.1 or \textit{bio}2.1 seedlings.

A more detailed and quantitative analysis of these double mutant phenotypes is shown in Figure 7. The cotyledons of many seedlings failed to expand and were too small to lift the seed coat (Figure 7a). However, even when the expanding cotyledons lifted the seed coat, they stayed tiny and failed to turn green (Figure 7c). Other seedlings had mis-shapen, only partially green cotyledons (Figure 7b), and the few seedlings with normally shaped cotyledons were smaller than wild-type seedlings (Figure 7d). A significant number of seeds did not germinate at all or only after prolonged incubation. A quantification of the observed phenotypes (Figure 7e) demonstrated that the percentage of mis-shapen seedlings or non-germinating and late-germinating seeds was negligible in the various homozygous single mutants, but very high in the double mutants.

Analyses of TAG content and fatty acid composition in seeds

As biotin-dependent enzymes are involved in central steps of fatty acid biosynthesis (Ohlrogge and Jaworski, 1997), biotin limitation not only affects seed and seedling morphology, but also reduces the capacity to synthesize TAG. If SUC5 acts as biotin transporter, an additional \textit{suc}5
mutation should further reduce these TAG levels. The seed yield from double mutants that were not supplemented with biotin was not high enough to perform TAG analyses. Therefore, we analysed seeds from wild-type plants, single and double mutants that were supplemented with 0.1 or 1 mM biotin (Figure 8a). For 0.1 mM biotin, the total TAG content of seeds from bio1.1 and bio2.1 single mutants was reduced to 10% (bio1.1) and 30% (bio2.1) of wild-type levels. For 1 mM biotin, the TAG content of the single mutants was still significantly lower than that of similarly supplemented wild-type plants (75% for bio1.1 and 78% for bio2.1). This reduction in TAG content was even greater in seeds from bio suc5 double mutants. The TAG content of bio1.1 suc5.4 seeds was only 6% (0.1 mM biotin) and 46% (1 mM biotin) of wild-type levels, and that of bio2.1 suc5.5 seeds was only 4% (0.1 mM biotin) and 49% (1 mM biotin) of wild-type levels. This stronger reduction also resulted in significant lower TAG contents between bio single and bio suc5 double mutant seeds (P ≤ 0.005, Student’s t-test). Only the difference between bio1.1 and bio1.1 suc5.4 supplemented with 0.1 mM biotin was not significant. Seeds from suc5.4 and suc5.5 single mutant plants supplemented with 0.1 or 1 mM biotin had TAG levels that were similar to the wild-type (Figure 8a).

Analyses of the fatty acid composition in the various TAG samples revealed that the reduced TAG contents in seeds of bio1.1 and bio2.1 plants (0.1 mM biotin) were paralleled by altered fatty acid compositions. Five- to sixfold higher contents of mono-unsaturated (n-7) fatty acids [16:1(n-7), 18:1(n-7) and 20:1(n-7)] and two- to threefold lower contents of mono-unsaturated (n-9) fatty acids [18:1(n-9) and 20:1(n-9); Figure 8b,c] were detected. Moreover, these seeds showed a higher percentage of the short-chain fatty acids 16:0 and 16:1(n-7). All of these alterations were more pronounced (up to twofold) in bio1.1 suc5.4 and bio2.1 suc5.5 seeds (Figure 8b,c). The percentage of the short-chain fatty acids 16:0 and 16:1(n-7) increased to over 20 mol% in suc5.5 bio2.1 in 0.1 mM biotin, in comparison with 8.5 mol% in wild-type, suc5.5 or bio2.1 in 0.1 mM biotin.
The fatty acid composition in seeds from bio1.1 or bio2.1 plants supplemented with 1 mM biotin were comparable to those in suc5 or wild-type seeds, but we did not observe complete restoration of the wild-type distribution in seeds of bio suc5 plants (Figure 8c). The seeds of suc5 or wild-type plants showed the same fatty acid composition under both conditions (Figure 8c).

**DISCUSSION**

The studies described here address the question of whether or not the Arabidopsis SUC5 protein acts as a biotin transporter _in planta_.

When we started our analyses, it was known that SUC5 transports both sucrose and biotin in yeast cells (Ludwig _et al._, 2000), that the SUC5 gene is expressed in the endosperm, and that suc5 mutants accumulate wild-type TAG levels in dry seeds (Baud _et al._, 2005).

Here we show that the SUC5 protein localizes to the plasma membrane (Figure 1j–m), confirming the predicted localization for a sucrose transporter of the SUT-1 clade (Reinders _et al._, 2012; Wolfenstetter _et al._, 2012). Moreover, SUC5 is also expressed in the epidermis of torpedo-stage or older embryos (Figure 1e–i), demonstrating that SUC5 is involved in transport of its substrate(s) across the plasma membrane of embryo epidermis cells. Our results also demonstrate that SUC5 is important for transport of biotin across these boundaries, and provide evidence that biotin transport by SUC proteins is physiologically relevant _in planta_.

**SUC5 is responsible for biotin transport _in planta_**

In agreement with our localization data (Figure 1), suc5 single mutants displayed reduced uptake of biotin but not sucrose into 8 DAF embryos in comparison with wild-type (Figure 3a,b), suggesting that either the role of SUC5 for the transport of sucrose into the embryo is negligible or the lack of sucrose transport activity is compensated for by the action of other SUCs. Of the nine members of the SUC transporter family in Arabidopsis, localization in the developing seed has been shown for SUC3 (embryo radicle; Meyer _et al._, 2004), SUC8 (whole seed; Sauer, 2007) and SUC9 (embryo cotyledons plus radicle; Sivitz _et al._, 2007). SUC9 in particular appears to be a good candidate for assuming a primary role in sucrose loading into the embryo, because it resembles SUC5 in terms of its transport kinetics for sucrose but has a tenfold lower K_m for sucrose (<0.1 mM for SUC9 versus 1 mM for SUC5; Ludwig _et al._, 2000; Sivitz _et al._, 2007). However, detailed analyses of _AtSUC9_ mutant embryos have not been performed, and nor has biotin transport activity for _AtSUC9_ been investigated yet.

However, the measured difference in biotin uptake suggests that, although the spatio-temporal expression patterns of the corresponding genes encoding the above mentioned SUCs overlap with that of SUC5, these transporters cannot fully compensate for the missing biotin transport activity of SUC5. The nonetheless measurable accumulation of radiolabelled biotin in suc5 mutant embryos may result from a lower transport affinity for...
biotin of these other SUCs (as shown for PmSUC2; Ludwig et al., 2000).

Apart from the reduced uptake of biotin into suc5.4 embryos (Figure 3), our comparisons of wild-type plants and suc5 mutants revealed no differences in embryo and seedling development (Figures 4−6), no significant decrease in TAG accumulation and no alteration in fatty acid compositions (Figure 8).

bio1.1 and bio2.1 single mutants had severe phenotypes, such as impaired seedling development (Figure 6), altered seed morphology and weight (Figures 4 and 5), reduced TAG content (Figure 8a), and altered fatty acid composition in dry seeds (Figure 8b,c). Most importantly, mutations in the SUC5 gene in the bio1 or bio2 mutant backgrounds led to a significant increase of all these defects. bio1 suc5 and bio2 suc5 double mutants that were not supplemented with biotin for one generation produced almost no seeds, and the few seeds obtained showed drastically impaired germination (Figure 7b). In fact, the bio1 suc5 and bio2 suc5 seeds (Figure 5) looked empty, had a wrinkled appearance, and resembled seeds of the low seed-oil mutant wrinkled1 (wri1; Focks and Benning, 1998), which has a defect in a transcription factor (WR11) that is involved in control of metabolism, particularly fatty acid synthesis, in developing seeds (Cernac et al., 2006; Baud et al., 2007). Seedlings that germinated from these bio1 suc5 and bio2 suc5 seeds exhibited strong deformations of their cotyledons, the main site of TAG synthesis and storage in the developing embryo (Figure 7a−c). The observed differences in cotyledon deformation may reflect variations in the biotin content of individual seeds caused by an unequal supply of biotin under conditions of biotin limitation. As in the bio1 and bio2 single mutants, supplementation with externally applied biotin established the wild-type phenotype in the bio1 suc5 and bio2 suc5 double mutants. However, full restoration of wild-type parameters, especially seed weight (Figure 5b), TAG content and TAG composition (Figure 8a−c) was not observed. This most likely reflects the lack of biotin transport activity in the embryo mediated by SUC5, and suggests that possibly even higher biotin concentrations are required for non-SUC5 mediated transport of biotin into the embryo.

When the bio or bio1 suc5 and bio2 suc5 mutants were supplemented with biotin, their otherwise embryo-lethal defects were rescued. Following uptake of biotin by the roots, and its translocation via the xylem, it is eventually unloaded at the integument, where both phloem and xylem vessels terminate (Stadler et al., 2005a). Transfer of biotin from the xylem into the phloem involves the apoplasm and is probably carrier-mediated, but it is unclear which carriers load biotin into sieve element/companion cell complexes. Complementation studies in yeast suggest an involvement of SUT1-type sucrose transporters, as shown for PmSUC2 (Ludwig et al., 2000). Export from the maternal tissues into the seed apoplasmic space is probably also carrier-mediated. Recently, a group of carriers has been described that catalyses the release of sucrose into the apoplasm (SWEET proteins, Chen et al., 2012). Whether these sucrose efflux transporters also accept biotin as a second substrate is unknown. However, our data suggest that import of biotin into the endosperm and the embryo epidermis is catalysed by the SUC5 protein. At what rate this import is catalysed is unknown, because the actual local biotin concentration at the integument and the outside of the embryo epidermis vary during embryo development. However, in biotin transporter-deficient yeast cells complemented with SUC5, biotin import was measured over a concentration range between 5 μM and 2 mM biotin, and such complemented yeast cells grow on medium with biotin concentrations as low as 10 nM (Ludwig et al., 2000).

**Sufficiently high biotin levels are essential for fatty acid and TAG biosynthesis**

In comparative analyses of embryo and endosperm fatty acid contents, Penfield et al. (2004) found that the endosperm contains proportionally higher levels (approximately 20%) of (n-7) long-chain fatty acids [16:1(n-7), 18:1(n-7) and 20:1(n-7)] than the embryo (2%). In contrast, 20:1(n-9) levels were shown to be proportionally higher in the embryo. The changes in the fatty acid compositions in bio versus suc5 seeds (Figure 8c) may therefore indicate that the reduced TAG levels (Figure 8a) in these seeds coincide with an increase in the endosperm-to-embryo ratio. The observed patterns of seed and embryo development shown in Figure 4 support this interpretation.

The embryo represents a strong sink for biotin, and our data suggest that fatty acid biosynthesis and acetyl CoA carboxylase activity are affected by changes in the availability of biotin. Reduced or absent SUC5 activity eventually leads to a lack of biotin in the embryo. This lack may cause the previously described transient decrease in fatty acid content in suc5 mutants at 8 DAF (the onset of fatty acid biosynthesis) (Baud et al., 2005). This lack of biotin is rapidly overcome by the biotin synthesis in developing wild-type seeds, but becomes increasingly important under conditions of biotin limitation (e.g. in bio1 or bio2 mutants). In addition to biotin biosynthesis, biotin supply from adjacent tissues is an alternative mechanism to adjust cellular biotin concentrations.

Our data suggest that the concentration of biotin is adjusted to the specific needs of an organ under various developmental conditions, and that SUC5 participates in this adjustment. An essential role for SUC5 in the supply of sucrose is rather unlikely, as, in suc5 embryos, neither the sucrose import is altered (Figure 3b) nor is the total fatty acid or TAG composition in dry seeds affected (Figure 8c) (Baud et al., 2005). The expression kinetics of SUC5 during seed development reported by Baud et al. (2005)
support a scenario in which the primary role of SUC5 is biotin transport. SUC5 shows peak expression at 7 DAF, which coincides with the onset of fatty acid synthesis. Shortly after, SUC5 expression decreases to almost zero and stays low during later seed development and ripening. Possibly, a short time frame, during which transport of the essential co-factor biotin across the embryo epidermis is catalysed by SUC5, is sufficient for proper fatty acid synthesis. Other genes encoding SUCs that are localized in the embryo, namely SUC8 and SUC9, are also active at later developmental stages (Sauer, 2007; Sivitz et al., 2007), and may provide the embryo with the carbon skeletons required for fatty acid synthesis. It remains to be shown whether or not biotin transport is a physiologically important property of other plant sucrose transporters also.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

*Arabidopsis thaliana* Col-0 and mutant plants were grown in a growth chamber on potting soil under a 16 h light/8 h dark regime at 22°C and 60% relative humidity, and watered with the indicated biotin concentrations. *bio1.1* (NASC stock number N6016), *bio2.1* (NASC stock number N6329) and *suc5* mutant lines (SAIL_367_D07, *suc5.4*; SALK_092412, *suc5.5*) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). *Agrobacterium tumefaciens* GV3101 (Holsters et al., 1980) was used for *Arabidopsis* transformation by floral dip (Clough and Bent, 1998). *Escherichia coli* strain DH5α (Hanahan, 1983) was used for all cloning steps.

**RNA isolation and cDNA synthesis**

RNA was isolated from 7 DAF siliques of wild-type and *suc5* mutant plants collected and dissected using fine forceps. Developing seeds were selected, and zygotic embryos at the upturned U stage were transferred into 25 mM sodium phosphate buffer, pH 7.0. For every uptake experiment, 50 embryos were incubated at 22°C in 200 μl solution containing 25 mM NaHPO4 (pH 5.5) and 2 mM CaCl2 plus the radiolabelled substrate. Indicated concentrations of 13C-biotin or 14C-sucrose were added. The incubation time was 6 h for biotin and 90 min for sucrose. Low biotin concentrations (10-50 μM) were chosen because 13C-biotin is in short supply. After incubation, samples were filtered on glass microfibre 696 filters (VWR, www.vwr.com), and washed 5 times for 5 min using an excess of distilled H2O to remove unincorporated radioactivity. Incorporated radioactivity was determined by scintillation counting. Incubation with each substrate and at each concentration was performed three times using independently isolated embryos.

**Generation of pSUC5/reporter lines**

For construction of pSUC5://GFP, 2030 bp of pSUC5 were amplified using primers AtSUC5-2030f and AtSUC5-1r (Table S1), and introduced into pEP/pUC19 (Imlau et al., 1999) via HindIII and NotI sites. From the resulting vector, pEP-SS-GFP, pSUC5://GFP was excised using HindIII and SacI, and cloned into the respective sites of pAF16 (Stadler et al., 2005b). This construct was used for *Arabidopsis* transformation.

For construction of pSUC5://tmGFP9, a genomic 1152 bp fragment encoding the 232 N-terminal amino acids of STP9 (Schneidereit et al., 2003) was excised from plasmid pMH4 (Stadler et al., 2005b) using Ncol and inserted into the unique Ncol site separating pSUC5 and the GFP open reading frame in pEP-SS-GFP. From the resulting plasmid, the 3916 bp pSUC5://tmGFP9 cassette was excised using HindIII/SacI, and cloned into the respective sites of pAF16, yielding pH31, which was used for Arabidopsis transformation.

**Confocal microscopy**

For detection of GFP fluorescence, images were produced using made a confocal microscope (Leica TCS SFI, www.leica-microsystems.com) as described previously (Stadler et al., 2005b). The excitation wavelength for GFP was 488 nm. Confocal images were processed using Leica confocal software 2.

**Analyses of TAG and fatty acids**

Fatty acid methyl esters of pooled Arabidopsis seeds were obtained and identified essentially as described by Hoffmann et al., 2008.
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SUPPORTING INFORMATION
Additional Supporting Information may be found in the online version of this article.

Figure S1. Young seedlings (a) and fully developed rosettes (b) of plants grown with supplementation of various amounts of biotin.

Figure S2. Seedlings (a) and fully developed rosettes (b) of the same plants germinated on biotin-containing (1μM) agar medium for 10 days, transferred to soils and supplemented with various amounts of biotin.

Table S1. Primer sequences.

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