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RESEARCH PAPER

Arabidopsis SUC1 loads the phloem in *suc2* mutants when expressed from the *SUC2* promoter

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Abstract

Active loading of sucrose into phloem companion cells (CCs) is an essential process in apoplastic loaders, such as *Arabidopsis* or tobacco (*Nicotiana* sp.), and is even used by symplastic loaders such as melon (*Cucumis melo*) under certain stress conditions. Reduction of the amount or complete removal of the transporters catalysing this transport step results in severe developmental defects. Here we present analyses of two *Arabidopsis* lines, *suc2-4* and *suc2-5*, that carry a null allele of the *SUC2* gene which encodes the *Arabidopsis* phloem loader. These lines were complemented with constructs expressing either the *Arabidopsis SUC1* or the *Ustilago maydis srt1* cDNA from the *SUC2* promoter. Both SUC1 and Srt1 are energy-dependent sucrose/H^+ symporters and differ in specific kinetic properties from the SUC2 protein. Transgene expression was confirmed by RT-PCRs, the subcellular localization of Srt1 *in planta* with an Srt1-RFP fusion, and the correct CC-specific localization of the recombinant proteins by immunolocalization with anti-Srt1 and anti-SUC1 antisera. The transport capacity of Srt1 was studied in *Srt1-GFP* expressing *Arabidopsis* protoplasts. Although both proteins were found exclusively in CCs, only SUC1 complemented the developmental defects of *suc2-4* and *suc2-5* mutants. As SUC1 and Srt1 are well characterized, this result provides an insight into the properties that are essential for sucrose transporters to load the phloem successfully.

Key words: Companion cell, phloem loading, Srt1, SUC1, SUC2, sucrose transport.

Introduction

Plants convert a major portion of their photosynthetically fixed CO_2 to sucrose, a molecule that is metabolically quite inert and, therefore, ideally suited for long-distance transport and long-term storage. In apoplastic loaders such as *Arabidopsis thaliana*, sucrose synthesized in the source leaf mesophyll is loaded into the phloem companion cells (CCs) by an energy-dependent H^+/sucrose symporter (Stadler *et al.*, 1995; Stadler and Sauer, 1996; Schmitt *et al.*, 2008). Sucrose transporter-mediated phloem loading was also observed in virus-infected melon plants (*Cucumis melo*; Gil *et al.*, 2011), a species clearly characterized to perform symplastic loading under normal growth conditions (Turgeon and Beebe, 1991). Although all plants analysed to date possess several genes for sucrose transporters (for reviews see Sauer, 2007; Ayre, 2011), studies on transporter mutants demonstrated that only one of these transporters is responsible for this loading step. *Arabidopsis* mutants with a T-DNA insertion in their *SUC2* gene (Gottwald *et al.*, 2000), which encodes the phloem loader of this species (Sauer and Stolz, 1994; Stadler and Sauer, 1996), show compromised carbon partitioning, fail to export sucrose from their source leaves, accumulate anthocyanin, are severely stunted, and only produce very few viable seeds (Gottwald *et al.*, 2000; Srivastava *et al.*, 2008; Srivastava *et al.*, 2009). Similar, although less severe phenotypes were observed in potato (*Solanum tuberosum*) plants carrying an antisense construct for *SUT1*, the gene encoding the phloem loader of this species (Kühn *et al.*, 1996).

In addition to this clear characterization of SUC2/SUT1-type sucrose transporters as phloem loaders, other sucrose transporters were reported to act as regulators of SUC2/SUT1-mediated phloem loading (Reinders *et al.*, 2002; Schulze *et al.*, 2003; Chincinska *et al.*, 2008; Kühn and

Grof, 2010). However, neither mutants in the genes for these putative interactors nor mutants in any other sucrose transporter gene resulted in phenotypes that came at least close to the strong effects observed with mutants defective in phloem loading (Hackel *et al.*, 2006; Sivitz *et al.*, 2007; Chincinska *et al.*, 2008; S Schneider *et al.*, unpublished data; Payyavula *et al.*, 2011).

There was speculation as to whether the SUC2/SUT1-type phloem loaders possess specific functional or structural properties that put them in a unique position over all other sucrose transporters, or whether the SUC2/SUT1-type phloem loaders can be replaced by other sucrose transporters normally responsible for different physiological tasks. Therefore, well-characterized sucrose transporters were sought that might be used as substitutes for SUC2 in *Arabidopsis* plants with a *suc2* null allele. Besides SUC2, six additional sucrose transporters were functionally characterized in *Arabidopsis*, SUC1, SUC3 (synonym SUT2), SUC4 (synonym SUT4), SUC5, SUC8, and SUC9. The *Arabidopsis SUC6* and *SUC7* genes are pseudogenes and do not encode intact transport proteins (Sauer *et al.*, 2006). SUC3 was excluded from our analyses, as (i) quite divergent K_m values have been published for this protein (1.9 mM: Meyer *et al.*, 2000; 11.7 mM: Schulze *et al.*, 2000) and (ii) it was reported to be involved in the regulation of phloem loading (Reinders *et al.*, 2002). SUC4 was also excluded as it is targeted to the tonoplast and not to the plasma membrane (Endler *et al.*, 2006).

Of the four remaining proteins, which all belong to the same phylogenetic group as SUC2 (Sauer, 2007), SUC1 seemed to be the best candidate, as it is perfectly characterized with respect to its plasma membrane localization (Sivitz *et al.*, 2008; Feuerstein *et al.*, 2010), its kinetic properties in baker's yeast [*Saccharomyces cerevisiae* (Sauer and Stolz, 1994)] and in *Xenopus laevis* oocytes (Zhou *et al.*, 1997), and the expression pattern of its gene. *SUC1* is expressed primarily in roots and the reproductive organs, but there is no evidence for *SUC1* expression in mature leaves or in the vasculature (Stadler *et al.*, 1999; Sivitz *et al.*, 2008; Feuerstein *et al.*, 2010; Hoth *et al.*, 2010). Studies of the K_m values for sucrose showed that SUC1 and SUC2 have similar affinities for sucrose (K_m SUC1: 0.4–0.5 mM, K_m SUC2: 0.8–1.4 mM; Sauer and Stolz, 1994; Zhou *et al.*, 1997; Chandran *et al.*, 2003). Interestingly, however, the two proteins respond quite differently to changes in the extracellular pH values. Whereas SUC2 exhibits a sharp optimum of sucrose transport at pH 4, retains 50% of its activity at pH 5, and has only marginal activities at pH 3 or 6, SUC1 is rather insensitive to changes in the extracellular pH. From its optimum at pH 3 to its minimum at pH 7, the transport rate only decreases by 50% (Sauer and Stolz, 1994; see Supplementary Fig. S1 at *JXB* online).

The second sucrose transporter chosen for our analyses was the Srt1 protein from *Ustilago maydis*, a biotrophic fungus that grows during its entire pathogenic development in the apoplast of its host plant (maize, *Zea mays*), where it feeds on extracellular sucrose. Deletion of the *srt1* gene leads to a loss of fungal virulence (Wahl *et al.*, 2010). While Srt1 is highly specific for sucrose, and while the pH-dependence of Srt1 is between that of SUC1 and SUC2, Srt1 has a significantly (20–70-fold) higher affinity for sucrose (K_m UmSrt1: 26 μM; Wahl *et al.*, 2010).

Detailed analyses are presented here of two different *Arabidopsis suc2* mutant lines, *suc2-4* and *suc2-5*, that express the *SUC1* or the *srt1* cDNA from the *SUC2* promoter and that target these proteins to the plasma membrane of their CCs. Interestingly, only recombinant SUC1 could complement the developmental defects of *suc2-4* and *suc2-5*, whereas *srt1*-expressing lines looked essentially as the untransformed mutants. The results are discussed against the background of the known kinetic properties of the deleted SUC2 protein and of the recombinant SUC1 and Srt1 proteins.

Materials and methods

Strains and growth conditions

Arabidopsis thaliana plants (Col-0) were used as wild-type (wt) controls. Col-0 plants, *suc2* mutant lines [*suc2-4* (SALK_038124) heterozygous seeds were provided by Brian Ayre, University of Texas; *suc2-5* (SALK_087046) heterozygous seeds were obtained from the Nottingham *Arabidopsis* Stock Centre] and complemented mutants were germinated and grown on soil under short-day conditions (8/16 h light/dark) at 22 °C and transferred to long day conditions (16/8 h light/dark) after 4 weeks for most applications. The *srt1*-expressing yeast strain (*Saccharomyces cerevisiae*) was described by Wahl *et al.* (2010). *Escherichia coli* strain DH5α (Hanahan, 1983) was used for all cloning steps. *E. coli* strain Rosetta™2(DE3) (Merck; Darmstadt, Germany) was used to express fusion protein. *Agrobacterium tumefaciens* strains C58C1 (Deblaere *et al.*, 1985) and GV3101 (Holsters *et al.*, 1980) were used for plant transformation.

T-DNA insertion lines

Seeds of heterozygous mutant lines were germinated on Murashige/Skoog (MS) agar medium (4.4 g MS salts with vitamins l^{-1}, 0.5 g l^{-1} MES, 8 g l^{-1} agar) and later transferred to soil. PCR genotyping of homozygous lines was performed using the primers 5′-GAC CGT TGC ACC TCA AGA TTC G-3′ (#1 in Fig. 1A) and 5′-CGA ATA GTT CGT CGA ATG GTC CAC-3′ (#2) for the *SUC2* wt allele, 5′-ATT TTG CCG ATT TCG GAA C-3′ (LB) and #1 for the *suc2-5* insertion and LB and #2 for the *suc2-4* insertion. PCRs were conducted with TaKaRa Ex Taq polymerase (Mobitec, Göttingen, Germany) according to the manufacturer's instructions. For RT-PCR analyses of transcript abundance, total RNA from mature leaves or (in the case of homozygous *suc2* plants) from whole seedlings was reverse transcribed and PCRs were performed with *Taq* polymerase. A truncated *SUC2* RNA fragment from upstream of the insertion site was amplified using the primers #1 and 5′-GAT ACC GAG GAT GGC GAA G-3′ (#3).

Constructs for stable and transient transformation

For *srt1* over-expression from the *35S* promoter or for CC-specific *srt1* expression, the *srt1* open reading frame (Wahl *et al.*, 2010) was cloned into pENTR™/D/TOPO® (Life Technologies; Darmstadt, Germany) and recombined into the Gateway®-compatible destination vectors pEARLEYGATE100 (p*35S*; Earley *et al.*, 2006) and pBSUC2 (p*SUC2*; Thompson and Wolniak, 2008) to obtain pKW41 (p*35S*::*srt1*) and pKW48 (p*SUC2*::*srt1*), respectively. pKW87 (p*SUC2*::*SUC1*) was obtained analogously.

For the investigation of the subcellular localization of Srt1 pENTR™/D/TOPO® containing the *srt1* open reading frame was

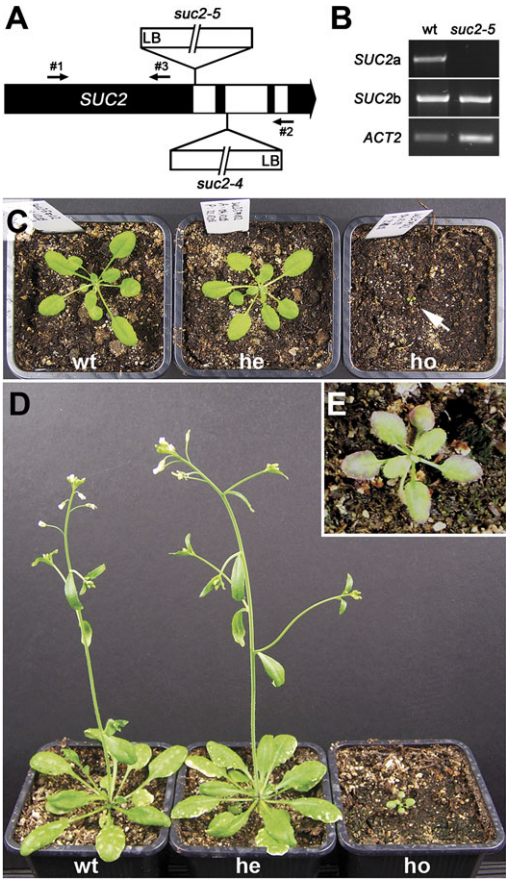

Fig. 1. Characterization of the *suc2-5* mutant. (A) Scheme of the *SUC2* gene with the confirmed insertion sites of *suc2-4* (SALK_038124) and *suc2-5* (SALK_087046). Black, exons; white, introns; LB, left border; small black arrows, primer binding sites and primer orientation. (B) Semi-quantitative RT-PCRs on total RNA from wt and *suc2-5* plants showing the abundance of a *SUC2* mRNA fragment spanning the insertion site (*SUC2*a, primers #1 and #2) and of an mRNA fragment upstream from the insertion site (*SUC2*b, primers #1 and #3). *ACTIN2* transcript (*ACT2*) was used as control for amounts of cDNA. (C, D) Phenotype of wt, heterozygous (he) and homozygous (ho) *suc2-5* plants at 27 d after germination (dag) (C) and at 41 dag (D). Arrow indicates the tiny homozygous plant. (E) Homozygous *suc2-5* plant (48 dag) with anthocyanin accumulation at the leaf margins. Edge length of pots: 6.5 cm.

recombined into the Gateway®-compatible destination vector pH7RWG2.0 (Karimi *et al.*, 2002) to obtain pKW45 (p*35S*::*srt1-RFP*). For the *GFP-INT4* control construct the *Arabidopsis INT4* coding sequence was amplified with the primers AtINT4-5-*Nco*I (5′-CCA TGG TGG AAG GAG GAA TTG-3′) and AtINT4-3-*Nco*I (5′-CCA TGG CAG CAG CAT CGA CTT CTT TGC-3′), which introduced *Nco*I sites at both ends and removed the stop codon. The resulting fragment was inserted into pJET1.2 (Fermentas; St Leon-Rot, Germany), sequenced and inserted into the unique *Nco*I site of pSS87 (S Schneider *et al.*, unpublished data) downstream of the *35S* promoter.

Generation and identification of transgenic plants

Transgenic plants were generated via *Agrobacterium*-mediated transformation of *Arabidopsis* Col-0 plants with C58C1 carrying pKW41 or GV3101 carrying pKW48 and pSOUP as a co-vector (Hellens *et al.*, 2000). The resulting plant lines were named KW41 (p*35S*::*srt1*) and KW48 (p*SUC2*::*srt1*). Transgenic plants were identified by Basta® resistance.

KW48 plants were crossed with heterozygous *SUC2*/*suc2* plants. *suc2*/*srt1*-transgenic lines were identified by PCR genotyping.

Heterozygous *SUC2*/*suc2* plants were transformed by GV3101 carrying pKW87 and pSOUP. *suc2*/p*SUC2*::*srt1*-transgenic lines were identified by Basta® resistance (T_1 generation) and subsequent generations by PCR genotyping. Primers #1 and #2 were used for the *SUC2* wt allele, 5′-GAC AAG CAC GGT CAA CTT CC-3′ and 5′-GAA GTC CAG CTG CCA GAA AC-3′ for the Basta® resistance gene, 5′-CAC CAT GGC GTC GTC TTC TCC-3′ and 5′-GCA GAT GTA CGC GTA AAC CG-3′ for the *srt1* gene, and 5′-CCT ACG CTA TAG ACA CAG CTC TG-3′ and 5′-GCT ACG TCG AGG ATC CAG AA-3′ for the p*SUC2*::-*SUC1* insertion.

RT-PCR analyses of transcript abundance in transgenic plants were performed with the primer 5′-ATT CAG ATG CCC AGA AGT CTT GTT-3′ and 5′-GAA ACA TTT TCT GTG AAC GAT TCC T-3′ for *ACTIN2* (*ACT2*) as control and with 5′-CTC TTC CTC CAC CAC TAC AAC CAC-3′ and 5′-GCT ACG TCG AGG ATC CAG AA-3′ for p*SUC2*-5′-UTR::*SUC1*. For the *srt1* mRNA levels the same primers were used as for plant genotyping.

Production and purification of antibodies

The sequence encoding the Srt1-C-terminus (69 amino acids) was amplified using the primers 5′-GAG AAT TCA GGA CTT TCT TCG AGA TC-3′ and 5′-CTG AAT TCT CAT TGT GGA CTC GGC-3′ containing *Eco*RI restriction sites, and cloned into *Eco*RI sites in the pMAL-c2 polylinker (New England Biolabs; Frankfurt, Germany) yielding a plasmid (pKW80) that encodes a maltose-binding-protein-(MBP)-Srt1-C-terminus fusion. *E. coli* Rosetta™2(DE3) cells were transformed with this plasmid, induced with 1 mM isopropyl-β-D-thiogalactopyranoside and harvested. The fusion protein was isolated from the cell extract by preparative SDS-PAGE (Laemmli, 1970), extracted from the gel and lyophilized as described by Sauer and Stadler (1993). Immunization of rabbits was done by Pineda Antikörper-Service (Berlin, Germany).

Affinity-purification of the anti-Srt1 antiserum (αSrt1) was carried out as described previously (Sauer and Stadler, 1993; Schmitt *et al.*, 2008). Analogously, purification of available anti-SUC1 raw serum (αSUC1) was done using a synthetic peptide (Feuerstein *et al.*, 2010).

Protein isolation and Western Blot analysis

Soluble and membrane protein fractions from plant leaves or baker's yeast were isolated as described previously (Sauer and Stolz, 2000; Drechsel *et al.*, 2010). SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and Western Blot analyses (Burnette, 1981) with affinity-purified αSrt1 (used in a 1:20 dilution) were performed as published.

Immunohistochemistry

Leaf tissue was fixed in ethanol:acetic acid (3:1 v/v) and embedded in methacrylate. Microtome sections (4 μm) were prepared as described by Stadler and Sauer (1996). Incubation with primary antibodies was performed overnight at 4 °C. Antibody dilutions in blocking buffer (50 mM TRIS/HCl pH 7.5, 150 mM NaCl, 1% skim milk powder, and 0.1% Triton X-100) were 1:20 for αSrt1, 1:5 for αSUC1, and 1:5 for the αRS6 antibody (Khan *et al.*, 2007). Secondary antibodies (anti-rabbit IgG-Cy2 and anti-mouse IgG-Cy3; Dianova; Hamburg, Germany) were diluted 1:50 in blocking buffer.

Confocal laser scanning microscopy

Confocal laser scanning microscopy for immunolocalizations was done as described by Schmitt *et al.* (2008). For colocalization of

Srt1-RFP and GFP-INT4 in transformed protoplasts, sequential scanning was performed using an excitation wavelength of 488 nm for GFP and 543 nm for RFP. Detection windows were 495–547 nm for GFP, 584–638 nm for RFP, and 675–767 nm for chlorophyll autofluorescence.

Protoplast techniques

Arabidopsis protoplasts were generated and transformed as described by Abel and Theologis (1994). For transport analyses, successful expression of reporter gene constructs was checked microscopically after 24 h. For each transport test, 700 000 protoplasts were harvested by centrifugation for 2 min at 50 *g*, resuspended in 0.5 ml W5 buffer (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES, adjusted to pH 5.2 with KOH), transferred to a 24-well cell culture plate, and ^{14}C-sucrose was added to a final concentration of 0.2 mM. After a 3 h incubation at 22 °C in the light, and after withdrawal of 50 µl of protoplast suspension for scintillation counting (total radioactivity), protoplasts were harvested (2 min, 50 *g*), washed twice with W5 buffer, and the radioactivity in the protoplast was determined.

Carbohydrate analyses

Plants were grown for 6 weeks under short-day conditions (8/16 h light/dark) at a proton flux density of 100 µmol m^{-2} s^{-1}. At the end of the light cycle, source leaves from 10 different wt plants (1 leaf per plant) or from 10 plants of a transgenic line were harvested and combined. Soluble carbohydrates were extracted for ion exchange chromatography (IC) as described by Schneider *et al.* (2008). The eluent was 500 mM NaOH; the run took 80 min. Three biological replicates were analysed.

Results

Characterization of the T-DNA insertion line SALK_087046

The T-DNA insertion lines SALK_038124 (*suc2-4*) and SALK_087046 (*suc2-5*) were used for our analyses. Whereas the position of the T-DNA insertion in the *suc2-4* line had previously been determined (Srivastava *et al.*, 2008), the position of the T-DNA insertion in the *suc2-5* mutant has only been predicted [SIGnAL: http://signal.salk.edu/cgi-bin/tdnaexpress; predicted insertion site: middle of the 1st exon (Lei *et al.*, 2011)] but not confirmed by sequencing. Therefore, the abundance of different *SUC2* mRNA fragments was studied by RT-PCRs on RNA from wt and homozygous *suc2-5* plants, and the T-DNA insertion site was sequenced. In contrast to the predicted insertion site (Lei *et al.*, 2011), our analyses identified the insertion in the 1st intron, 1265 base pairs (bp) downstream from the start codon in the genomic sequence (Fig. 1A). Whereas a truncated sequence upstream from this insertion site could be amplified from homozygous *suc2-5* mutant plants, no full-length *SUC2* transcript could be detected (Fig. 1B). A protein translated from this truncated *suc2-5* mRNA would encode 419 of the 512 amino acids of the intact SUC2 protein. A sucrose transport activity of the resulting truncated suc2-5 protein can be excluded, as even the slightly longer but also truncated suc2-4 protein was shown to be functionally inactive (Srivastava *et al.*, 2009).

The *suc2-5* phenotype matches that of previously described *suc2* mutants (Gottwald *et al.*, 2000; Srivastava *et al.*, 2008; Lei *et al.*, 2011). Homozygous plants showed delayed development, stunted growth, and the accumulation of anthocyanin in the leaf margins (Fig. 1C, D, E). Despite these severe defects, *suc2-5* plants were able to complete their life cycle and generated viable seeds. Heterozygous plants did not differ phenotypically from the wt.

Generation and genotyping of suc2/*p*SUC2*::*srt1 *and* suc2/*p*SUC2*::*SUC1 *plants*

For complementation of *suc2-4* and *suc2-5* mutants with an *srt1*-containing construct, wt plants were transformed with the construct pKW48 (Fig. 2) and these wt/p*SUC2*::*srt1* plants were crossed with heterozygous *suc2* mutants. The resulting *suc2*/p*SUC2*::*srt1* plants should express *srt1* from the *SUC2* promoter (p*SUC2*) in the respective mutant background (*suc2-4*/p*SUC2*::*srt1* or *suc2-5*/p*SUC2*::*srt1*). The absence of a wt *SUC2* allele from homozygous *suc2*/p*SUC2*::*srt1* plants, and the presence of a copy of the *srt1* gene and of the *BAR* gene for Basta® resistance was confirmed by PCR (Fig. 3A). The abundance of the *srt1* transcript was checked by RT-PCR on total RNA from transgenic and wt plants. As an additional control, RNA was included from wt plants expressing *srt1* from the *35S* promoter (wt/p*35S*::*srt1* plants). The *srt1* transcript could be detected in all transgenics but not in the wt (Fig. 3B).

Transgenic lines expressing *SUC1* from the *SUC2* promoter in the *suc2* background (*suc2*/p*SUC2*::*SUC1*) were generated by *Agrobacterium*-mediated transformation of heterozygous *suc2-4* and *suc2-5* plants with the construct pKW87 (Fig. 2). Plants carrying the *suc2*/p*SUC2*::*SUC1* construct were identified by Basta® selection and genotyped (Fig. 3C). Again, homozygous *suc2* plants did not contain *SUC2* mRNA. However, they contained novel *SUC1* mRNA that resulted from the p*SUC2*::*SUC1* insertion as demonstrated by a primer combination specific for this transgene. To avoid amplification of *SUC1* transcripts encoded by the wt *SUC1* allele present in the *suc2* mutants, one of the primers was chosen to bind to the p*SUC2*-derived 5'-UTR of the p*SUC2*::*SUC1* transgene (Fig. 3D). Of the crosses obtained (*srt1*) or transformants (*SUC1*), seven *suc2-5*/p*SUC2*::*srt1* lines, two *suc2-4*/p*SUC2*::*SUC1* lines, and three *suc2-5*/p*SUC2*::*SUC1* lines were used for further analyses).

Phenotypes of suc2/*p*SUC2*::*srt1 *and* suc2/*p*SUC2*::*SUC1 *plants*

Although both sucrose transporter genes, *SUC1* and *srt1*, were expressed in *suc2*/p*SUC2*::*SUC1* and *suc2*/p*SUC2*::*srt1* plants, respectively (Fig. 3B, D), they showed strong morphological differences. Whereas *suc2* mutants expressing *srt1* from the *SUC2* promoter looked essentially like untransformed *suc2* mutants (Fig. 4A, B, C), *suc2*/p*SUC2*::*SUC1* plants looked like wt plants and did not show any recognizable developmental defect (Fig. 4D, E). This suggested that transformation with the pKW87 construct (p*SUC2*::*SUC1*) but not with the pKW48 (p*SUC2*::*srt1*) construct (Fig. 2) leads to successful complementation of the *suc2* phenotype.

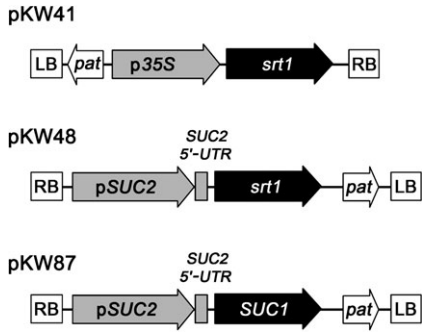

Fig. 2. Constructs used to generate transgenic plants. RB, right border; LB, left border; p*SUC2*, *SUC2* promoter; p*35S*, *35S* promoter; *pat*, phosphinotricin acetyltransferase (Basta® resistance gene); *SUC2 5'-UTR*, *SUC2* 5' untranslated region.

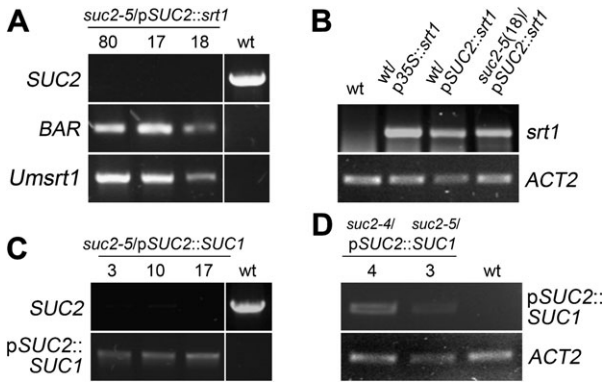

Fig. 3. Genotyping of *suc2*/p*SUC2*::*srt1* and *suc2*/p*SUC2*::*SUC1* plants and determination of transcript abundance. (A) PCR analyses with genomic DNA from *suc2*/p*SUC2*::*srt1* showing the absence of the *SUC2* wt allele and the presence of the *BAR* gene and of *srt1* in three different *suc2*/p*SUC2*::*srt1* lines (#17, #18, #80). Control PCRs were performed on wt genomic DNA to visualize the *SUC2* gene fragment, or on wt/p*SUC2*::*srt1* genomic DNA to show the identity of the amplified *BAR* and *Umsrt1* fragments. (B) Comparative RT-PCR analyses of *srt1* transcript abundance on total RNA from wt, wt/p*35S*::*srt1* and wt/p*SUC2*::*srt1* source leaves or from entire *suc2*/p*SUC2*::*srt1* plants showing *srt1* transcripts only in transgenics. *ACT2* levels are shown as controls. (C) PCR analyses on genomic DNA from three different *suc2*/p*SUC2*::*SUC1* lines (#3, #10, #17) showing the absence of the *SUC2* wt allele and the presence of the p*SUC2*::*SUC1* insertion. Control PCRs on genomic DNA from wt plants identified the *SUC2* gene and failed to amplify the mutant *SUC1* allele. (D) RT-PCR analyses on total RNA from source leaves of two different *suc2*/p*SUC2*::*SUC1* lines (#3, #4) and of wt plants identifying *SUC1* mRNA transcribed from the p*SUC2*::*SUC1* insertion only in transgenics. *ACT2* transcript levels are shown as controls.

An Srt1-RFP fusion localizes to the plasma membrane and is a functionally active transporter in planta

The observed lack of complementation in *suc2*/p*SUC2*::*srt1* plants might indicate (i) that the identified *srt1* mRNA (Fig. 3B) is not translated, (ii) that the Srt1 protein is not targeted to the plasma membrane, (iii) that the Srt1 protein is not functional in the plant plasma membrane, or (iv) that the p*SUC2*::*srt1* construct is not expressed in the correct cell type, i.e. in the CCs. To exclude the first three options, the subcellular localization of an Srt1-RFP fusion in plant cells was checked, the presence of Srt1 protein was tested on Western blots, and the sucrose transport capacity was studied in *Srt1-RFP*-expressing *Arabidopsis* protoplasts.

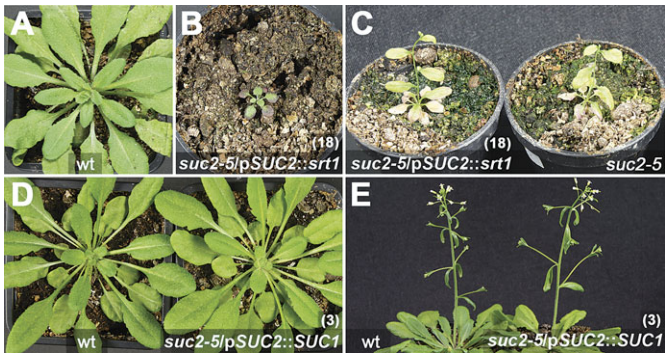

Fig. 4. Phenotype of *suc2*/p*SUC2*::*srt1* and *suc2*/p*SUC2*::*SUC1* plants. (A) wt and (B) *suc2-5*/p*SUC2*::*srt1* (line 18) plant at 46 dag. (C) *suc2-5*/p*SUC2*::*srt1* (line 18) and *suc2-5* flowering plants at 80 dag. (D) and (E) *suc2-5/pSUC2:SUC1* (line 3) and wt plant at 88 dag (D) and flowering at 99 dag (E). Edge length of squared pots: 6.5 cm, diameter of round pots: 6 cm.

To determine the subcellular localization of Srt1, co-localization analyses were performed in *Arabidopsis* protoplasts co-transformed with constructs for *Srt1-RFP* and *GFP-INT4* fusions. INT4 is an inositol transporter of the *Arabidopsis* plasma membrane and a GFP fusion was previously shown to be targeted to the plasma membrane (Schneider *et al.*, 2006). In confocal sections from *Srt1-RFP* and *GFP-INT4* co-expressing protoplasts, both the red Srt1-RFP fluorescence (Fig. 5A) and the green GFP-INT4 fluorescence (Fig. 5B) labelled the plasma membrane, which is most obvious in a merge of these images (Fig. 5D).

To test, if Srt1 is a functional sucrose transporter in plant cells, the capacity to import ^{14}C-labelled sucrose of *Srt1-RFP* or *GFP-INT4*-expressing protoplasts was compared. From comparative analyses of infection rates obtained with a *U. maydis* wt strain, a *U. maydis* Δ*srt1* mutant, and a *U. maydis* Δ*srt1* mutant that had been complemented with an *srt1-GFP* fusion it was known that the fusion of a fluorescent reporter to the Srt1 C-terminus does not affect the functionality of the transporter in the fungus (Wahl *et al.*, 2010). When the capacity to transport ^{14}C-labelled sucrose (initial concentration 0.2 mM) of *Srt1-RFP*-expressing and *GFP-INT4*-expressing *Arabidopsis* protoplasts was compared, significantly larger amounts of sucrose uptake into *Srt1-RFP*-expressing protoplasts was observed. In summary, these data demonstrate that the Srt1 protein is synthesized from its mRNA, that it is targeted to the plasma membrane, and that it is functionally active in *Arabidopsis*.

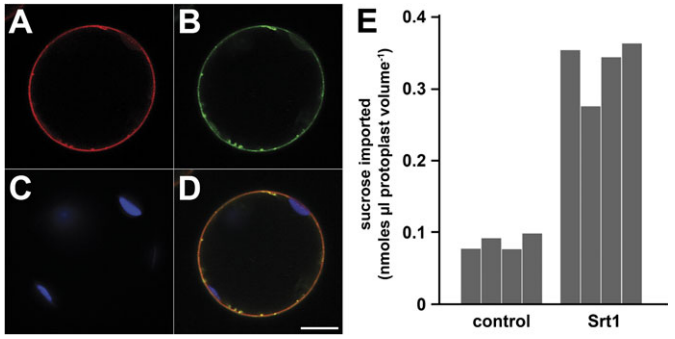

Fig. 5. Srt1-RFP localizes to the plasma membrane of *Arabidopsis* mesophyll protoplasts where it catalyses the uptake of radiolabelled sucrose. (A–C) Optical sections of an *Arabidopsis* protoplast cotransformed with an Srt1-RFP and an GFP-INT4 construct. (A) Localization of Srt1-RFP. (B) Localization of GFP-INT4. (C) Detection of chloroplasts by chlorophyll autofluorescence. (D) Merge of (A–C). Bar=10 µm. (E) Uptake of ^{14}C-sucrose into *Arabidopsis* protoplasts expressing GFP-INT4 (control) or Srt1-RFP (Srt1). Srt1 and control data show results from two different protoplast transformations and two transport tests per transformation, respectively.

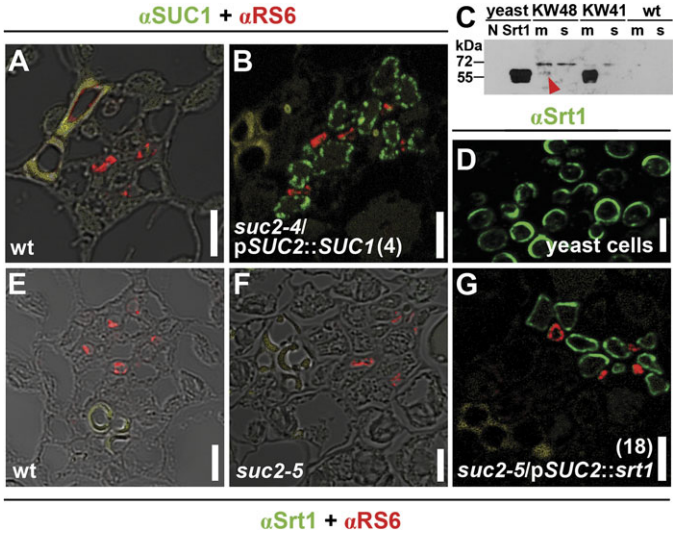

Fig. 6. Detection of SUC1 and Srt1 proteins in plants and yeast. (A), (B), and (E–G), Immunohistochemical stainings of 4 µm sections showing leaf veins from wt [(A) and (E)], *suc2-4*/p*SUC2*::*SUC1* (line 4) (B), *suc2-5* (F), and *suc2-5*/p*SUC2*::*srt1* (line 18) (G) plants. Green fluorescence of anti-rabbit-Cy2 in CCs corresponds to αSUC1 [(A) and (B)] or αSrt1 [(E–G)]. Red fluorescence of anti-mouse-Cy3 corresponds to anti-RS6 labelling of sieve elements. For (A), (E), and (F) the images of the fluorescence signals were merged with the corresponding bright field images. Yellow staining in (A), (B), and (G) shows xylem autofluorescence. (C) Western blot analyses using αSrt1 to detect the 60 kDa Srt1 protein in extracts from yeast strain SEY2102 (Emr *et al.*, 1983) (N, control yeast cells transformed with the empty vector; Srt1, yeast cells expressing *srt1*) or in extracts from source leaves of wt/p*SUC2*::*srt1* (KW48), wt/p*35S*::*srt1* (KW41), and wt plants (m, membrane fraction; s, soluble fraction). The red arrowhead shows the weak Srt1-derived signal in wt/p*SUC2*::*srt1* plants. (D) Immunostaining of 4 µm sections of *srt1*-expressing yeast cells. Green fluorescence of anti-rabbit-Cy2 corresponds to αSrt1. Yellowish colour in (A), (B), (E), and (G) shows autofluorescence of cell-wall phenolic compounds in xylem vessels. Bars=5 µm.

Analyses of recombinant SUC1 and Srt1 proteins in suc2/*p*SUC2::SUC1 *and* suc2/*p*SUC2::srt1 *plants*

The presence of recombinant proteins in CCs was studied with αSUC1 and αSrt1 antisera. The αSUC1 antiserum has been described before (Feuerstein *et al.*, 2010). For the αSrt1 antiserum, antibodies were raised in rabbits against the 69 C-terminal amino acids of Srt1, which had been fused to the maltose-binding protein. After affinity purification of the raw serum, the αSrt1 fraction was tested on Western blots with membrane proteins from the *srt1*-expressing yeast (*Saccharomyces cerevisiae*) strain described by Wahl *et al.* (2010) and with membrane protein and soluble protein fractions from wt, KW48 and KW41 plants (Fig. 6). KW41 plants represent controls that express *srt1* from the *35S* promoter in the wt background (p*35S*; Fig. 2).

The αSrt1 antiserum yielded a strong signal at the expected molecular mass of about 60 kDa in the membrane fraction from *srt1*-expressing yeast cells but not from control yeast cells (Fig. 6C), and a signal of comparable intensity and of the same size was detected in wt/p*35S*::*srt1* controls (KW41 in Fig. 6C). By contrast, no signals were detected in the soluble-protein fraction of wt/p*35S*::*srt1* controls or in the membrane or soluble fraction from wt plants. In wt/p*SUC2*::*srt1* plants, where *srt1* is expected to be expressed in CCs, only a significantly weaker signal could be detected in the membrane extract; no signal was seen in the soluble protein fraction (Fig. 6C).

The weak signal in wt/p*SUC2*::*srt1* plants might reflect the comparatively small number of CCs in leaves (probably less than 1% of all cells). To test this hypothesis, immunolocalization studies were performed on sections of methacrylate-embedded, *srt1*-expressing yeast cells to test the capacity of the αSrt1 antiserum to label Srt1 protein after fixation and embedding (Fig. 6D). To this end, thin sections of embedded cells were treated with αSrt1 and with anti-rabbit-Cy2 2nd antibody. As expected, green Cy2 fluorescence could be observed in the cell periphery of the yeast sections (Fig. 6D) indicating that αSrt1 labels Srt1 in immunolocalizations. No fluorescence was detected in control cells carrying the empty vector (not shown).

Sections from source leaves of *suc2*/p*SUC2*::*srt1* plants, that had been fixed and embedded essentially as the yeast cells shown in Fig. 6D, were analysed next. These leaf sections were treated with αSrt1, which was expected to label the CCs, and simultaneously with a sieve element (SE)-specific antiserum (αRS6) that was previously shown to label the SEs of *Arabidopsis* with high specificity (Meyer *et al.*, 2004; Khan *et al.*, 2007; Hoth *et al.*, 2008). The αSrt1 and αRS6 signals were detected with anti-rabbit-Cy2 (green fluorescence) and anti-rabbit-Cy3 (red fluorescence) 2nd antibodies, respectively. In sections from *suc2*/p*SUC2*::*srt1* source leaves, the Srt1-specific Cy2 fluorescence could be

detected in cells that could be classified as CCs as (i) they were in the immediate vicinity of the αRS6-labelled SEs, (ii) they had a significantly larger diameter than these SEs, and (iii) there were more CCs than SEs, which is typical for minor or medium-sized veins (Fig. 6G; Esau, 1969; Schmitt *et al.*, 2008). By contrast, no αSrt1-derived Cy2 fluorescence was detected in wt (Fig. 6E) or *suc2-5* plants (Fig. 6F), where the SEs could be labelled by αRS6. These data demonstrate that, as expected by the known specificity of the *SUC2* promoter, Srt1 is present in source leaf CCs of *suc2*/p*SUC2*::*srt1* plants.

A similar result was obtained, when a combination of αSrt1 and αRS6 was used on thin sections of fixed and embedded source-leaf material from *suc2*/p*SUC2*::*SUC1* and wt plants (Fig. 6A, B). Again, the SUC1 and RS6 antigens were detected by Cy2 and Cy3 fluorescence, and again αRS6 labelled the SEs in sections from all plants analysed. SUC1-specific Cy2 fluorescence, however, was only seen in *suc2*/p*SUC2*::*SUC1* plants (Fig. 6B), while no SUC1-specific fluorescence could be observed in sections from wt plants (Fig. 6A). Thus, *suc2*/p*SUC2*::*srt1* and *suc2*/p*SUC2*::*SUC1* plants have their respective recombinant sucrose transporter specifically localized in the CCs.

Carbohydrate analysis of wt and suc2/*p*SUC2::SUC1 *plants*

In contrast to Srt1, SUC1 restored wt development in *suc2* mutants when expressed in source leaf CCs. However, besides this macroscopically detectable developmental defect, a lack of sucrose export (in the *suc2-1* mutant; Gottwald *et al.*, 2000) and, consequently, an accumulation of sucrose (more than 20-fold compared with the wt in the *suc2-4* mutant; Srivastava *et al.*, 2008) has been observed in the source leaves of *suc2* mutants analysed before. These increased sucrose concentrations lead to the production of protective anthocyanins, a phenotype also clearly visible in the newly characterized *suc2-5* mutant (Fig. 1E). It was examined whether SUC1 in the CCs of *suc2* null mutants can restore this biochemical phenotype as well. To this end, the carbohydrate content in source leaves of 6-week-old *suc2*/p*SUC2*::*SUC1* and wt plants was compared by ion exchange chromatography. Figure 7 shows that the levels for glucose, sucrose, *myo*-inositol, fructose, and raffinose were comparable in most *suc2*/p*SUC2*::*SUC1* and wt plants. However, in two of the *suc2*/p*SUC2*::*SUC1* lines [*suc2-5* (3) and *suc2-5* (17) in Fig. 7] the source-leaf sucrose levels or the sucrose plus glucose levels were significantly (30%) lower than in the wt plants suggesting that phloem loading in these lines might be even more effective than in wt plants.

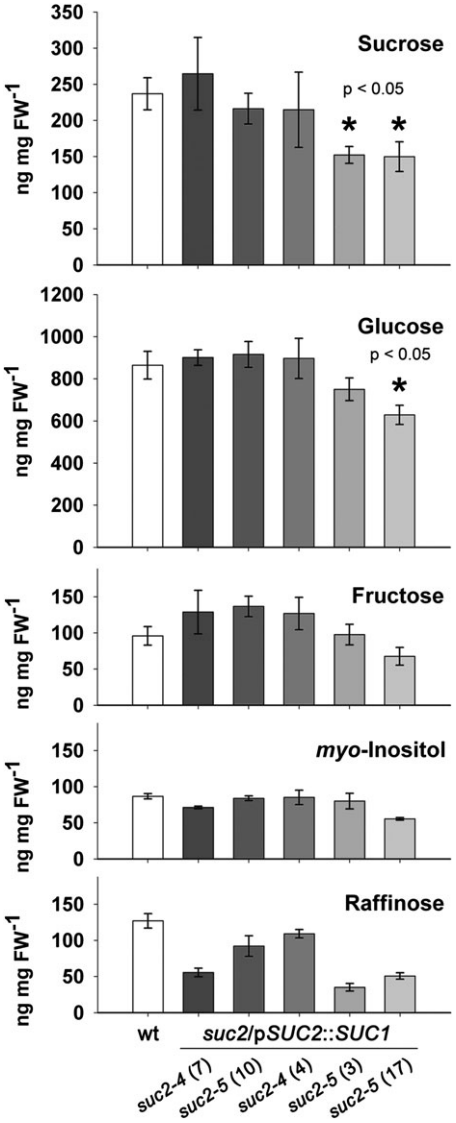

Fig. 7. Carbohydrate analyses of wt and *suc2*/p*SUC2*::*SUC1* plants. Source leaves of 6-week-old wt plants and five different *suc2*/p*SUC2*::*SUC1* lines [*suc2-4* (#7), *suc2-5* (#10), *suc2-4* (#4), *suc2-5* (#3), and *suc2-5* (#17)] grown under short-day conditions were analysed by ion exchange chromatography to determine amounts of the indicated carbohydrates (n=3 ±SE). Asterisks show significantly decreased sucrose or glucose concentrations based on Student's t tests.

Discussion

Arabidopsis knockout mutants harbouring a T-DNA insertion in their *SUC2* gene or potato plants expressing antisense constructs for their *SUT1* gene, fail to export photoassimilates from their source leaves, show feedback inhibition of their photosynthetic activity, and form stunted, often tiny plants (Kühn *et al.*, 1996; Gottwald *et al.*, 2000; Srivastava *et al.*, 2008; Srivastava *et al.*, 2009; this paper). In the present study, *Arabidopsis* lines were generated and analysed that had SUC2, their companion cell-specific phloem loader, replaced either with SUC1, another *Arabidopsis* sucrose transporter that is usually expressed in pollen, anther connective tissue, developing ovules, or roots of seedlings (Stadler *et al.*, 1999; Sivitz *et al.*, 2008; Feuerstein *et al.*, 2010), or with Srt1 from the corn smut fungus *U. maydis* (Wahl *et al.*, 2010). Transcription and translation of the transgenes were followed by RT-PCR and with specific antisera, respectively. CC-specific expression was confirmed in immunohistochemical analyses.

Although both SUC1 and Srt1 were synthesized specifically and exclusively in CCs, only SUC1 complemented the strong developmental defects of the *suc2* mutant lines.

SUC1 complements all defects described for suc2 *mutants*

SUC1-complemented *suc2* mutants developed and flowered like wt plants (Fig. 4D, E) and showed no accumulation of carbohydrates in their source leaves (Fig. 7). For several reasons, this successful replacement of SUC2 by a non-phloem sucrose transporter was not predictable. Firstly, although SUC1 and SUC2 have comparable affinities for their substrate sucrose, they respond differently to changes in the extracellular pH. This had already been demonstrated during the initial characterization of these proteins (Sauer and Stolz, 1994) and confirmed in more detail during the present study (see Supplementary Fig. S1 at *JXB* online). Secondly, in different publications, phloem loading by SUC2-type transporters was reported to be regulated by physical interaction with other transporters, for example, with SUC3 (synonym SUT2) and SUC4 (synonym SUT4) in *Arabidopsis* (Reinders *et al.*, 2002; Schulze *et al.*, 2003; Kühn and Grof, 2010). While an interaction with SUC4 can, meanwhile, be excluded, as SUC4-transporters were characterized as tonoplast proteins (Endler *et al.*, 2006; Schulz *et al.*, 2011), the regulatory interaction of SUC2 and SUC3 (SUT2) is still under discussion.

Our results demonstrate that, quite obviously under the growth conditions analysed, the different pH-sensitivities of SUC1 and SUC2 are of no or only of minor importance. It may well be, however, that this altered pH-sensitivity of SUC1 becomes important during the adaptation of phloem loading to environmental changes or to certain stress conditions. In fact, it has been discussed only recently that changes in the extracellular pH might represent a tool to regulate the competition for sucrose at the host/pathogen interface (Wippel *et al.*, 2010).

Despite their different pH responses, SUC1 and SUC2 share about 80% identical amino acids. It might, therefore, well be that CC-specific regulatory mechanisms that possibly modulate the activity of SUC2 in WT plants will also act on SUC1. Such regulatory mechanisms could be post-translational modifications or protein/protein interactions.

In summary, the successful replacement of SUC2 by SUC1 indicates that the phloem loader SUC2 does not contain a specific domain or a special functional property that puts it in a unique position compared with all other non-phloem plant sucrose transporters. The important role of the *SUC2* gene for plant growth and development rather depends on its promoter that directs and limits the function of the SUC2 protein to the CCs of WT plants or of SUC1 in the transgenic lines analysed in the present study.

Srt1 cannot complement the defects of suc2 *mutant lines*

In a second approach we replaced SUC2 by the *U. maydis* Srt1 protein. It was shown that the *srt1* gene is transcribed (Fig. 3B), checked that the Srt1 protein is synthesized and made specifically in CCs (Fig. 6G), confirmed that it is targeted to the plasma membrane (Fig. 5D), and demonstrated that recombinant Srt1-RFP acts as functional sucrose transporter in *Arabidopsis* protoplasts (Fig. 5E). Nevertheless, and in contrast to SUC1, Srt1 cannot replace SUC2 (Fig. 4B, C).

The extracellular sucrose concentrations at the mesophyll/CC interface should be more than sufficient to drive sucrose uptake by Srt1. In apoplastic loaders like *Arabidopsis*, bulk apoplastic sucrose concentrations were found to be 2–6 mM (López-Millán *et al.*, 2000; Voitsekhovskaja *et al.*, 2000; Lohaus *et al.*, 2001), concentrations that are nearly saturating for SUC2 (K_m 0.8–1.4 mM) and completely saturating for Srt1 (K_m 26 μM). Nonetheless, the lack of complementation might result from this much lower K_m value of Srt1, as low K_m values (or high affinities) typically come along with low transport capacities or vice versa. Therefore, low-affinity/high-capacity (LAHC) transporters and high-affinity/low-capacity (HALC) transporters have been described in numerous systems (Delrot and Bonnemain, 1981; Maynard and Lucas, 1982; Russell, 1990; Weise *et al.*, 2000; Geiger, 2011). Although SUC2-type transporters are usually described as HALC transporters [although LAHC activities were measured *in planta* (Delrot and Bonnemain, 1981; Maynard and Lucas, 1982), sucrose transporters with LAHC activities have not been identified so far], the 50-fold lower K_m of Srt1 clearly characterizes Srt1 as a transporter with very low capacity, which may be too low to replace the missing activity of SUC2.

Alternatively, the lack of complementation might result from a specific difference in the transport properties reflecting different physiological roles of Srt1 in *U. maydis* and of SUC2 in *Arabidopsis* CCs. Although fungal sucrose transporters belong to the major facilitator superfamily (MFS) of transporters described almost 20 years ago by Marger and Saier (1993), Srt1 and SUC2 differ significantly with respect to their physiological tasks. Whereas sucrose imported by Srt1 into *U. maydis* cells is used for cellular metabolism, sucrose loaded by SUC2 into CCs is accumulated to generate the osmotic driving force for long-distance mass flow (Münch, 1930). Therefore, although Srt1 is an energy-dependent H^+-symporter and although it can accumulate sucrose to intracellular concentrations that exceed the concentrations in the extracellular lumen, it catalyses the permanent exchange of accumulated sucrose already at relatively low concentrations (Wahl *et al.*, 2010). This exchange flux is a well-known property of transporters that do not accumulate their substrates under physiological conditions (Komor *et al.*, 1972; Eddy, 1982).

Together, the low transport capacity of Srt1 (predicted from its high affinity) and the catalysis of an exchange flux already at low intracellular concentration might be the reason for the unsuccessful complementation of *suc2* mutants. Both factors will reduce the capacity to accumulate sucrose inside CCs to concentrations that are high enough to initiate long-distance transport and to remove photoassimilates from the source leaves.

Srt1 and SUC1 are differentially distributed in CCs

A direct comparison of the immunohistochemical images of leaf sections obtained after the treatment with αSrt1 (Fig. 6G) or αSUC1 (Fig. 6B) antisera revealed a difference in the distribution of Srt1 and SUC1. Whereas αSrt1-decoration of Srt1 results in a uniform labelling of the CCs (Fig. 6G), quite likely showing the plasma membrane (Fig. 5D), αSUC1-decoration of SUC1 results in a patchy distribution of the fluorescence at the surface of the CCs (Fig. 6B). This resembles the similarly patchy distribution observed for other plant sucrose transporters in CCs (Sauer, 2007; Schmitt *et al.*, 2008), and may point towards a concentration of these proteins within large subdomains of the CC plasma membrane. As SUC1–GFP fusions show uniform labelling of the plasma membrane in *Arabidopsis* mesophyll protoplasts (Feuerstein *et al.*, 2010), just like the Srt1–RFP fusion (Fig. 5D, E), this may be a CC-specific phenomenon. Whether or not this contributes to the successful complementation of *suc2* plants remains to be analysed.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Differences in the pH-dependences of SUC1 and SUC2.

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