Phloem Mobility and Stability of Sucrose Transporter Transcripts

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Abstract: Sucrose transporters are essential membrane proteins for the distribution of photoassimilates in higher plants. In Solanaceae species the proteins of all known sucrose transporters are co-localized in enucleate sieve elements and undergo permanent turnover. The mRNA of the sucrose transporter StSUT1 is localized in both, sieve elements and companion cells. Sucrose transporter mRNAs have been detected in the phloem sap of several species. Here, we analyzed the mobility of sucrose transporter transcripts in grafted plants and between host and parasitic plants. Phloem-mobility was found when a c-myc tagged SUT1-fusion construct without untranslated regions (UTRs) was expressed under the CaMV 35S promoter. We conclude that neither 3′-nor 5′-UTRs are essential for mRNA transport through plasmodesmata. Cycloheximide, which inhibits translation, has also effects on SUT transcript stability. Whereas SUT1 transcripts are destabilized when translation is inhibited, SUT2 and SUT4 transcripts accumulate up to 4-fold under these conditions. Inhibitor studies revealed post-transcriptional regulation of SUT2 and SUT4 transcript accumulation. A model is proposed explaining the coordination of SUT expression in Solanaceae.

INTRODUCTION

Sucrose transport in Solanaceae was studied intensively in the past decades. Three different sucrose transporters have been identified from Solanum tuberosum and Lycopersicon esculentum [1-3]. Antisense inhibition of LeSUT1 and StSUT1 revealed an important role of SUT1 in phloem loading [4,5]. LeSUT2 from tomato was found to be expressed in pollen and pollen tubes and to be essential for pollen germination and pollen tube growth [6]. All three known transporters from potato, StSUT1, StSUT2 and StSUT4, have been co-localized in phloem sieve elements [7], but the phloem specific role of SUT2/SUT4 proteins still needs further elucidation. A dual function of the LeSUT2 protein is discussed [6]. Phylogenetic analysis shows redundancies among the major family of dicotyledonous SUT1 transporters with as much as 7 members in Arabidopsis thaliana, whereas only one single member of sucrose transporters can be found in SUT2 or SUT4 subfamilies per species [8] suggesting a function different from SUT1 for these proteins.

Whereas the SUT1 protein was detected immunocytochemically in the sieve elements of the phloem, the mRNA of the SUT1 transporter was localized electron microscopically in both, the sieve elements and the companion cells of the phloem [9]. Since the use of the companion cell-specific rolC promoter inhibited successfully the expression of the StSUT1 gene, it was concluded that the SUT1 mRNA is transcribed in phloem companion cells and translocated via the branched plasmodesmata connecting companion cells and sieve elements [5]. Indeed, the StSUT1 mRNA molecules were preferentially associated to these branched plasmodesmal structures [9]. The ability of the StSUT1 mRNA to move from one cell into another via plasmodesmata was confirmed by microinjection experiments with or without the RNA-binding phloem protein CmPP16 [10]. In many plant species, SUT1 mRNA was detected in the phloem sap collected by aphid stelyctomy via RT-PCR, whereas amplification of other mRNAs i.e. from the potassium transporter HAK1 failed [11-13].

The phloem mobility of many phloem specific mRNAs and proteins has been analyzed in detail via interspecific graft experiments [12]. So far, nothing is known about the phloem mobility of sucrose transporter mRNAs. By interspecific graft experiments using transgenic potato and tobacco plants we were able to show that indeed the mRNA of the sucrose transporters StSUT1 from potato and NtSUT1 from tobacco are phloem mobile between grafted plants and between host plants and the holoparasite Cuscuta reflexa.

mRNA molecules require protection against nucleolytic degradation during transport through plasmodesmata and during long distance transport in the phloem. So is known that the StSUT1 protein decreases within only 4 h of CHX treatment [9] and the accumulation of sucrose transporter mRNA follows a diurnal rhythm at least in Solanaceae and carrot plants [9, 14]. This indicates an apparent short half life of sucrose transporter mRNA and protein.

We performed a detailed analysis of the transcript stability. Inhibitor studies revealed that expression of members of the SUT2 and SUT4 family undergo completely different regulation as described for sucrose transporters belonging to the SUT1 subfamily. Whereas SUT1 is mainly under transcriptional control, SUT2 and SUT4 transcript accumulation depends on a post-transcriptional mechanism as well. New aspects will help to elucidate the coordination of sucrose transporter expression.
METHODS

Plant material. Transgenic plants were kindly provided by G. Leggewie [15] and A. Weise [16,17]. Cuscuta reflexa was kindly provided by Kirsten Krause, University of Kiel. Cuscuta reflexa was grown in potato and tobacco plants for at least two weeks before harvest. To avoid contamination, RNA samples from Cuscuta reflexa were taken in a 2 cm distance from haustoria between host and parasite.

Plant growth conditions and tissue culture. Potato plants in sterile culture were grown on 2MS-medium (MS-medium according to reference [18] with 2% sucrose) in tissue culture chambers at 24°C, at 50% humidity and 1000 μmol photons m⁻² sec⁻¹ with a light/dark cycle of 16 h/8 h. Following transformation, leaf discs were put on 2MS with 1 μg/l naphthyl acetic acid and 0.1 μg/l benzyl amino purine. For the selection of transformant tissue, 3MS with 2 μg/l zeatin and 35 μg/l kanamycin was used. The root induction of plantlets was performed on 2MS with 2 μg/l indol butyric acid and 50 μg/l kanamycin. After 2 weeks, plantlets were placed on 2MS containing 50 μg/l kanamycin.

Greenhouse. Transgenic plants were amplified in tissue culture and 60 plants were transferred to soil and grown in a cycle of 16h light (22°C) and 8h darkness (15°C) in 60% humidity. The mean photosynthetic photon flux density (PPFD; 400–700 nm) was about 150 μmol photons m⁻² sec⁻¹ and additional illumination was provided by high-pressure sodium lamps SON-T Green Power and metal halide lamps MASTER LPI-T Plus (Philips Belgium, Brussels).

Grafting. Plants had 5-6 leaves when grafted. The experiment was performed as described in reference [19,20]. RNA was isolated from stem section with 1, 5, and 10 cm distance from the graft union.

RNA Quantification by Real-Time PCR

RNA was isolated from different organs of greenhouse grown Lesculentum Moneymaker, S. tuberosum Désirée or N. tabacum SNN as described [21]. Reverse transcription was performed with the Qiagen Omniscript RT Kit according to the manual. Optimised conditions included using oligo(dT) primers for the initial reverse transcription reaction on approximately 2 μg of total RNA after digestion with RNase-free DNase (Qiagen).

Aliquots of 0.5 μl of the 20 μl RT-reaction were used for the subsequent PCR reaction in the presence of SYBR Green with Hot Goldstar Polymerase (Eurogentec) in a Rotor Gene Cycler (LTF) using the Rotor Gene Software Version 4.6.94. The best products were obtained with the following program: denaturation at 95°C for 30 sec, annealing for 30 seconds at 61°C and elongation for 30 sec at 72°C, in a program of 40 cycles in 25 μl reaction volume. Relative quantification of LeSUT1 and LeSUT2 transcript amounts was always calculated in relation to the respective ubiquitin3 transcript level and given in % of ubiquitin or in % of the water control. Primers were designed to obtain a 50-150 bp amplicon using Primer3 software (http://frodo.wi.mi.edu/cgi-bin/primer3/primer3 www.cgi).

Primer sequences: Ubiquitin fw: CAC CAA GCC AAA GAA GAT CA; Ubiquitin rev: TCA GCA TTA GGG CAC TCC TT; LeSUT1 fw: TTC CAT AGC TGC TGG TGT TC; LeSUT2 rev: CAC CAA TGG GTC CAC AA; LeSUT1 rev: TAC CAG AAA TGG GTC CAC AA; LeSUT2 fw: CCT ACA GCG TCC CTT TCT CT; LeSUT2 rev: GGA TAC AAC CAT CTG AGG TAC AA, LeSUT4 fw: TCT CCG CTG ATA TTG GAG GG; LeSUT4 rev: GCA ACA TCG AGA AGC CAA AA; SiSUT2 fw: GCA ATG CAT TCG GTC TCT AT; SiSUT2 rev: CGG GTC CCC ATG ATA GAC TT; SiSUT1 fw: CAA TTT TGT GGT GCC CT; SaSUT1 rev: AGT AGC CGA CAA CTG GT GA, NtSUT1 fw: TTG GGG CTG TTT AAC CT; NtSUT1 rev: GCA AAC AGC CAA CAC GAA AT; GUS fw: CAT GTC GCG CAA GAC TGT AA; GUS rev: GTC CAG TTG CAA CCA CCT GT; TEF fw: TGG AAC TGT CCC TGT TGC TT; TEF rev: ACA TGG TCA CCA GGG AGT GC; SoSUT1-RT-fw: CCC CCT GAA GCT AAA ATT GG; SoSUT1-RT-rev: AGT TGA GGT CTT CTT CCG AGA TTA G; LeSUT1 primers for semi-quantitative RT-PCR: SiSUT1 fw: TTT AGG TAC CAT GGA GAA TGG TAC AAA AAG; SiSUT1 rev: GAG AGA TAT CCC ATG GAC CAG.

Real time PCR data were corrected by calculation of the PCR efficiency individually using the LinReg PCR software.

Feeding Experiments and Inhibitor Studies

Plants were grown in the greenhouse with a 16-hr-light/8-hr-dark cycle, and leaves were harvested from 3-month-old plants. Petioles of detached leaves were re-cut while submerged in water, 2.5 mM EDTA was added to inhibit callose formation, and the cut petioles were transferred to inhibitor or phytohormone containing solutions where they were kept for the indicated period of time under greenhouse conditions. Phytohormones (2,4-D or BAP) were used in a final concentration of 5 μM. The inhibitors CHX or MG132 were used at a concentration of 10μM, actinomycin was used at 2 μM and the final cordycepin concentration was 50μg/ml. All experiments were repeated several times independently.

The transcript amount of each messenger RNA was determined before and after the experiment by real time PCR. The messenger decay rate during actinomycin D or cordycepin treatment was used to calculate the half life of sucrose transporter mRNAs (Table 1).

RESULTS

Phloem Mobility Between Host Plant and Parasite: Detection of SiSUT1 and NiSUT1 Transcripts in Cuscuta reflexa

Cuscuta reflexa (dodder) is a holoparasite able to form haustoria to the host plants in order to collect photoassimilates (Fig. 1). Hyphae of the parasitic plant build up symplasmic connections to the phloem cells of the host plant allowing the transport of macromolecules as shown by GFP movement from transgenic tobacco plants into C. reflexa [22]. C. reflexa uses potato as well as tobacco plants as a host (Figs. 1B, C), whereas tomato plants are resistant against the parasite [23]. We used C. reflexa to analyze the movement of SiSUT1 and NiSUT1 mRNA from host to parasite. Both, the full length SiSUT1 transcript as well as the NiSUT1 mRNA were detectable via RT-PCR in C. reflexa RNA grown for 2 weeks on potato or tobacco host plants respectively (Figs. 1B, 1C). No RT-PCR amplicon was detectable when Cuscuta was grown on other host plants like
SUT1 Transcripts are Phloem Mobile Between Grafted plants

*StSUT1* mRNA cell-to-cell movement through plasmodesmata was first shown by microinjection experiments with or without the RNA-binding protein CmPP16 from *Curcurbita maxima* [10]. In order to test whether *StSUT1* mRNA mobility is also detectable in cells, where the short lived *StSUT1* transcripts naturally occur, we performed intra- and interspecific grafts. Transgenic potato plants overexpressing a c-myc-tagged version of the *SoSUT1* gene were used to answer the question whether untranslated regions of the *SUT1* transcript are required for phloem mobility (Fig. 2A). The *SoSUT1*-c-myc gene does not contain untranslated regions of the mRNA since only the coding region of the cDNA was used for the construct [15]. Although neither the 5'UTR, nor the 3'UTR is included, phloem mobility of *SoSUT1*-c-myc mRNA was detectable by semi-quantitative RT-PCR (Fig. 2B). Thus, untranslated regions (UTRs) of *SUT1* transcripts are not required for mRNA movement from one graft partner into the grafted WT potato plants.

Phloem mobility of *LeSUT1-GUS* mRNA

Phloem mobility of *SUT1* transcripts was not only detected when the cDNA was expressed under a strong constitutive CaMV 35S promoter, but also when the tomato gene *LeSUT1* was expressed under its own promoter in transgenic tobacco plants (Fig. 2C). We used a *LeSUT1* promoter::*LeSUT1-GUS* fusion construct containing exon as well as intron sequences of *LeSUT1* [17]. When the *LeSUT1-GUS* fusion protein was expressed under its own promoter, again phloem mobility of the corresponding mRNA could be observed in the grafted WT scion by RT-PCR (Fig. 2C). Thus, even the larger mRNA is able to move in the phloem of grafted tobacco plants and phloem-mobility occurs not only if the gene is overexpressed under the constitutive 35S promoter.

In Silico Promoter Analysis of Genomic Sucrose Transporter Sequences

The expression of sucrose transporters is under phytohormonal control. The expression of *StSUT1* is inducible by auxin and by cytokinin as shown by Northern Blot Analysis [24] and *StSUT4* is shown to be induced by ethylene and gibberellins [25]. The transcript level of all three known sucrose transporters from potato oscillates even under constant light conditions [25]. Promoter analysis by the Web Signal Scan Program (http://www.dna.affrc.go.jp/sigscan/signal1.pl) was performed with the genomic *LeSUT1* and *LeSUT2* sequences. The *LeSUT1* promoter region contains consensus sequences involved in sugar repression, circadian expression, ethylene response, pollen specific expression, light regulation, guard cell specific expression, and induction by salicylic acid. Some of the genomic cis-regulatory elements are located in intron sequences such as the auxin responsive element (AuxRE), which represents the binding site of the auxin response factor ARF and which is located in the 3rd intron of *LeSUT1*. A promoter sequence element for circadian evening genes is known, called evening element EE with the consensus AAAATATCT [26]. According to Harmer and Kay, an imperfect evening element of circadian genes is present in the *LeSUT1* promoter [26].
The orthologous gene from Arabidopsis, AtSUT2/AtSUC3, is known to be expressed in pollen and to be essential in pollen development and pollen tube growth [6]. The orthologous gene from Spinacia oleracea under the control of an enhanced CaMV35S promoter was used for transformation of Beta vulgaris (accession no: At2g02860), the Arabidopsis ortholog in Beta vulgaris, BvSUT1: 108 min. [29]. The half life of SUT2 and SUT4 mRNAs from potato and tomato show a longer half life of about 130 min as shown by transcription inhibition using actinomycin D or cordycepin (Table 1, Fig. 3). Since transcript accumulation of all three known sucrose transporters from potato follow a diurnal rhythm [9,25], transcript amounts have been related to the corresponding water control after the indicated period of time.

The stability of the sucrose transporter protein StSUT1 is rapidly affected by CHX treatment shown by western blot analysis [9]. Sucrose transporter transcript accumulation was now investigated in the presence of cycloheximide and quantified by real time PCR analysis. The mRNA levels of both, StSUT1 from potato as well as LeSUT1 from tomato rapidly decreased within 4 h upon CHX application (Figs. 3A, 3B). Thus, in case of SUT1 not only the protein stability is affected by inhibition of translation, but also the transcript amounts.

In a very similar experiment analyzing the transcript accumulation of SUT2 and SUT4 transporters from potato and tomato in the presence of CHX, the inhibitor had the opposite effect on transcript amounts as shown for SUT1 mRNA. In contrast to SUT1 mRNAs, the levels of the SUT2 and SUT4 mRNAs from potato and tomato are stabilized in the presence of CHX: the mRNA amount increases strongly within the very short period of only 2 h upon CHX treatment (Fig. 4A, 4B). This is in agreement with the transcript amount of AtSUT2/AtSUC3 (accession no: At2g02860), the SUT2 ortholog in Arabidopsis, as analysed by microarray analysis available from the Arabidopsis co-response database (http://csbdb.mpimp-golm.mpg.de/csbdb/bcor/ath/ath_txp.html). The level of AtSUT2 transcripts increases more than 4fold after 3h treatment with 10µM CHX. Thus inhibition of translation either increases transcriptional efficiency and/or the protein stability of both SUT2 and SUT4 genes in tomato, potato and in case of SUT2 also in Arabidopsis.

**SUT Regulation at the Transcriptional and Post-Transcriptional Level**

Translational inhibition by CHX can affect the accumulation of sucrose transporter transcripts by two different processes: either reducing amounts of short-lived transcriptional regulators modify transcriptional activity or the stability of the already transcribed messenger RNAs is affected post-transcriptionally by RNA-binding factors. It is possible, that
these factors may specifically stabilize SUT1 mRNA or de-stabilize SUT2 and SUT4 mRNA.

In order to distinguish between transcriptional and post-transcriptional control, experiments with translational and transcriptional inhibitors have been performed simultaneously (Figs. 3B, 4). In the presence of both, transcriptional and translational inhibitors the half life of StSUT1 mRNA decreased compared to the untreated control (Fig. 3B) as already observed using both inhibitors alone.

### Table 1. Half life of different sucrose transporter transcripts as determined via real time PCR on mRNA isolated after feeding experiments with appropriate inhibitors. RNA quantification was performed in relation to the amount of ubiquitin transcripts as internal standard. Water treated plant material was taken as a reference. The standard deviation is given.

<table>
<thead>
<tr>
<th>Gene</th>
<th>mRNA half life</th>
<th>Inhibitors</th>
<th>Reference</th>
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<tbody>
<tr>
<td>LeSUT1</td>
<td>88.1 ± 13 min</td>
<td>cordycepin</td>
<td>this work</td>
</tr>
<tr>
<td>StSUT1</td>
<td>69.3 ± 3 min</td>
<td>cordycepin; act D</td>
<td>this work</td>
</tr>
<tr>
<td>BvSUT1</td>
<td>108 min</td>
<td>cordycepin</td>
<td>[29]</td>
</tr>
<tr>
<td>LeSUT2</td>
<td>131.6 min</td>
<td>cordycepin</td>
<td>this work</td>
</tr>
<tr>
<td>LeSUT4</td>
<td>131 min</td>
<td>cordycepin</td>
<td>this work</td>
</tr>
<tr>
<td>StSUT4</td>
<td>131 min</td>
<td>act D</td>
<td>this work</td>
</tr>
</tbody>
</table>
In contrast, the half life of StSUT2 and StSUT4 mRNA was significantly prolonged in the presence of both inhibitors in comparison to the treatment with only the transcriptional inhibitor actinomycin D and the amount of StSUT2 and StSUT4 transcripts increased compared to the untreated control (Fig. 4). Thus, transcript half life is prolonged by CHX even when transcription is blocked.

For further confirmation of the StSUT1 regulation at the transcriptional level, transgenic plants overexpressing the c-myc tagged SoSUT1 gene under the constitutive 35S-promotor have been analyzed [15]. Plants overexpressing SoSUT1 under control of the constitutive 35S promoter do not follow the diurnal regulation of the mRNA (Supplement 1). Analysis of the 35S::SoSUT1 overexpressing potato plants showed furthermore, that the transcripts of the overexpressed c-myc tagged SUT1 gene is not destabilized in the presence of cycloheximide as previously shown for SUT1 expressed under the endogenous promoter (see above) arguing for transcriptional control of SUT1 mRNA and requirement of cis-acting promoter elements for its diurnal SUT1 regulation (Supplement 1). Diurnal mRNA oscillation due to post-transcriptional mRNA decay is thus unlikely in case of SUT1.

Auxin Induces StSUT1 Expression

Real time analysis of potato leaves pre-incubated with 2,4-D for 2 to 4 h revealed a strong increase of StSUT1 mRNA amount (Fig. 5A). Not only mRNA accumulation is observed, but also the StSUT1 protein content follows the increasing SUT1 mRNA accumulation transiently upon treatment with 5 μM 2,4-D and progressively upon treatment with 5 μM BAP (Fig. 5B). The inducibility of StSUT1 by phytohormones such as auxin or cytokinin at the transcriptional level was previously shown [24].

If auxin is supplied and simultaneously, the translation is blocked by CHX as shown in Fig. 5A, no StSUT1 transcript accumulation can be observed (Fig. 5A). It is concluded, that auxin induction of StSUT1 requires de novo protein synthesis since the effect is completely abolished in the presence of CHX. Therefore, the analyzed sucrose transporter StSUT1 belongs to the so-called late auxin responsive genes.

In contrast to StSUT1, StSUT4 is only marginally induced by auxin, and transcript accumulation is strongly increased upon CHX application (Fig. 5C). Similar observations have been done with StSUT2 transcript levels (data not shown). Simultaneous application of 2,4-D and CHX results in low transcript amounts (Fig. 5C).
Phloem Mobility and Stability of Sucrose Transporter Transcripts

**CONCLUSIONS**

Phloem Mobility of SUT1 mRNA

From several independent studies the presence of SUT1 mRNA in the phloem sap of several species was known [11,12,13] and the StSUT1 mRNA was localized electron microscopically in the companion cells as well as in the sieve elements of the phloem in potato plants [9]. For many of the known mRNAs present in the phloem sap, the phloem mobility has been analyzed via interspecific graft experiments [12]. The mobility of the sucrose transporter mRNA has not yet been analyzed. We were able to show phloem mobility of StSUT1, NtSUT1, LeSUT1-GUS and SoSUT1-myc transcripts either between parasite and host plants or between grafted plants. Thereby, we confirmed previous investigations regarding the phloem mobility of sucrose transporter mRNA from Cucurbita maxima from the host into the dodder plant Cuscuta pentagona Engelm [31].

The detailed analysis of the mobility of pumpkin and tomato phloem transcripts from the host plants into the dodder plant Cuscuta pentagona by microarray and RT-PCR analysis revealed species-specific differences. Only three out of the eight mentioned phloem mobile pumpkin transcripts, were detectable in the parasite, namely, CmNACP, CmRKYP and CmSUT1P [31]. This argues for the specificity of the mRNA transport through symplasmic connections between host plant and parasite, since the ability to be transported into the dodder plants is not related to the transcript amount in the host plant [31]. It is discussed whether the mobility of specific mRNAs allows interspecific communication and whether Cuscuta can recognize, use, and respond to specific plant mRNAs.

**Fig. (5).** Expression of the sucrose transporter StSUT1 is induced by auxin (5 μM 2,4-D) both at the mRNA and the protein level. A. Real time-PCR quantification of StSUT1 upon 2 and 4 h treatment with 5 μM 2,4-D and in the presence of 5 μM 2,4-D and 10 μM CHX or 10μM CHX alone. Auxin induction of StSUT1 transcript accumulation is abolished by CHX. B. Western Blot Analysis with microsomal membranes separated by SDS-PAGE and immunodetected with StSUT1-specific antibodies. Potato leaves were treated with either 5μM 2,4-D or 5μM BAP and 15μg of total membrane proteins were loaded per lane. StSUT1 protein amount increases upon cytokinin treatment (BAP) and transiently upon auxin treatment. C. Real time quantification of StSUT4 transcripts upon the same treatment shown in A. StSUT4 mRNA increases only marginally upon auxin treatment. Cycloheximide treatment increases StSUT4 mRNA half life significantly. The stabilizing effect of CHX is completely abolished in the presence of auxin (2,4-D + cyclo).

It is known, that AUX/IAA genes belong to the early auxin responsive genes and encode in most cases very short lived repressors of transcription. Repression is achieved by forming ARF heterodimers preventing ARF activation by binding to the AuxRE cis-element. AUX/IAA proteins are degraded by the ubiquitin-mediated 26S proteasome pathway.

In order to test whether SUT transcription depends on short-lived proteins which are degraded via the 26S protea-

some pathway, transcript quantification was performed in the presence of the inhibitor of the 26S proteasome, MG132 [30].

The inhibitor of the 26S proteasome MG132 does not affect transcript stability or accumulation, whereas CHX leads to a decreased StSUT1 transcript amount (Fig. 6A).

Simultaneous treatment with CHX and MG132 lead to low StSUT1 transcript amounts as with CHX alone. Therefore the inhibitor of the 26S proteasome is not able to stabilize StSUT1 mRNA in the presence of CHX. It is concluded that proteins responsible for high StSUT1 transcript levels under normal conditions (in the absence of CHX) are not degraded by the 26S proteasome since StSUT1 transcripts disappear efficiently within 4h of incubation with both inhibitors.

CHX has the opposite effect on SUT2 and SUT4 transcript accumulation, which increase within only 2h of treatment (Figs. 4A, 4B, 5C, 6B). MG132 has no significant effect on StSUT2 transcript amounts (Fig. 6B). But the inhibitor of the 26S proteasome, MG132, is able to prevent the stabilising CHX effect on StSUT2 transcripts leading to similar transcript amounts as seen in the untreated control (Fig. 6B). Therefore, negative effectors on StSUT2 transcript accumulation are most likely degraded via the ubiquitin-mediated 26S proteasome pathway since inhibition of their degradation abolished the effect of the inhibition of their synthesis.
general feature of phloem-specific RNAs and into the shoot apex is a selective process [32]. It was shown that the RNA entry only three out of eight phloem-specific mRNAs were detected in graft scions [12]. It was shown that the RNA entry into the shoot apices of grafted plants, since different tomato transcripts identified in the parasite and most of these mRNA play a role in mediating plant response to the environment [31].

Also heterograft experiments argue for specificity of the mRNA delivery into the shoot apices of grafted plants, since only three out of eight phloem-specific mRNAs were detected in graft scions [12]. Note that relative quantification is not related to the corresponding water control as in Fig. (3-5) but to the amount of ubiquitin transcripts after 4 h of incubation (A, StSUT1) and 2 h of incubation (B, StSUT2).

Tomato microarray analysis revealed as much as 474 different tomato transcripts identified in the parasite and most of these mRNA play a role in mediating plant response to the environment [31].

Also heterograft experiments argue for specificity of the mRNA delivery into the shoot apices of grafted plants, since only three out of eight phloem-specific mRNAs were detected in graft scions [12]. It was shown that the RNA entry into the shoot apex is a selective process [32].

Thus phloem-mobility of mRNAs is seemingly not a general feature of phloem-specific RNAs and SUT1 mRNA is one of the specific phloem mobile components in grafted plants as well as between host and parasitic plants.

The ability of StSUT1 mRNA to move through plasmodesmata has previously been shown in microinjection experiments [10]. Co-injection of StSUT1 mRNA together with the RNA-binding phloem protein CmPP16 significantly increased the efficiency of plasmodesmal transport. In 10 out of 10 experiments co-injected fluorescently labelled StSUT1 mRNA was able to move from the microinjected into neighbouring cells when co-injected with CmPP16. The phloem mobility of both the CmPP16 mRNA and protein was shown by heterograft experiments [12]. For many other phloem-specific genes, mobility of only their mRNA, but not of the corresponding protein was observed. This is the case for CmNACP, CmWRKYP, CmRINGP, CmGAIP, CmRABP, CmSTP etc. In all cases, a regulatory role of these phloem-mobile mRNAs has been postulated [12].

In contrast, phloem mobility of the two structural phloem proteins PP1 and PP2 are detectable in the graft partner in heterografts, whereas their mRNA is obviously not phloem mobile [33]. This argues again for the selectivity of phloem transported mRNAs.

Transcriptional control of SUT1, post-transcriptional control of SUT2 and SUT4

We showed that the half life of solanaceous sucrose transporter mRNAs ranges between 60 and 130 min. (this work). The half life of the BvSUT1 mRNA was determined to be 108 min and repression by sucrose takes place at the transcriptional level as shown by nuclear run off analysis [29].

Thus, sucrose transporter mRNAs are very short lived and accumulation is tightly controlled at various levels. Whereas, transcriptional control of the diurnally expressed StSUT1 gene seems to have high impact on its accumulation, StSUT2 and StSUT4 transcript accumulation is highly dependent on the de novo biosynthesis of negative acting regulators indicated by the enhanced stability in the presence of CHX within only 2 h. Prolongation of mRNA half life by inhibition of transcription and translation argues for a tight post-transcriptional control of these two sucrose transporter-related genes in a sequence-specific manner.

It was shown previously, that the LeSUT1 mRNA follows a diurnal expression pattern [9]. In contrast to carrot, where DcSUT1 and DcSUT2 are diurnally regulated in leaves, but expressed at a constantly low level in tap roots [14], the diurnal rhythm of sucrose transporter expression in potato plants is also detectable in sink organs such as flowers (Chincinska, Kühn, unpublished). Sucrose transporter mRNA oscillation is not only detectable under dark/light cycles, but also under constant light conditions [25] arguing for circadian regulation.

For several circadian genes, a specific post-transcriptional mRNA decay mechanism has been postulated [34]. The circadian control of messenger stability was found to be associated to a sequence specific decay pathway [34,35]. Instability determinants of clock controlled mRNAs might be located within untranslated regions, introns or within the coding region as shown for mRNA of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) [36]. Here we show that both, the StSUT2 and the StSUT4 mRNA undergo sequence-specific post-transcriptional mRNA decay as previously shown for other circadian genes [34]. The half life of the two transcripts is increased by CHX even in the presence of transcriptional inhibitor actinomycin D (Figs. 4A, 4B).

RNA stability is triggered by elements like the well characterized AUUUA motif, which is known to accelerate mRNA decay of many eukaryotic transcripts [37]. StSUT2 mRNA contains one of these motifs in the 3’UTR, the StSUT1 mRNA contains one and the StSUT4 mRNA two AUUUA motifs within the coding region (Supplement 2).

In a project where two million small RNAs from Arabidopsis seedlings and inflorescences were sequenced, new micro RNAs were identified [10]. Among the set of 75.000 non-redundant sequences most of the small RNAs were...
miRNAs and more than half represented low abundant small interfering RNAs (siRNAs) matching genes or intergenic regions. Micro RNAs matching sucrose transporter genes were found for all known sucrose transporter genes from *Arabidopsis* except for *AtSUT2* and *AtSUT4* (according to the supplementary files of [38]), the orthologous genes of *StSUT2* and *StSUT4*. Thus, the control of *SUT2* and *SUT4* genes via micro RNAs is unlikely and it is assumed that very short lived RNA-binding proteins play an important role in the regulation of the *SUT2* and *SUT4* expression level.

**Auxin Regulation of Sucrose Transporters**

Auxin induced genes fall into two classes: early auxin genes are primary response genes which are insensitive to protein synthesis inhibitors such as CHX. Some of these early genes encode proteins that regulate the transcription of secondary or late response genes that require de novo protein synthesis, their expression can be blocked by CHX. A conserved auxin response element (AuxRE) can be found in the promoter region of *LeSUT2*. It represents the binding site of the auxin response factor ARF, a transcriptional activator, which is regulated by short-lived Aux/IAA proteins acting as repressor of ARF [39].

Aux/IAA proteins are known to have a half life of only a few minutes and to be degraded via the proteasome pathway. This degradation is promoted by auxin. A negative feedback mechanism induces AUX/IAA transcription to ensure a short term auxin effect.

A transcriptional activator seems to be required for *SiSUT1* induction which is auxin-dependent and short-lived, but not degraded via the ubiquitin-mediated proteasome pathway, since the inhibitor of the 26S proteasome did not lead to transcript stabilisation in the presence of CHX (Fig. 6A). In case of *SUT2/SUT4* the mRNA stabilizing effect of CHX is prevented by MG132 arguing for a ubiquitin-mediated degradation of the short-lived inhibitor of *SUT2* and *SUT4* transcript accumulation via the 26S proteasome (Fig. 6B).

The change of *SUT1* transcript levels upon CHX occurs within 4 h upon CHX treatment, whereas the increase of *SUT2* and *SUT4* mRNA is immediately detectable within only 2 h after CHX treatment (Figs. 3, 4, 5 and 6). The non-redundancy of *SUT2* and *SUT4* sucrose transporters, the low and ubiquitous expression level in the phloem, together with the low codon bias and the tight short term regulation of their expression pattern makes these genes potential candidates for regulatory processes. Auxin-dependent regulation of the transcriptional efficiency of sucrose transporter genes together with regulation of the transcript stability by a sequence specific mRNA decay mechanism might represent a possibility to coordinate the interplay of sucrose transporters of higher plants as suggested in the hypothetical model of Fig. (7).

The model in Fig. (7) summarises the positive effect of auxin on the transcriptional activity of the *SUT1* genes via transcriptional activators, whereas *SUT2* and *SUT4* genes are obviously negatively affected by short-lived proteins either at the transcriptional level potentially via AUX/IAA proteins or at the post-transcriptional level via RNA-binding proteins. The fact that *SUT2* and *SUT4* mRNA half life is indeed affected at the post-transcriptional level (as shown in Fig. 4) points on the important regulatory function of phloem-specific RNA-binding proteins and sequence-specific mRNA degradation.

In summary, *SiSUT1, NiSUT1* as well as *SoSUT1-c-myc* mRNA and *LeSUT1-GUS* mRNA are phloem mobile although they are very short-lived with a half life between 1
and 2 h. StSUT1 regulation required cis-acting promoter elements to show diurnal oscillation as well as decreased transcript amounts in the presence of the translational inhibitor CHX. In contrast, SUT2 and SUT4 from potato and tomato are regulated at the post-transcriptional level.

Thus, mRNA binding proteins are necessary to enhance mRNA stability during mRNA transport through the phloem. Sequence-specific RNA binding proteins might play a role in the selective stability of phloem mobile transcripts since it was shown, that not all of the transcripts present in the phloem sap are able to move within grafted plants or between host and parasitic plants [31].

As we found RNA-binding protein-dependent stability of SUT mRNA, we began to screen for mRNA-binding proteins using a yeast three hybrid system and sucrose transporter mRNAs as bait. These proteins might be involved in either stabilisation of the SUT1 mRNA during long distance transport in the phloem in form of ribonucleoprotein particles (RNPs) or might represent RNA-binding proteins able to affect the stability of SUT2 or SUT4 transcripts. These proteins could also contribute to the circadian control of the latter sucrose transporter mRNAs.

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ABBREVIATIONS

ARF = auxin response factor
SUT = sucrose transporter
GA = gibberellic acid
ABA = abscisic acid
actD = actinomycin D
CHX = cycloheximide
TEF = transcription elongation factor
GUS = ß-Glucuronidase
RT-PCR = reverse transcription polymerase chain reaction

REFERENCES

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Real time PCR analysis of SoSUT1-myc expression in transgenic potato plants, where SoSUT1 expression is under control of the 35S promoter. SoSUT1 expression does not follow a diurnal expression pattern as it is shown for the endogenous StSUT1 gene. Inhibition of translation by cycloheximide has no destabilizing effect in SoSUT1 transcripts as shown for the endogenous StSUT1 gene.