

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 Solanaceous 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 4fold 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 plasmodes-

mal 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 styletomy 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 intraspecific 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.

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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 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ with a light/dark cycle of 16 h/8 h. Following transformation, leaf discs were put on 2MS with 1 $\mu\text{g/l}$ naphtyl acetic acid and 0.1 $\mu\text{g/l}$ benzyl amino purine. For the selection of transformant tissue, 3MS with 2 $\mu\text{g/l}$ zeatin and 35 $\mu\text{g/l}$ kanamycin was used. The root induction of plantlets was performed on 2MS with 2 $\mu\text{g/l}$ indol butyric acid and 50 $\mu\text{g/l}$ kanamycin. After 2 weeks, plantlets were placed on 2MS containing 50 $\mu\text{g/l}$ 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 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ 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 *L.esculentum* 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.mit.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; 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 GAT GG; LeSUT4 rev: GCA ACA TCG AGA AGC CAA AA; StSUT2 fw: GCA ATG CAT TCG GTT CTC AT; StSUT2 rev: CGG GTC CCC ATG ATA GAC TT; StSUT1 fw: CAA TTT GGT TGG GCT CTT CA; StSUT1 rev: AGT AGC CGA CAA CTG GCT GA. NtSUT1 fw: TTG GGG CTG TTG AAC TC; 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 TGG TC; TEF rev: ACA TTG TCA CCA GGG AGT GC; SoSUT1-RT-fw: CCC CCT GAA GCT AAA ATT GG; c-myc-RT-rev: AGT TGA GGT CTT CTT CGG AGA TTA G. StSUT1 primers for semi-quantitative RT-PCR: StSUT1 fw: TTT AGG TAC CAT GGA GAA TGG TAC AAA AAG; StSUT1 rev: GAG AGA TAT CCC ATG GAA ACC GCC CAT GGC GAC.

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 10 μM , actinomycin was used at 2 μM and the final cordycepin concentration was 50 $\mu\text{g/ml}$. 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 *StSUT1* and *NtSUT1* 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 *StSUT1* and *NtSUT1* mRNA from host to parasite. Both, the full length *StSUT1* transcript as well as the *NtSUT1* 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

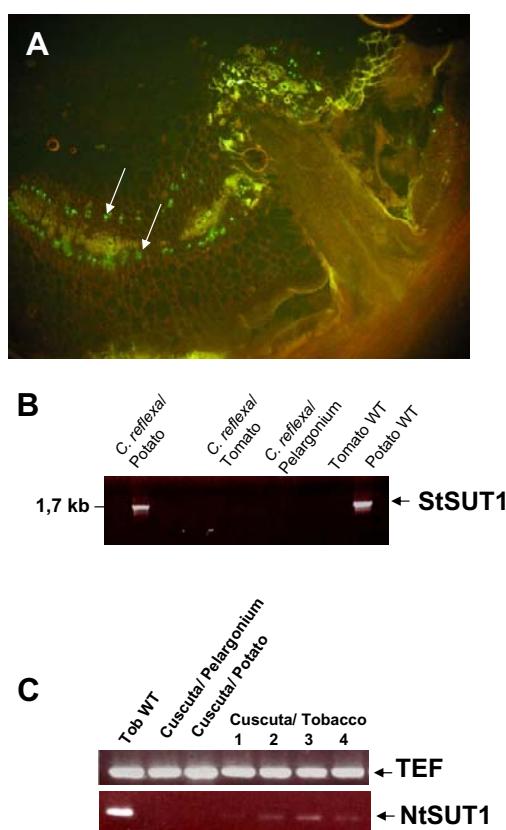


Fig. (1). **A.** Cross section of a *Cuscuta reflexa* haustorium on potato plants immunolabeled with RS21, a monoclonal antibody against a phloem-specific protein [40]. RS21 immunolabeling is detectable in the sieve elements of the b collateral vascular bundle of the potato stem tissue and visualized by FITC-coupled secondary antibodies (arrows). **B.** RT-PCR with *StSUT1*-specific primers. Cuscuta RNA was isolated after 2 weeks of growth on the host plants and RT-PCR was performed using *StSUT1* specific primers. Full length *StSUT1* transcripts are only detectable in RNA from potato plants (right lane) and in *Cuscuta* if grown on potato plants (left lane). Specificity of the *StSUT1*-primers has been tested in tomato RNA and RNA from *Cuscuta* grown on *Lycopersicon esculentum* or *Pelargonium*. **C.** RT-PCR with RNA from *Cuscuta reflexa* grown for 2 weeks on tobacco or *Pelargonium* plants. Quality and amount of the reversely transcribed mRNA was tested using standard primers (translation elongation factor TEF). *NtSUT1* specific primers were able to amplify *NtSUT1* on RNA from tobacco plants and *Cuscuta reflexa* grown on tobacco plants, whereas no amplicon was detected with the negative control (*Cuscuta* grown on *Pelargonium*).

Pelargonium or in the no template control. To avoid contamination, *Cuscuta* samples for mRNA preparation were taken in at least two cm distance from haustoria between dodder and host plant. Thus, *SUT1* mRNA movement was observed between host and parasite and requires passage through symplasmic connections between the parasitic and the host plants. In case of *StSUT1*, the full length transcript of 1.7 kb was detectable arguing for mRNA stabilization during long distance transport. The mRNA encoding the translation elongation factor TEF was used as a positive control for RNA and cDNA quality (Figs. 1B, 1C).

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 *Cucurbita 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].

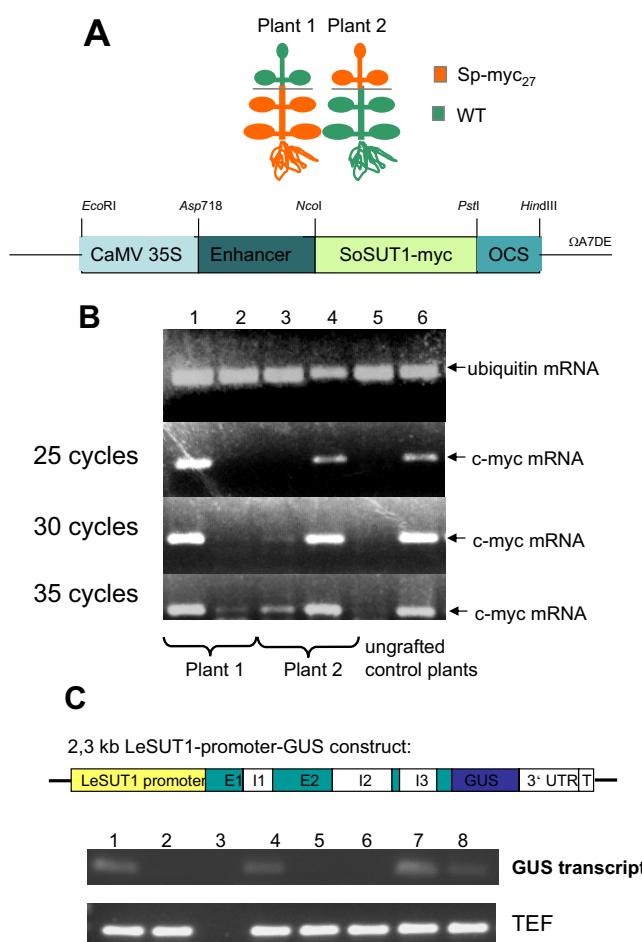


Fig. (2). A. Schematic representation of the intraspecific graft experiment (above) using transgenic potato plants overexpressing a tagged version of the *SoSUT1* gene of *Spinacia oleracea* under the control of an enhanced CaMV35S promoter as indicated in the construct (below) used for transformation of *Solanum tuberosum* [15]. B. Semi-quantitative RT-PCR of the tagged *SoSUT1* gene in grafted plants. Total RNA was assayed from the following tissues: Line 1 Sp-myc27 stock; line 2 Wild type scion; line 3 Wild type stock; line 4 Sp-myc27 scion; line 5 ungrafted Sp-myc27 potato; line 6 wild type potato. After 25 PCR cycles, the tagged *SoSUT1*-myc gene is detectable only in the transgenic graft stock of plant 1 and in the transgenic graft scion of plant 2. After 30 PCR cycles, the *SoSUT1*-myc gene is also detectable in the WT graft partner. Ubiquitin was used as internal control. C. Phloem mobility of *LeSUT1*-GUS transcript expressed under the endogenous *LeSUT1* promoter. Transgenic tobacco plants expressing a *LeSUT1*-GUS fusion construct under control of the 2,3 kb promoter fragment of *LeSUT1* were grafted with WT tobacco plants [17]. *LeSUT1*-GUS transcripts are detectable in grafted wild type tobacco plants via RT-PCR. Line 1, transgenic *LeSUT1*-GUS plant line 38; line 2, tobacco wild type; line 3 no-template control; line 4, transgenic *LeSUT1*-GUS 38 stock; line 5, grafted wild type scion 1>10cm from graft union; line 6, grafted wild type scion 2>5cm from graft union; line 7, grafted wild type scion 3>=1cm from graft union; line 8, grafted wild type scion 4<1cm close to graft union. TEF (translation elongation factor) was used as internal control.

LeSUT2 is known to be expressed in pollen and to be essential in pollen development and pollen tube growth [6]. The orthologous gene from *Arabidopsis*, *AtSUC3/AtSUT2* is wound inducible [27]. Analysis of the genomic *LeSUT2* se-

quence revealed cis-elements necessary for induction by ABA or SA, regulation by GA, ethylene, light, wounding, salt and water stress and for pollen specific expression. Additionally, two auxin response elements (AuxRE) are located in the *SUT2* promoter region and in the 10th intron arguing for a regulation via the auxin response factor (ARF).

Half Life of Sucrose Transporter mRNAs

The half life of sucrose transporter mRNAs was determined by help of actinomycin D as inhibitor of transcription and of cordycepin as inhibitor of polyadenylation. Most sense mRNA degradation follows first-order kinetics [28] and sucrose transporter transcript degradation can be fitted with first-order kinetics (R-squared greater than 0.9). The half life of *LeSUT1* mRNA was 88.1 ± 13 min and for *StSUT1* mRNA 69.3 ± 3 min (Table 1, Fig. 3). This is in close proximity to what was found for the *SUT1* mRNA from *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. 4). 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/dbcor/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 transcript 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

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

Gene	mRNA half life	Inhibitors	Reference
LeSUT1	88.1 ± 13 min	cordycepin	this work
StSUT1	69.3 ± 3 min	cordycepin; act D	this work
BvSUT1	108 min	cordycepin	[29]
LeSUT2	131.6 min	cordycepin	this work
LeSUT4	131 min	cordycepin	this work
StSUT4	131 min	act D	this work

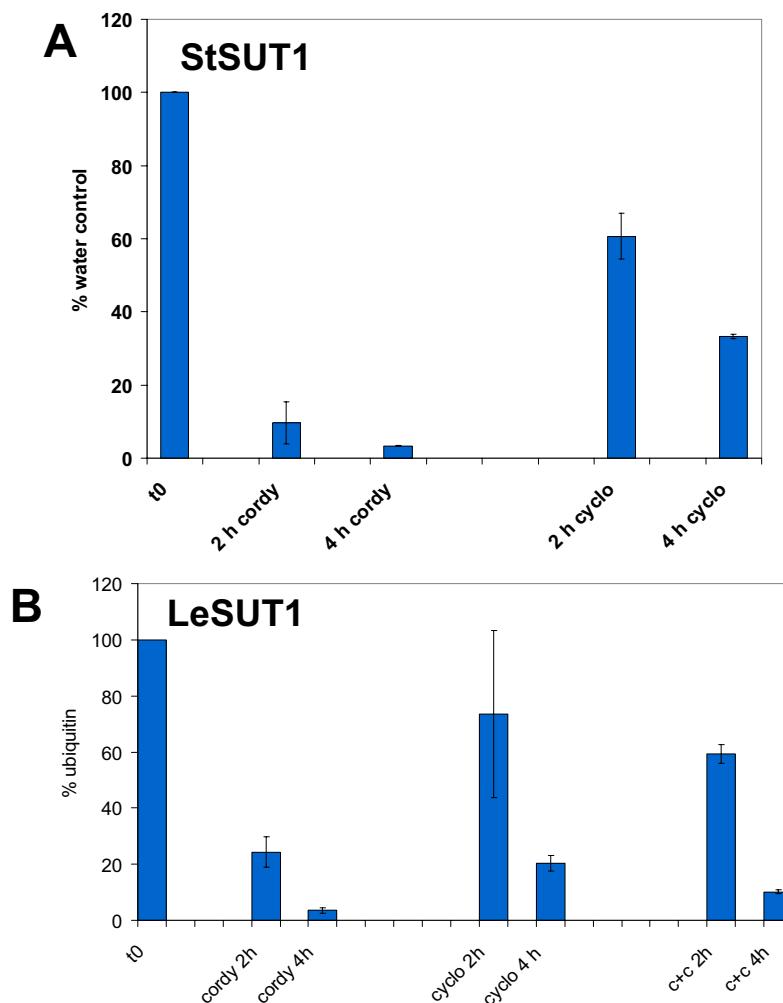


Fig. (3). Real time PCR analysis of sucrose transporter *SUT1* mRNA stability in the presence of transcriptional and translational inhibitors. **A.** Determination of *StSUT1* half life by cordycepin (cordy) and CHX (cyclo). Half life of *StSUT1* mRNA was calculated based on real time quantification and presented in table 1. **B.** Half life of *LeSUT1* mRNA as determined by cordycepin treatment. 6 h after cordycepin supply no mRNA is detectable (not shown). *StSUT1* and *LeSUT1* mRNA half life decreases in the presence of the translational inhibitor CHX. Simultaneous incubation with cordycepin and CHX (c+c) does not lead to significant prolongation of *LeSUT1* mRNA half life. Quantification of transcripts is relative to the amount of ubiquitin transcripts. In order to exclude diurnal changes of transcript levels, the values are given in % of the corresponding water control after the indicated period of time.

these factors may specifically stabilize *SUT1* mRNA or destabilize *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.

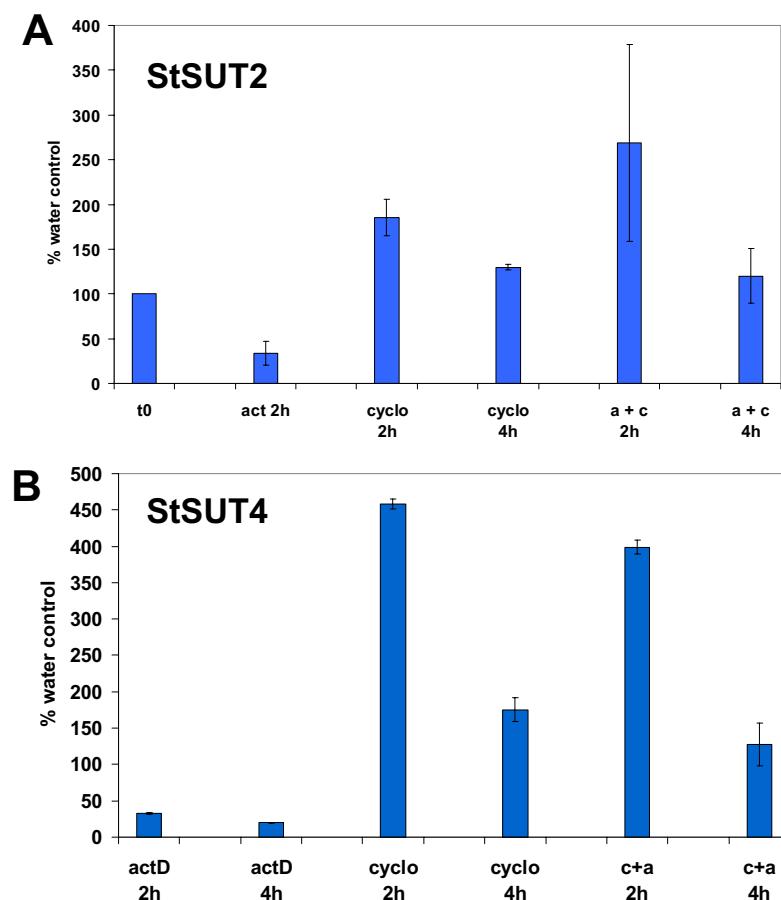


Fig. (4). Real time PCR analysis of sucrose transporter *SUT2* and *SUT4* mRNA stability in the presence of transcriptional and translational inhibitors. **A.** *StSUT2* mRNA quantification in the presence of actinomycin D (actD), CHX (cyclo) and both inhibitors (c+a). Half life of *StSUT2* mRNA was calculated based on real time quantification and presented in table 1. *StSUT2* mRNA accumulates upon 4 h CHX treatment. *StSUT2* mRNA half life is prolonged by CHX even if transcription is blocked (c+a). **B.** *StSUT4* mRNA half life was calculated by actinomycin D treatment and presented in table 1. Inhibition of translation increases *StSUT4* transcript accumulation 4.5 fold within 2 h. *StSUT4* mRNA half life is prolonged by simultaneous treatment with translational and transcriptional inhibition (c+a).

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 over-expressed 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).

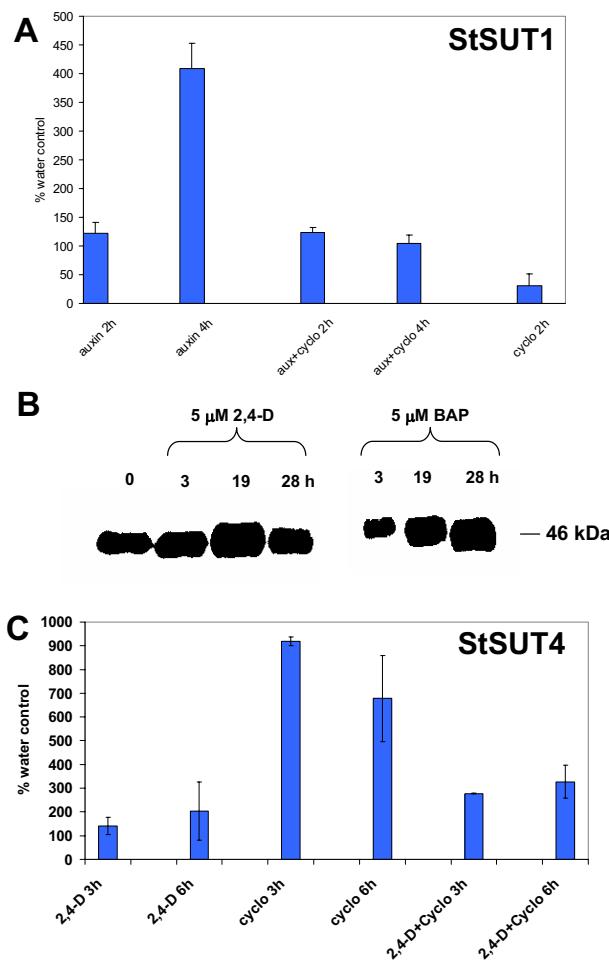


Fig. (5). Expression of the sucrose transporter *StSUT1* is induced by auxin ($5 \mu\text{M}$ 2,4-D) both at the mRNA and the protein level. **A.** Real time-PCR quantification of *StSUT1* upon 2 and 4 h treatment with $5 \mu\text{M}$ 2,4-D and in the presence of $5 \mu\text{M}$ 2,4-D and $10 \mu\text{M}$ CHX or $10 \mu\text{M}$ 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 $5 \mu\text{M}$ 2,4-D or $5 \mu\text{M}$ BAP and $15 \mu\text{g}$ 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 by 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.

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 translocated 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.

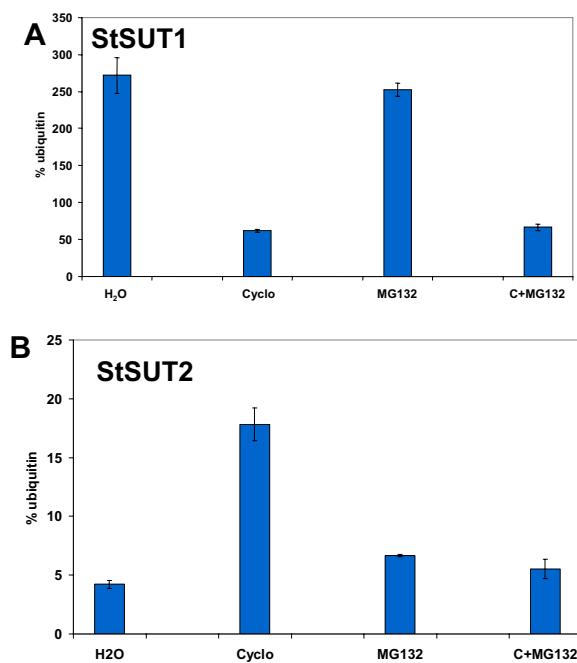


Fig. (6). Effects of the 26S proteasome inhibitor MG132 on sucrose transporter mRNA half life. **A.** 4 h CHX treatment affects *StSUT1* transcript stability. This effect is not decreased in the presence of the 26S proteasome inhibitor MG132. **B.** *StSUT2* mRNA half life increase upon 2 h of CHX treatment. This stabilizing effect is abolished by the 26S proteasome inhibitor MG132. 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

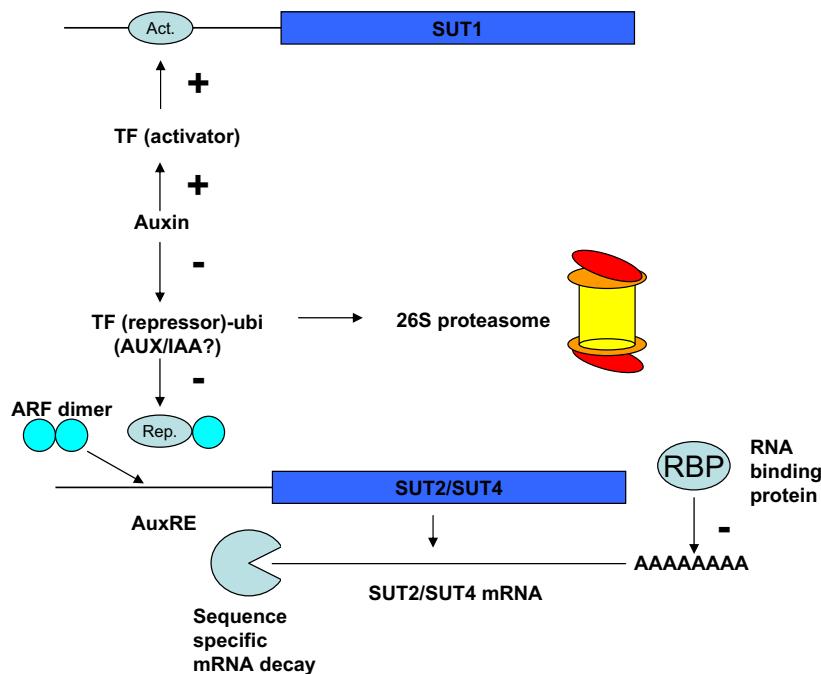


Fig. (7). Hypothetical model for the transcriptional control via auxin and post-transcriptional mRNA decay of *SUT2* and *SUT4* by short-lived mRNA-binding proteins which are degraded via the 26S proteasome pathway. TF: transcription factor; Ubi: ubiquitin; ARF: auxin response factor; AuxRE : auxin responsive element, RBP: RNA binding protein.

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 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 *StSUT1* 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, *StSUT1*, *NtSUT1* 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.

ACKNOWLEDGEMENTS

This work was supported by grants from the DFG to C.K. and A.H. (SFB 429) and to H.H. (SPP1108). We gratefully acknowledge Angelika Pötter for the care of greenhouse plants.

ABBREVIATIONS

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

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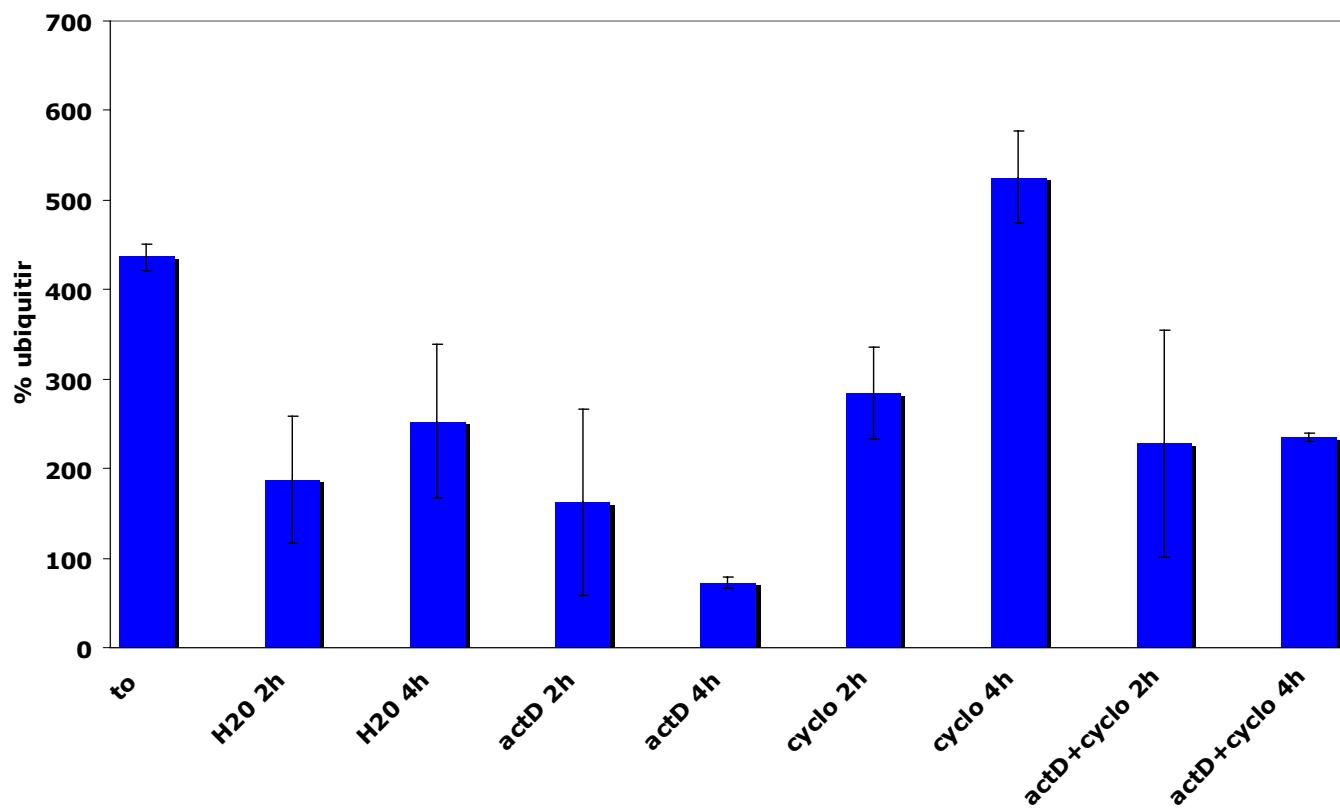
Received: December 23, 2007

Revised: February 21, 2008

Accepted: March 20, 2008

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Supplement 1



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.

Supplement 2

Sequence alignment of StSUT1, StSUT2, and StSUT4 across 1040 positions. The alignment shows highly conserved regions with some variations, particularly around positions 410-420 and 570-590 where StSUT1 contains a red-highlighted ATTAA sequence.

Position	StSUT1.seq	StSUT2.seq	StSUT4.seq
1	StSUT1.sec AAAATGGACATGGTACAAAAGACAAGGTTAACGAACTTCGACTTCTCTCTCAAGTGAACAGCGTTAG	StSUT2.sec ATGGATCCGGTAT---CGTTCAGACTACGGUATAAGAAUCTCA-ACGACCA-	StSUT4.sec aca---TAGAACAGCAGGCGAACCGACCGCGAATTGAGAACCGT-G-
90	StSUT1.sec CACCACTCAAACCATATGGAAATTATTAGTTGTAGCTTCCATGGTGTGTCCTCAATTGGTGGGCTCTTCACGCTCT	StSUT2.sec GTTGATGAA-TCAAGGTTTCACAGTTGGAGGCCG-----TAGCTCTTCCTGATTCCTAGGGCTCTPAGGAA	StSUT4.sec TACCAATGAGACTAATTCG-----TTCGA-GTAGCTTCCGGTGAATTCAATTICGGTTGGGTTACACATATCA
170	StSUT1.seq TTGCTTACACCTTATGTCATATTGCTGGAAATTCTCTATGATTTGGTTGTTGACGTTGAGGCGGTTTC	StSUT2.seq ATCAAAGATTCCTCAACAC-----CTC-----TTCCCTCTCGCCTTAAGCACACATTGCT-----AACCTTGATTCCTAG	StSUT4.seq CTGCTACACCTTATGTCGAACGACTGGAAATTGAGAACCGCATGTTGGGACCCATATGGCTCTGTGGACGGTTTC
250	StSUT1.seq TAGATGTTCAGCCAGTGTGGCGACTACAGTGAATTGGTCCCTCCCGGTTCCGGTGCCTGCCGCCATTCAATTGGCG	StSUT2.seq TTGACACCGTC--GGTGGCCGTGTTGCACTTTGGATGGCTTGGCAACTATCTTCCTTA-CACCTTATAATTCA-GGAA	StSUT4.seq TTACAGGGTCAAGCCTTATGTCAGGTTAGTGGGACACGUGCAACAGTCGGTTCCGGTGCCTGCCGCCATTCAATTGGCG
330	StSUT1.seq CCGGAGCCGATCTGTTATGATTGGCGTTTCTCTATCGGTTGGCGGCCGCTTGGTCACTCCGGTACAGCTCCG	StSUT2.seq CTG-----GCATAGACCATCTCTCTCTT-TTATGCGCTTCTCCCTATTATA-CTGGCTTGTGCTACACCTT	StSUT4.seq CCGGAGCACTATGATCATGATTGGCGTTCTGGATTATCGGTTCTCCGGTGTGATTGGATGGCTTGGAG
410	StSUT1.seq CGAACGCCATTAAATTGAGCTAACAAAGCTCAATTCAAATAGGAAAGGCTCTCAATTGATTTGGCTCTCGTACAGCTTC	StSUT2.seq GTCTAGGTTATAAGGAGTCATAAAAGCTCAATTCAAATAGGAAAGGCTCTCAATTGATTTGGCTCTCGTACAGCTTC	StSUT4.seq GAGGUCAATTAAAGCTGCTATAGGGCGCTTGGCTAGGGTTTGGCTTCGATGTTGGCAAAATATTGCTCA
490	StSUT1.seq GGCCCCATCGAGAGGACTACTGCTGATCTCCCGGGGAATTCCGGAGG-----ATGAGAACAGCAAAATGGATTTTC	StSUT2.seq ATCTCTCAATTGCTGATTAATGCGTTTCCTCTCAGA-CATAGCAACCTTATTGGGGCAACAAAGGATTCG	StSUT4.seq AGGACCTTGCAGAGGCTCTGGTCTGCTGATCTCAACAAAGGAGCATAGAGA-----ACCCGGGTA-GCAAAATGGATTTTC
570	StSUT1.seq TCATTTCATGGCCGCGGAAACATTCTGGGTACGGCCCGGTTGATATTCTCACCTCTTAAAGTATTCCCTTCTC	StSUT2.seq ACCACTTCAAACTCA-----GGTCAACAGCA-GGCAATTCTGAGG-----GGTTTGATGCTCAATTGCTG	StSUT4.seq CCTTATTAAATTGAGCTAACAACTCTCTGGGTTTGGCTAGGGATCTACACTCTGGTTCAAGAGCTTCCCCTTAC
650	StSUT1.seq AAAPACCAGGCTGGACATGTACTGCGCAATTCTGAAGAGCTGTTTCTCATCGCTAT-ATTCTCTTCTCTCCT	StSUT2.seq TAATAACTCTGCAAGGG-----CCGGCTCGAG-----CCTTTGGAGAG-----ATTTCAGCAGGTCTCTGATCA	StSUT4.seq TCTCAACTACTGGATGCAACATCAACTGTCGAATCTRAAGGCCTGCTTTATTATCGAGATTATTATTGCAACACTA
730	StSUT1.seq AACAAACCTTACCCATTACCTTGTGCGGAAACAGGCTCCCGAGAGAACAGACGACAAAGAAATGGACGAGAAATTG	StSUT2.seq AACAAATCTGCAAGGAG-----AGGCTCTGGTGTGAGAACATTTCTGGAATTTCCTGCA	StSUT4.seq ATGCAATTGCTATACCCATTGTCAGGACGCTCTAGATCCCAAGTCTGTTCTCTCATACCAAGAGAGAATTG
810	StSUT1.seq CGCGCCGGAA-AATGCA-AAGGACCTTTTTCGGTAAATTGGGCTTGGGTTGAAACAAATTCTGACAGCTGTGGAT	StSUT2.seq CAGTCAGGTGCGCAAGAGCTGG-----TTTCTGCAAA-----AGAGCTCTGGTGTGACCTGTTGAA	StSUT4.seq GATCAACCCATTGTCAGAAGGACGCTTCTGCGGAGTCTTGGGTTTTCAGGATCTCCCAAGGCTTGTGCT
890	StSUT1.seq TCTTCATGAGTAACTGTTGACCTGGATCCCTGGTTTCCGTTTCTCTCTGATGATACTGAGATGGAGGGCTAAGG	StSUT2.seq ATCTCAAACTCTCTGACTGCTGCTCTGAGAACCTCTGAGCTACTCTGACTGCTGCTGCTGCTGCTGCTG	StSUT4.seq GATCCCTGAGCTGCACTGCGCTGAGCAATTGGATTGATGGTTCTCTCATACCAAGAGAGAATTG
970	StSUT1.seq TTTCCGGGACAACTGGTGTGATGGCGACCTTCTACGAAATTGGTGTGAGACCCGCTGGTCAATTGGGATTAACGGTCAATCT	StSUT2.seq CCC---ACTGTCACCAAGCAATTAACGCTCTT-----CAGAT-CTCTGCT-----CTGACTCTCTACTCTGCAAAAGAAC	StSUT4.seq GATCCCTGAGCTGCACTGCGCTGAGCAATTGGATTGATGGTTCTCTCATACCAAGAGAGAATTG
990	StSUT1.seq TTTCCGGGACAACTGGTGTGATGGCGACCTTCTACGAAATTGGTGTGAGACCCGCTGGTCAATTGGGATTAACGGTCAATCT	StSUT2.seq CCC---ACTGTCACCAAGCAATTAACGCTCTT-----CAGAT-CTCTGCT-----CTGACTCTCTACTCTGCAAAAGAAC	StSUT4.seq GATCCCTGAGCTGCACTGCGCTGAGCAATTGGATTGATGGTTCTCTCATACCAAGAGAGAATTG
995	StSUT1.seq TTTCCGGGACAACTGGTGTGATGGCGACCTTCTACGAAATTGGTGTGAGACCCGCTGGTCAATTGGGATTAACGGTCAATCT	StSUT2.seq CCC---ACTGTCACCAAGCAATTAACGCTCTT-----CAGAT-CTCTGCT-----CTGACTCTCTACTCTGCAAAAGAAC	StSUT4.seq GATCCCTGAGCTGCACTGCGCTGAGCAATTGGATTGATGGTTCTCTCATACCAAGAGAGAATTG

