# Introns control expression of sucrose transporter LeSUT1 in trichomes, companion cells and in guard cells

Andreas Weise · Sylvie Lalonde · Christina Kühn · Wolf B. Frommer · John M. Ward

**Abstract** In solanaceous plants such as tomato and tobacco, the sucrose transporter SUT1 is crucial for phloem loading. Using GUS as a reporter, the promoter and other regulatory *cis* elements required for the tomato *LeSUT1* expression were analyzed by heterologous expression of translational chimeric constructs in tobacco. Although *LeSUT1* is highly expressed at the RNA level, GUS expression under the control of a 1.8 kb *LeSUT1* promoter resulted in few plants expressing GUS. In GUS-positive transformants, expression levels were low and limited to leaf phloem. Increasing or decreasing the length of *LeSUT1* promoter did not lead to a significant increase in positive transformants or higher expression levels. Translational fusion of GUS to the LeSUT1 C-terminus in a construct containing all exons and introns and the 3′-UTR led to a higher number of positive transformants and many plants with high GUS activity. *LeSUT1* expression was detected in ab- and adaxial phloem companion cells, trichomes and guard cells. The role of individual introns in *LeSUT1* expression was further analyzed by placing each *LeSUT1* intron into the 5′-UTR within the 2.3 kb LeSUT1 promoter construct. Results showed remarkable functions for the three introns for *SUT1* expression in trichomes, guard cells and phloem cells. Intron 3 is responsible for expression in trichomes, whereas intron 2 is necessary for expression in companion cells and guard cells. The combination of all introns is required for the full expression pattern in phloem, guard cells and trichomes.

**Keywords** Companion cell · Intron · Guard cell · Regulation of expression · Sucrose transporter · Trichome

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## Introduction

The phloem is the vascular tissue responsible for transport of photoassimilate from autotrophic (source) leaves to heterotrophic tissues (sinks). The sucrose transporter SUT1 in solanaceous plants (and orthologs in other plants) is required to load sucrose into the phloem in source leaves (Riesmeier et al. 1994; Bürkle et al. 1998; Kühn et al. 1996). RNA in situ hybridization studies showed localization of *SUT1* mRNA in minor veins (Riesmeier et al. 1993). In addition to the cell-specific expression pattern, the control of *SUT1* expression by light, hormones, and developmental cues makes *SUT1* an interesting model for studying transcriptional regulation. For example, the expression of *StSUT1* from potato and *AtSUC2* from Arabidopsis both follow the sink-to-source transition (Riesmeier et al. 1993; Truernit and Sauer 1995). The hormone auxin increases the rate of phloem loading (Lepp and Peel 1970) and induces an increase in *SUT1* mRNA levels (Harms et al. 1994). *StSUT1* expression is induced

by light at both mRNA and protein levels (Kühn et al. 1997) and expression of *ZmSUT1* in maize is also light dependent (Aoki et al. 1999). Here we address the function of individual introns in controlling expression of tomato sucrose transporter *LeSUT1*. Promoter-GUS analysis of an isolated *LeSUT1* promoter from tomato (Kühn et al. 2003) showed weak expression by comparison to blots that showed very high levels of expression. This indicated that in contrast to the AtSUC2 promoter (Truernit and Sauer 1995; Sivitz et al. 2007), the tomato SUT1 upstream sequences used for the promoter GUS fusions did not contain all necessary information for full expression.

Introns can affect both expression pattern and the level of gene expression. For example, removal of the large leader intron from sucrose synthase *Sus3* and *Sus4* genes of potato resulted in altered expression patterns and levels (Fu et al. 1995a; 1995b). For the MADS box gene *AGAMOUS* from *Arabidopsis*, spatial and temporal gene expression was demonstrated to be dependent on intragenic sequences containing the target sites for regulatory trans-factors known to be important in controlling *AGAMOUS* expression (Sieburth and Meyerowitz 1997). However, in this case the authors did not determine if the observed expression effects were due to introns, exons, or both. Exon sequences can also have an influence on gene expression. For example, the first intron of the maize *shrunken1* gene enhances transgene expression 100-fold, but if its native upstream exon was included in a chimeric construct gene expression was increased 1,000-fold (Maas et al. 1991). The mechanisms by which introns mediate enhanced or regulated gene expression are largely unknown. For intron-mediated enhancement (IME) of gene expression post-transcriptional regulation is generally postulated (Rose and Last 1997; Simpson and Filipowicz 1996; Jeong et al. 2007). It was shown that splicing per se is not necessarily required for intron-mediated RNA accumulation (Rose and Beliakoff 2000). The expression of Arabidopsis sucrose transporters *AtSUC9* and *AtSUC1* were recently shown to be dependent on intragenic sequences (Sivitz et al. 2007). In the case of *AtSUC1*, promoter GUS analysis showed expression in pollen and trichomes while whole-gene GUS fusions showed additional sucrose-inducible expression in roots (Sivitz et al. 2008). However, the contributions of individual introns or exons in controlling expression of *AtSUC1* and *AtSUC9* were not determined.

The stability of mRNAs is in general determined by the $m^7$Gppp cap at the 5′-end and the poly (A) tail at the 3′-end of mRNAs. The 3′-UTRs of genes also can have strong influence on gene expression by influencing the mRNA stability. The 3′-UTR can regulate gene expression for example by determining mRNA instability for transcripts with very short half-lives. This was shown for the S̲mall A̲u̲p R̲NA *SAUR-AC1* gene from *Arabidopsis* (Gil

and Green 1996). The reverse situation, that the 3′-UTR can enhance gene expression by stabilizing the mRNA, has also been suggested for plant genes, for example the *Sus4* gene from potato (Fu et al. 1995a) or the small subunit *rbcS* gene from *Arabidopsis* (Ingelbrecht et al. 1989).

Promoter-GUS analysis of an isolated *LeSUT1* promoter from tomato (Kühn et al. 2003) showed weak expression by comparison to RNA blots that showed very high levels of expression. To determine whether intragenic sequences contribute to LeSUT1 expression, we isolated the genomic sequence of the sucrose transporter gene *LeSUT1* from tomato. By generating different constructs with the GUS reporter, we found that the promoter region of *LeSUT1* alone is insufficient for proper *SUT1* expression and that regulatory *cis* elements in introns are involved in the expression of *SUT1*.

## Methods

### Isolation of the *LeSUT1* promoter region

Genomic clone of *LeSUT1* was isolated by screening a genomic library from *Lycopersicon esculentum* cv. VFN8 constructed in the EMBL-3 vector (Clontech, Palo Alto, USA) (Kühn et al. 2003). To isolate an extended promoter region of *LeSUT1*, a DIG-labelled probe (Roche Diagnostic GmbH, Mannheim, Germany) containing the 5′-end of the *LeSUT1* promoter (Kühn et al. 2003) was hybridized with DNA extracted from phage lysates and digested with different restriction enzymes. A positive 0.8 kb *Eco*RI fragment was subsequently cloned into pBlueskript SK$^+$ (Stratagene, La Jolla, USA). Sequencing of the fragment verified that it represents an extended promoter region of *LeSUT1*.

### Generation of promoter GUS constructs

#### SUT1 promoter-GUS series

For the generation of a translational promoter-GUS fusion, a 2.1 kb *Bam*HI genomic *LeSUT1* fragment was used. By PCR, a *Sma*I site was introduced in frame at the 3′-end of the genomic *LeSUT1* fragment. The resulting *Bam*HI-*Sma*I fragment (1,672 bp) was digested and ligated in the same sites of the plant binary vector pBI101.3 (Jefferson 1987). The reading frame of this translational fusion, 1.7 kb promoter-GUS, was confirmed by sequencing. The translation fusion corresponds to the 5 amino acids of *LeSUT1*, 7 amino acids encoded by the polylinker of pBI101.3 and followed by GUS (Fig. 1a).

For the generation of the 1.5 kb promoter-GUS construct, the 1.7 kb promoter-GUS construct was digested
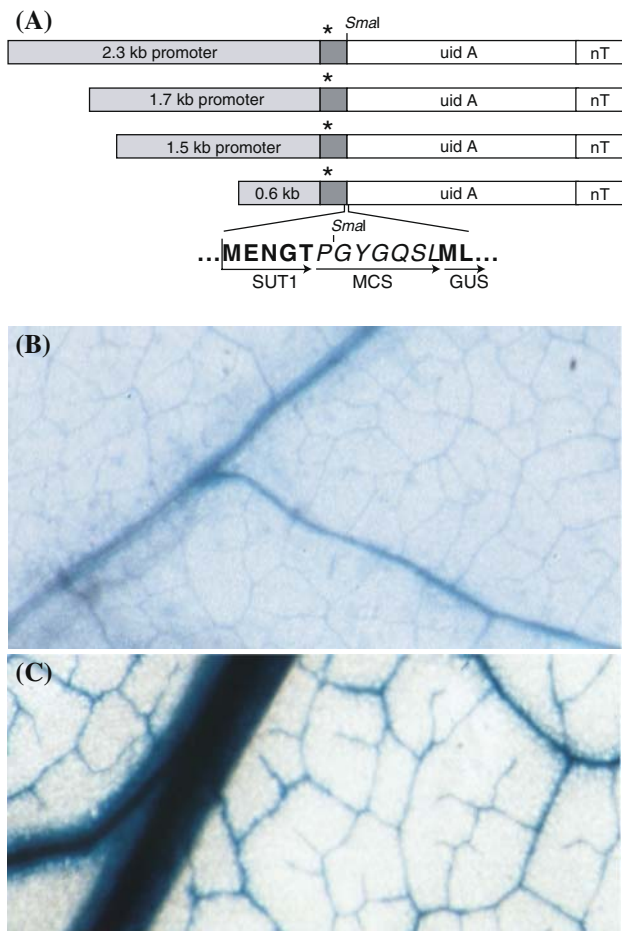
**Fig. 1** LeSUT1 promoter deletion constructs. (**a**) 0.6 kb, 1.5 kb, 1.7 kb and 2.3 kb promoter fragments were translationally fused to the GUS gene (uidA) within the plant binary vector pBI101.3 (Jefferson 1987). nT, nopaline synthase terminator; star indicate the position of the start codon. (**b**) Representative GUS staining pattern of 2.3P-*GUS* expression in tobacco plants which was observed for all four different promoter constructs, (**c**) Strong staining pattern observed in very few plants in the different promoter-*GUS* lines

*Xba*I and religated. For the 0.6 kb promoter-GUS construct, the 1.7 kb promoter-GUS construct was digested with *Hind*III and religated. For the 2.3 kb promoter-GUS construct the 0.8 kb *Eco*RI fragment of the extended 5′-region of the *LeSUT1* promoter was cloned into the *Eco*RI site in pBlueskript SK+. This fragment cloned into the *Sal*I/*Xba*I digested 1.7 kb promoter-*GUS* construct.

### 2.3 kb Promoter-SUT1-GUS construct

A genomic 7.1 kb *Eco*RI fragment containing 1.5 kb of the promoter region, the whole genomic sequence (4 exons and 3 introns) of the *SUT1* gene and further upstream sequence was cloned into pBlueskript SK+. Because of a lacking suitable restriction site around the stop codon of *SUT1*, a 1.4 kb fragment was amplified by PCR with primers containing restriction sites. This PCR product was cut with *Bcl*I/*Sma*I and cloned into the *Bcl*I/*Sma*I digested 7.1 kb *Eco*RI fragment in pBlueskript SK+. To this clone, the 0.8 kb *Eco*RI 5′-end was ligated into the *Eco*RI site. The final 6.0 kb fragment containing 2.3 kb of the promoter region and the genomic sequence ending 6 amino acids before the stop codon of *LeSUT1* was then digested *Sal*I/*Sma*I and cloned into the plant binary vector pBI101.3. The maintenance of the reading frame was confirmed by sequencing.

### 2.3 kb Promoter-SUT1-GUS-3′-UTR construct

The 3′-UTR of *LeSUT1* was isolated by PCR with restriction sites from genomic DNA. The 1.2 kb product was *Eag*I/*Sal*I digested and cloned into pBlueskript SK+ cut with *Eag*I/*Sal*I. The fragment was then cut out with *Eag*I and *Eco*RV. After filling *Eag*I end with Klenow, this blunt fragment was then cloned into the *Sst*I site between the *uidA* gene and the nos terminator of pBI101.3. The 6.0 kb fragment, containing 2.3 kb of the promoter region and the genomic sequence ending 6 amino acids before the stop codon of *LeSUT1* was then *Sal*I/*Sma*I digested and cloned into this 3′-UTR containing pBI101.3 cut with *Sal*I/*Sma*I.

### 2.3 kb Promoter intron-1/2/3-GUS-3′-UTR constructs

For the insertion of introns into the 5′-UTR of *LeSUT1*, an *Xho*I restriction site was introduced. By PCR a 650 bp fragment of the 3′-end of the 2.3 kb promoter was amplified using a reverse primer containing *Sma*I site and further upstream an *Xho*I site (leading to an exchange of 3 base-pairs) and a forward primer containing an endogenous *Bsp*1407I site which cuts twice in the *GUS* gene. The PCR product was digested with *Bsp*1407I/*Sma*I and used in a triple ligation step together with the excised 5′*Sal*I/*Bsp*1407I fragment of the 2.3 kb promoter fragment and the *Sal*I/*Sma*I cut pBI101.3 vector containing the 3′-UTR of *LeSUT1*. All three introns were PCR amplified using the primers containing *Xho*I sites at both ends. The introns were then cloned into the *Xho*I site in the modified 5′-UTR of the 2.3PXhoI-*GUS*-3′-UTR construct leading to I-1 (intron 1), I-2 (intron 2) and I-3 (intron 3) constructs. The correct orientation of the introns was confirmed by sequencing. As a control, a construct was also used (C-1) that contains the *Xho*I site in the 5′-UTR but lacks introns.

### Plant transformations

Plants were transformed by *Agrobacterium*-mediated gene transfer using *Agrobacterium tumefaciens* strain GV2260 (Deblaere et al. 1985). Transformation of *Nicotiana*

*tabacum* was performed as described by Rosahl et al. (1987). Transgenic plants were regenerated on kanamycin containing media and multiplied in vitro. All plants rooting on kanamycin-containing medium were tested in 3-way PCR using specific primers for *nptII* (plant kanamycin resistance marker), *nptIII* (bacterial kanamycin resistance marker) and NtSUT1 as a positive control.

### Histochemical localization of GUS activity

For the localization of the GUS activity transgenic plants or parts of transgenic plants were infiltrated with 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) in 50 mM sodium phosphate buffer pH 7.2 containing 0.5% Triton X-100 and incubated overnight at 37°C (15, 23). In some cases 1 mM potassium ferri-/ferrocyanide was added to the infiltration buffer. After incubation, plant material was cleared with 70% ethanol and photographically documented. For the localization of GUS on the cellular level, plant material was first incubated in X-Gluc solution as described above. After staining, plant material was fixed overnight under a slight vacuum in 4% (v/v) glutaraldehyde. After fixation, leaves were cut into small pieces (~4 mm$^2$) and dehydrated in ascending ethanol concentration and then gradually infiltrated in LR White. Polymerization of LRwhite was performed for 20 h at 58°C. Four μm thin sections were prepared using a microtome.

### Determination of GUS activity

Quantitative GUS assays were performed as described by Jefferson et al. (1987). Transgenic plants were transferred from sterile-culture to the greenhouse and grown for 10 weeks. The 5th expanded source leaf from the top of each plant was cut and frozen in liquid nitrogen. Leaf material was ground in a mortar to a fine powder to achieve an equal distribution of the vascular and mesophyll tissue. 200–300 mg samples was then used in the GUS assay as described by Gallagher (1992). Fluorescence was measured in a Kontron SFM 25 spectrofluorometer. The linearity of the reaction was shown by measuring GUS activity of two plants, showing strong GUS activity in the histochemical analysis, after 0, 30 and 60 min. Wild type plants showed a negligible activity between 0 and 1 nmol MU/mg protein/min. Protein concentrations were determined by the method of Bradford (1976) using BSA as standard.

### Immunolocalization

Immunodetection with StSUT1 (Lemoine et al. 1996) antisera was performed as described previously (Kühn et al. 1997). Plant material was fixed in ethanol: acetic acid (3:1) for 1 h at 4°C. After three washes with 70% EtOH, fragments were dehydrated in an ethanol series. Following overnight incubation in 1:1 ethanol methacrylate (75% [v/v] butylmethacrylate, 25% [v/v] methylmethacrylate, 0.5% benzoine ethylether, 10 mM DTT) material was embedded in 100% methacrylate. Polymerization took place overnight under UV (365 nm) at 4°C. Semithin sections (1 μm) of embedded material were cut with a diamond knife (Diatome), and were mounted on adhesion slides (Starfrost) and dried at 60°C. For removal of methacrylate, slides were incubated for 30 s in acetone and blocked for 30 min with 2% BSA in PBS (100 mM sodium phosphate, pH 7.5, 100 mM NaCl). After overnight incubation in a humid chamber at 4°C with affinity purified antibody or preimmunsera at same protein concentration (as determined by Bradford), slides were washed twice in PBS-T (PBS with 0.1% Tween) and once in PBS, followed by 1 h incubation with anti-rabbit IgG-FITC conjugate. After three washes with PBS-T and PBS, Cityfluor (Plano) was applied to prevent bleaching. Antibody binding was analyzed with a CLSM TCS-SP2 (Leica, Bensheim).

## Results

### Expression of *LeSUT1* promoter GUS deletion constructs in *Nicotiana tabacum*

The GUS activity detected in transgenic lines transformed with the 1.7 kb *LeSUT1* promoter construct did not correspond to the high level of SUT1 observed in source tissues by RNA blot analysis in tobacco, potato or tomato (Kühn et al. 2003). To test whether different promoter lengths would affect expression levels, four promoter-*GUS* constructs were designed and tested in stably transformed tobacco (Fig. 1a). A series of constructs with promoter lengths of 0.6, 1.5, 1.7 and 2.3 kb were produced as translational GUS fusions in pBI101.3 (Fig. 1a).

In the case of the 2.3 kb, 1.7 kb and 0.6 kb promoter constructs, between 40 and 50% of the plants showed GUS staining (Table 1). Only 16% of the lines transformed with the 1.5 kb promoter construct showed GUS activity. The average intensity of staining was very low for all constructs even after 48 h of incubation in X-Gluc solution. In all four constructs the weak staining was mostly restricted to major veins. Figure 1b shows a representative example. In lines from all constructs a few plants showed strong expression in all veins: three lines for the 2.3 kb promoter construct, one each for the 1.7 kb and 1.5 kb promoter constructs, and three lines for the 0.6 kb construct (Fig. 1c). T-DNA insertion into the tobacco genome and the presence of the

**Table 1** Number of transgenic tobacco plants analyzed for each *LeSUT1* reporter construct

| Construct | Transgenic plants | Plants showing GUS staining | Plants showing GUS staining (%) |
|---|---|---|---|
| 0.6 kb Promoter | 36 | 19 | 50 |
| 1.5 kb Promoter | 31 | 5 | 16 |
| 1.7 kb Promoter | 36 | 14 | 39 |
| 2.3 kb Promoter | 41 | 16 | 39 |
| 2.3P-SUT1-GUS | 20 | 11 | 55 |
| 2.3P-SUT1-GUS-3′-UTR | 46 | 36 | 78 |
| I-1 (Intron 1) | 30 | 0 | 0 |
| I-2 (Intron 2) | 8 | 6 | 75 |
| I-3 (Intron 3) | 20 | 17 | 85 |
| C-1 (Control) | 24 | 7 | 30 |

*GUS* gene fused to the promoter was confirmed for all plants by PCR.

## Influence of intragenic sequences and the 3′-UTR of *LeSUT1* on the expression of GUS

The low number of transformants showing GUS staining (Table 1) as well as the low levels of expression indicated that the isolated promoter sequences were insufficient to provide the expected high level of expression (Riesmeier et al. 1993; Kühn et al. 2003). Therefore, two additional constructs were generated to test if regions downstream of the first ATG have a function in controlling *LeSUT1* gene expression (Fig. 2a). The *LeSUT1* gene contains four exons and three introns. In one construct (2.3P-*SUT1-GUS*) the longest promoter fragment together with the entire coding sequence of *LeSUT1* ending 6 amino acids before the stop codon was fused to the *GUS* gene (Fig. 2a). The second construct (2.3P-*SUT1-GUS*-3′-UTR) included, in addition, a 1.2 kb fragment containing the 3′-UTR of *LeSUT1* placed between the *GUS* gene and the nopaline synthase terminator (Fig. 2a).

For the 2.3P-*SUT1-GUS* construct, 20 transgenic plants were obtained of which 11 plants showed GUS expression. All positive lines showed a clearly stronger and more consistent blue staining than the plants transformed with any of the promoter deletion constructs without introns described above (Fig. 1). For the 2.3P-*SUT1-GUS*-3′-UTR construct 46 transgenic plants were obtained of which 36 (78%) plants showed GUS expression. The expression level judged from the intensity of blue staining in these lines, appeared to be the highest and expression was detectable in all leaf veins, trichomes and guard cells (Fig. 2b, c, d). The same expression pattern was observed for the construct without the 3′-UTR.
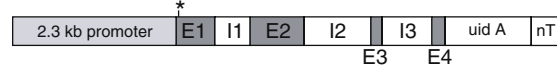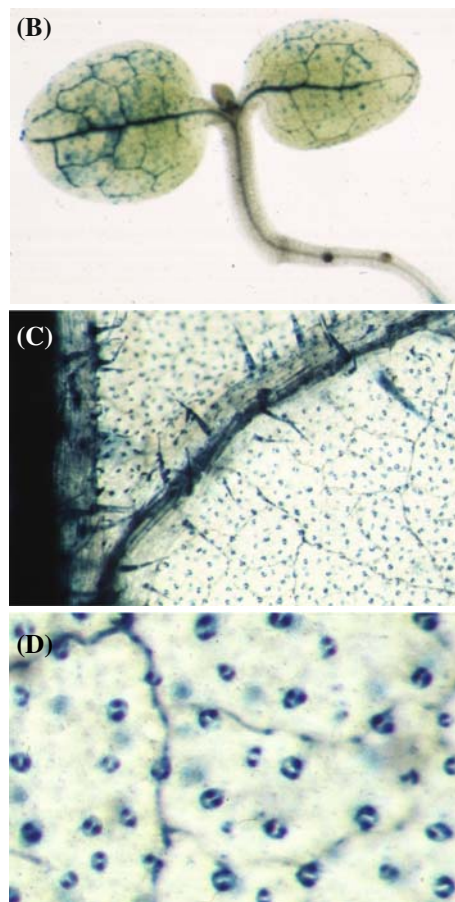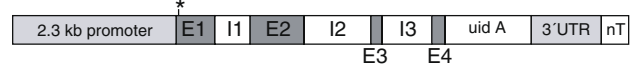
**(A)**

2.3P-SUT1-GUS

2.3P-SUT1-GUS-3'UTR

**Fig. 2** Structures of chimeric constructs 2.3P-*SUT1-GUS* and 2.3P-*SUT1-GUS*-3′-UTR in pBI101.3. The introns are marked I1-I3, the exons are marked E1-E4. The 3′ untranslated region is marked as 3′-UTR. (**b–d**) GUS pattern in lines transformed with 2.3-SUT1-GUS-3′-UTR. (**b**) 10 day-old seedlings. (**c**) Part of a source leaf showing the midvein, second-order vein and minor veins with surrounding leaf tissue. (**d**) Closeup of C showing guard cell expression

## Quantification of GUS activity

$\beta$-Glucuronidase activity in the transgenic lines was quantified using MUG (4-methyl umbelliferyl $\beta$-D-glucuronide) as a substrate (Jefferson 1987; Gallagher 1992). All transgenic tobacco lines with the 2.3P-*GUS*, the 2.3P-*SUT1-GUS* and the 2.3P-*SUT1-GUS*-3′-UTR constructs were transferred to soil and grown in a greenhouse. After 10–12 weeks of growth, fully expanded source leaves were cut from the plants approximately 1 cm below the leaf

base, frozen in liquid nitrogen and tested for $\beta$-glucuronidase activity. A few lines showed extremely high GUS activity, probably due to positional effects, and were considered not representative of the expression level due to the transgene (three 2.3P-GUS lines and four 2.3P-SUT1-GUS-3'-UTR lines).

Average $\beta$-glucuronidase activity was determined for all lines (Fig. 3a) and the values were normalized to percentage of plants showing enzyme activity in different activity intervals (Fig. 3b). The average $\beta$-glucuronidase activity for the 2.3P-GUS construct was 47.4 ± 13.4 pmol MU/mg protein/min ($n = 39$) (Fig. 3a). The activity of GUS in plants containing the 2.3P-SUT1-GUS construct was 97.0 ± 24.2 pmol MU/mg protein/min ($n = 18$) and significantly (t-test, $\alpha = 0.05$) higher than for the 2.3P-GUS construct. The highest mean activity was observed for the 2.3P-SUT1-GUS-3'-UTR construct: 115.5 ± 21.5 pmol MU/mg protein/min. ($n = 43$). The higher average activity of lines transformed with this construct compared with the

2.3P-SUT1-GUS construct was statistically not significant (t-test, $\alpha = 0.05$).

Normalizing $\beta$-glucuronidase activities into activity intervals (Fig. 3b) showed that the majority (73%) of the 2.3P-GUS lines had low enzyme activities (<32 pmol MU/mg protein/min) and only 16% of the lines were in the range of 100–316 pmol MU/mg protein/min. The distribution of the 2.3P-SUT1-GUS lines shows a greater percentage in a higher activity range (33% in the range of 100–316 pmol MU/mg protein/min). A higher percentage of 2.3P-SUT1-GUS-3'-UTR lines showed activities of 100–316 pmol MU/mg protein/min (42%). Both the means of $\beta$-glucuronidase activities and their distributions indicate that both intragenic and 3'-UTR sequences contribute to enhanced GUS activity.

## Histochemical analysis of heterologous expression of the 2.3P-SUT1-GUS-3'-UTR construct in *Nicotiana tabacum*

Of the 36 2.3P-SUT1-GUS-3'-UTR lines, 34 showed the same expression pattern with varying intensities. Eleven 2.3P-SUT1-GUS lines showed the same expression pattern as the 3'-UTR construct but with generally lower intensity. Therefore, the histochemical expression analysis presented here is restricted to the 2.3P-SUT1-GUS-3'-UTR tobacco lines.

The expression pattern in 2.3P-SUT1-GUS-3'-UTR lines was first analyzed in leaves of tobacco plants grown in sterile-culture. The staining was clearly different compared with plants expressing GUS under the control of only the 2.3 kb promoter fragment (Fig. 1b, c). GUS expression in 2.3P-SUT1-GUS-3'-UTR lines was observed in all leaf veins, in trichomes and in guard cells of leaves (Fig. 2b, c, d). The expression pattern in leaves of plants grown in the greenhouse was indistinguishable. Seeds were collected from the primary transformants and used to analyze GUS expression. No GUS activity was found in mature seeds while upon germination very strong GUS activity could be observed. GUS staining was first visible under the testa in the radicula and plumula (Fig. 4a) and increased in the developing root and hypocotyl (Fig. 4b, c). At later stages, GUS was expressed in the expanding cotyledons when they turned green as well as in the growing hypocotyl (Fig. 4d, e). The staining pattern found in roots was also detectable in older plants. In 10-day-old seedlings, GUS activity was observed in the veins of the green cotyledons, trichomes and guard cells, developing primary (sink) leaves, vascular tissue of the hypocotyl and of the roots in the root hair zone (Fig. 4f), in lateral root primordia (Fig. 4g), and in the root tip (Fig. 4h). In the pistil, GUS expression was restricted to the stigma (Fig. 4j). In the ovules and later in the developing fruits no GUS activity was found. GUS expression in
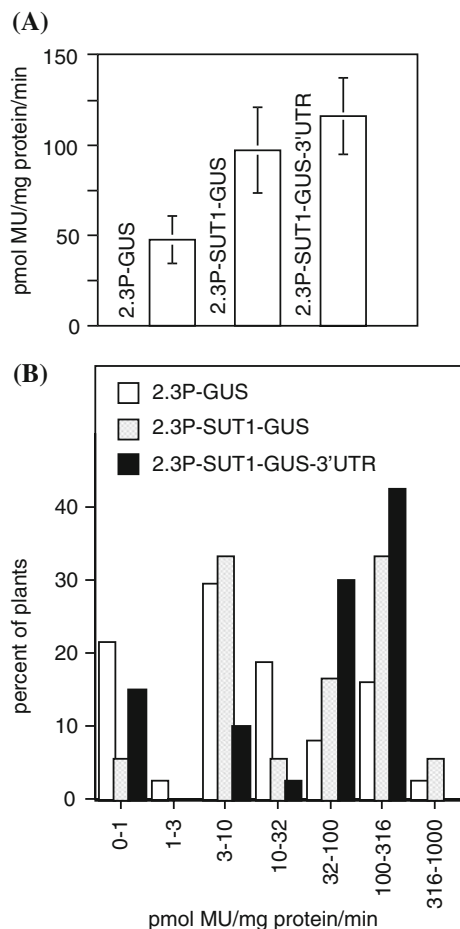


**Fig. 3** GUS activity in source leaves of 10–12 week-old tobacco plants containing different *GUS* fusion constructs. (**a**) Mean values of all transgenic plants for the different constructs, mean ± SE, (**b**) logarithmic histogram of GUS enzyme activities. The percentage of plant lines with GUS activity in the indicated ranges is shown
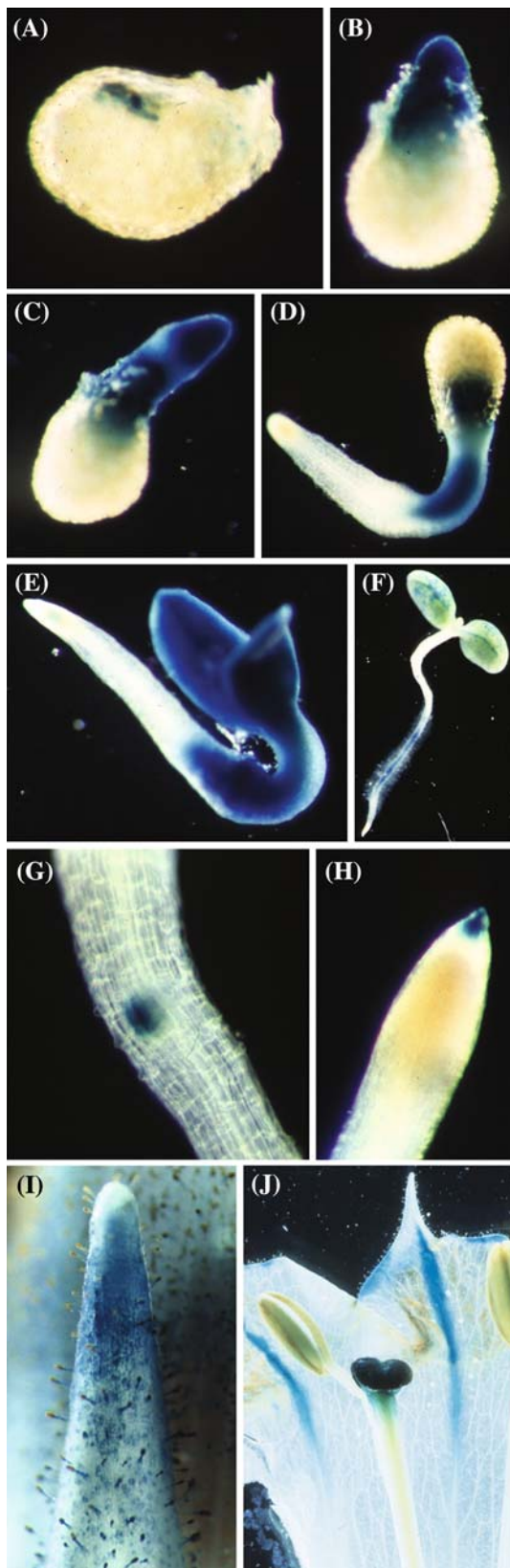
**Fig. 4** Expression of 2.3P-*SUT1-GUS*-3′-UTR in tobacco. (**a–e**) GUS activity at different developmental stages of seedlings; (**f**) GUS activity in a 10 day old tobacco seedling; (**g**) lateral root bud; (**h**) root tip; (**i**) sepal; (**j**) upper part of petal, pistil and anthers

flowers could be observed in trichomes, guard cells, and the cells surrounding guard cells of sepals and, in the fused petals, at the end of the major veins (Fig. 4i).

Guard cell expression of SUT1 in source leaves (Fig. 5a) was confirmed by immunolocalization. Potato leaf tissue was embedded, sectioned and probed using an antibody against StSUT1 as described by Kühn et al. (1997). Figure 5c and d show the immunolocalization of StSUT1 in guard cells in leaves. Figure 5b shows the bright field image corresponding to Fig. 5c. Preimmune controls (Fig. 5e) only showed background fluorescence at the same gain settings and magnification as used in Fig. 5d.

Companion cell localization for the LeSUT1-GUS fusion was previously demonstrated (Lalonde et al. 2003). Cross sections of tobacco stem were analyzed to determine which areas of the phloem showed GUS expression. GUS staining was detectable in the internal (adaxial) and stronger in the external (abaxial) phloem of mid veins (Fig. 6)

Role of the introns of *LeSUT1* in GUS expression

As shown in Fig. 2, sequences downstream of the translation start codon of *LeSUT1* had a strong influence on GUS expression. The intragenic sequences (exons/introns) also had an effect on the spatial expression pattern compared to the 2.3P-*GUS* construct in which only 5′-upstream promoter sequence was included. Therefore the role of the introns in controlling *LeSUT1* expression was further analyzed. The individual introns were amplified by PCR and, to allow a high efficiency of intron splicing (Klinz and Gallwitz 1985), they were inserted into the 5′-UTR and consensus splice sites were maintained (Fig. 7a, b). The 3′-UTR was included in the intron constructs between the *GUS* gene and the nopaline synthase terminator (Fig. 7a, b) to maintain the enhancing effect of the 3′-UTR.

The three intron constructs I-1, I-2 and I-3 as well as the control construct (C-1) lacking an intron but containing the 5′-UTR were introduced into tobacco plants. Thirty transgenic lines for the intron 1 construct (I-1), 8 for the intron 2 construct (I-2), 20 for intron 3 construct (I-3) and 24 for the control construct (C-1) were analyzed (Table 1). Seventeen I-3 lines (85%) showed GUS activity, of which 16 had GUS activity exclusively in trichomes (Table 1, Fig. 7g, h). Six I-2 lines (75%) showed GUS expression; GUS activity was only detectable in guard cells and veins (Fig. 5e, f). None of the 30 I-1 lines showed detectable GUS staining after overnight incubation in X-Gluc (Fig. 5c, d). As expected, seven tobacco lines (30%) transformed with the control construct showed weak GUS staining comparable to plants with the 2.3 P-*GUS* construct (data not shown).

**Fig. 5** Analysis of SUT1 expression in guard cells. (**a**) GUS staining in tobacco leaf of 2.3P-*SUT1-GUS*-3′-UTR transgenic line; (**b–e**) thin sections from potato leaf showing guard cells; (**b**) transmission image of section in C; (**c**) immunofluorescence image showing plasma membrane localization of StSUT1; (**d**) immunofluorescence image of potato guard cell (as in **c**); (**e**) preimmune serum control at same gain and magnification as **d**)
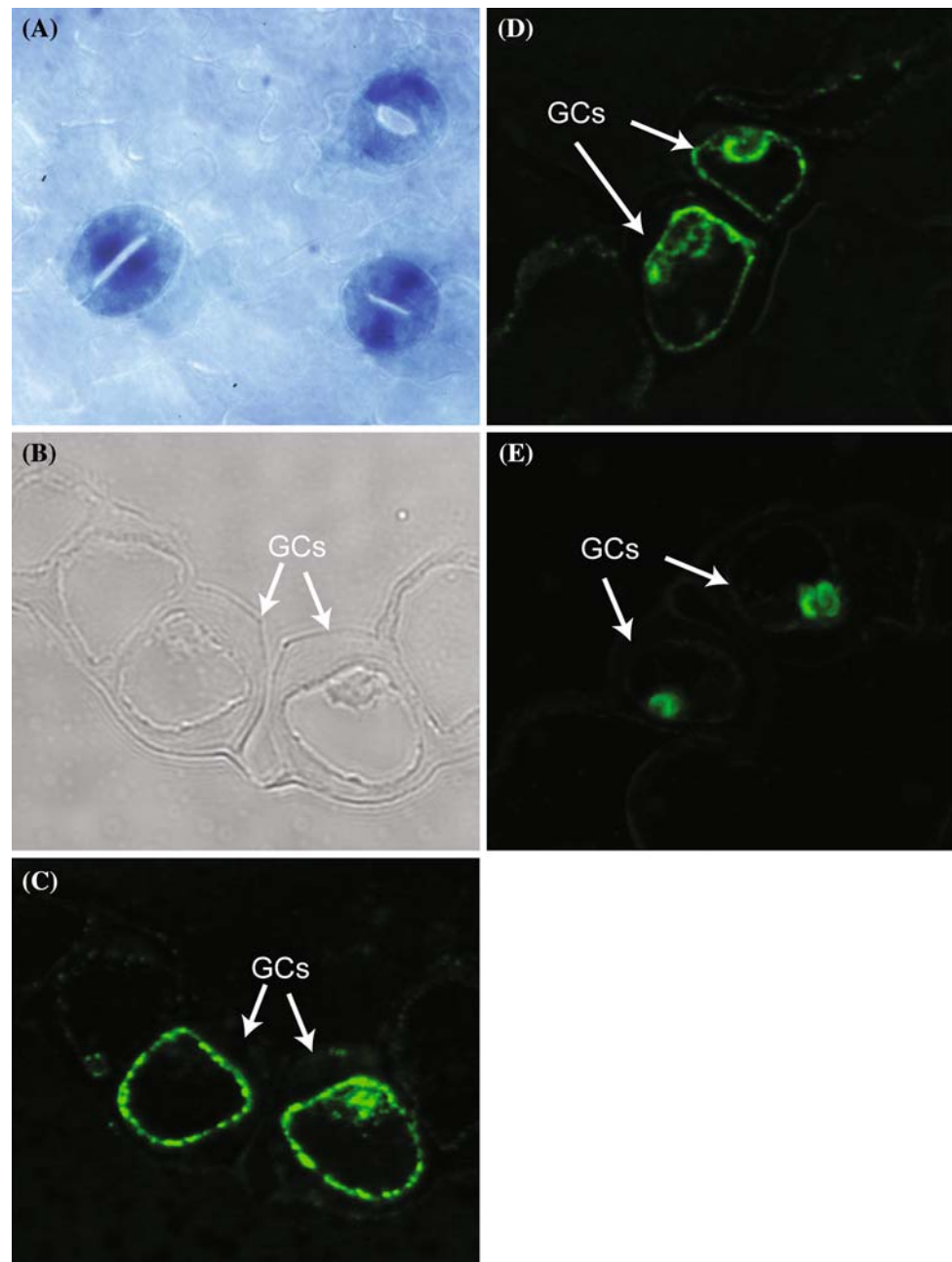
## Discussion

### SUT1 expression

RNA blot analysis had suggested that *SUT1* is strongly expressed in source leaves (Riesmeier et al. 1993; Bürkle et al. 1998). Therefore, it was surprising that only a low percentage of transgenic lines expressing *GUS* driven by *LeSUT1* promoters of different length showed detectable GUS activity (Table 1, Fig. 1). A small fraction of the promoter-*GUS* transgenic lines showed strong expression in veins (Fig. 1c), indicating that the promoter is sufficient

to direct vascular-specific expression when positioned in the vicinity of an enhancer element. A construct containing all intragenic sequences as well as the 3′-UTR (2.3P-*SUT1-GUS*-3′-UTR) should reflect the native expression pattern of the *SUT1* gene. Lines with this construct consistently showed high *GUS* expression within the vascular tissue. In addition, expression was also observed in trichomes and guard cells, a finding that had not been previously reported. Guard cell sucrose uptake activity and expression of sucrose transporter genes at the RNA level in guard cells have been previously reported (Ritte et al. 1999; Reddy and Rama Das 1986; Talbot and Zeiger 1996; Kang et al.
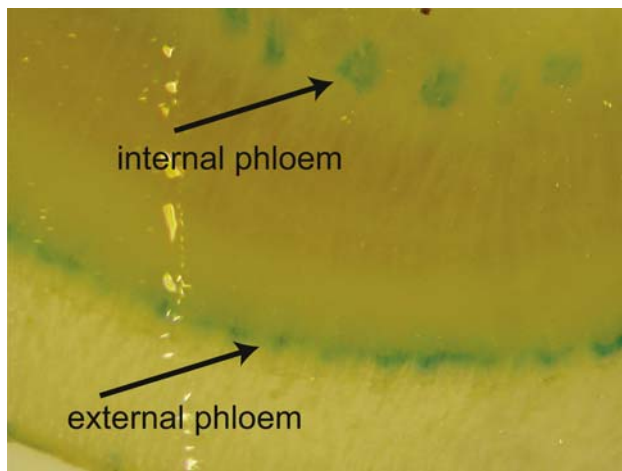
**Fig. 6** Analysis of vascular expression of 2.3P-*SUT1*-GUS-3′-UTR in tobacco. Cross section of tobacco stem showing GUS activity in adaxial (inner) and abaxial (outer) phloem

2007). This expression pattern was similar for constructs with and without the *LeSUT1* 3′-UTR. These results indicated that elements existed within *LeSUT1* introns and/or exons that control transcriptional activity within guard cells and trichomes and enhance expression within companion cells of the phloem.

Differential control by *LeSUT1* introns

To further identify intergenic elements controlling expression, each of the three *LeSUT1* introns was inserted separately into the 5′-UTR of the 2.3 kb promoter-*GUS* construct. The constructs were designed so that the introns could be spliced out, as in the native gene. Each of the introns was found to exert a unique effect on *GUS* expression. The construct containing the third intron (I-3) resulted in trichome-specific *GUS* expression. Interestingly, vascular expression was not observed for I-3 indicating that the third intron does not act as a general transcriptional enhancer but rather contained an element that enhances expression specifically in trichomes. It is not known if specific interaction with elements in the putative promoter region of *LeSUT1* was required for trichome expression driven by intron 3 or if this element functions autonomously. However, since trichome expression was not observed in any lines lacking intron 3, and in particular not in the few promoter-*GUS* lines with high expression presumably due to positional effects, it is likely that intron 3 is sufficient to allow trichome expression. Therefore, one component of the expression pattern observed for the 2.3P-*SUT1*-GUS-3′-UTR construct, trichome expression, can be uniquely assigned to activity of the third intron.

The construct containing the second intron (I-2) resulted in strong expression in vascular tissue and guard cells.
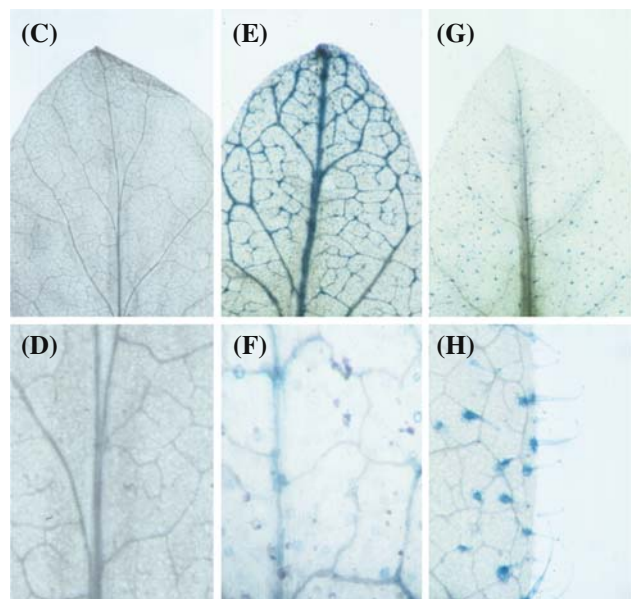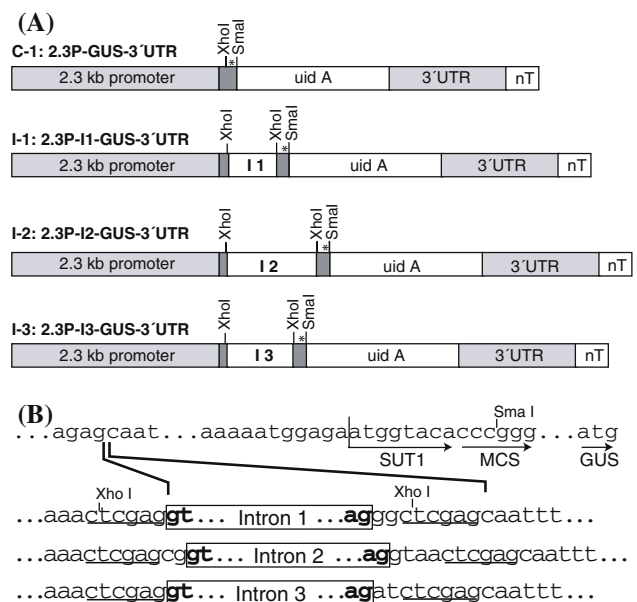


**Fig. 7** Chimeric promoter-intron-*GUS*-3′-UTR constructs used to analyze function of the *LeSUT1* introns. (**a**) Models of constructs: the 5′-UTR (hatched) was modified by the introduction of an *Xho*I restriction site for the insertion of the introns (labeled I1-I3). (**b**) Details of the intron insertion sites. Letters in bold indicate resulting modifications within the 5′-UTR of *LeSUT1* sequence remaining after intron splicing. (**c**, **d**) Representative GUS expression for I-1, (**e**, **f**) I-2 and (**g**, **h**) I-3 constructs in leaves of sterile-culture grown tobacco plants

Since guard cell expression was not observed in any lines not containing intron 2, elements within this intron are presumably sufficient to allow guard cell expression. Strong expression of GUS within the vascular tissue indicates that, in addition, intron 2 contains a general transcriptional enhancer. Based on the finding that a few

*LeSUT1* promoter-*GUS* transgenics showed strong vascular expression, it is likely that intron 2 functions to enhance expression in companion cells as directed by the *LeSUT1* promoter.

Surprisingly, none of the lines transformed with the intron 1 construct (I-1), which contains the *LeSUT1* promoter and the first intron, showed *GUS* expression. This indicates that the first intron acts as a repressor of the weak vascular expression exhibited by lines transformed with *LeSUT1* promoters-*GUS* constructs. To summarize the effects of the three introns on GUS expression, transcriptional repression by intron 1 and activation by intron 2 are likely to be a result of modulation of *LeSUT1* promoter activity. However, expression in guard cells and trichomes, mediated by intron 2 and 3, respectively, may be independent of the *LeSUT1* promoter. To determine whether intron 2 and intron 3 contain autonomous elements will require further investigation. It is also unknown whether splicing is important for the effects of the LeSUT1 introns on expression. It is known that splicing is not necessary for intron-mediated enhancement (IME), however, the results presented here for LeSUT1 show some distinct differences compared to IME (Rose et al. 2008).

IME is defined as an increase in mRNA levels due to introns and has been described for a variety of genes (Rose and Last 1997). The mechanism has not been determined but appears to be post transcriptional (Jeong et al. 2007) and is most commonly associated with promoter-proximal (first) introns (Rose et al. 2008). Enhanced vascular expression attributed here to the second intron in LeSUT1 may occur by IME. In addition, LeSUT1 introns functioned in expansion of expression to guard cells and trichomes, two cell types that did not show GUS expression from constructs that contained only promoter sequences. The strict dependence of guard cell and trichome expression on the intron sequences indicates that the introns use a mechanism other than IME. Evidence that intragenic sequences can regulate gene expression on the transcriptional level has been presented for the *AGAMOUS* gene, which requires intragenic sequences for correct developmental expression (Sieburth and Meyerowitz 1997. The second intron in AGAMOUS is a target for transcription factors Apetela 2 (AP2) and Leunig (LU) (Deyholos and Sieburth 2000).

### Putative function of *SUT1* in guard cells and trichomes

Guard cells are symplasmically isolated from their surrounding tissue as necessitated by their function in controlling stomatal aperture. Strong evidence for a potential role of sucrose for guard cell regulation was reported by Talbott and Zeiger (1996). They showed that the $K^+$ concentrations in guard cells increase in the first half of the day accompanied by an increased stomatal opening. During the second half of the day, $K^+$ concentration decreases but stomatal aperture further increased and was accompanied by a strong increase of sucrose concentration suggesting a direct osmotic function for sucrose. In this study, GUS expression data and immunolocalization indicate that *SUT1* is expressed in guard cells (Figs. 2 and 5), suggesting that sucrose is transported into guard cells by SUT1. In uptake measurements with isolated guard cell protoplasts of *Pisum sativum*, Ritte et al. (1999) observed a saturable sucrose uptake component with a $K_m$ varying up to 0.8 mM sucrose. SUT1 has a $K_m$ for sucrose of 1 mM when expressed in yeast and *Xenopus* oocytes (Boorer et al. 1996), which correlates to this component. A second non-saturable sucrose uptake component was observed in isolated guard cell protoplasts (Ritte et al. 1999), indicating the possibility of another sucrose transporter being involved in sucrose uptake into guard cells.

In solanaceous species, trichomes are symplasmically isolated from surrounding tissue (Derrick et al. 1992) and are non-photosynthetic. Therefore, trichomes are dependent on transmembrane transport for delivery of solutes and assimilates for the production of secondary metabolites, especially in gland trichomes. Our results indicate that SUT1 could function in sucrose uptake into trichomes.

It is interesting to compare the expression pattern of SUT1 in Solanaceous plants to AtSUC2 in Arabidopsis. Both are expressed in companion cells and are necessary for phloem loading of sucrose. AtSUC2 expression is limited to the phloem and AtSUC2 introns do not appear to contribute to expression (Sivitz et al. 2007). In contrast, as shown here, LeSUT1 introns contribute to expression and, in addition to vascular expression, SUT1 expression was identified in guard cells and trichomes. Interestingly, in Arabidopsis a different sucrose transporter (AtSUC1) is expressed in trichomes (Sivitz et al. 2007).

### Conclusions

Comparison of transgenics expressing *LeSUT1* promoter-GUS and whole gene-GUS indicated that *LeSUT1* is expressed in guard cells and trichomes in addition to companion cells. Guard cell expression of SUT1 (in potato) was confirmed by immunolocalization. This indicates that SUT1 in solanaceous plants could have a role in sucrose uptake into these symplasmically isolated cells. In addition, we identified the second intron as functioning as a general enhancer and specifically in producing guard cell expression. Intron three was identified as producing trichome expression. Interestingly, the first intron appears to function as a repressor. In conclusion, we have demonstrated that all three *LeSUT1* introns contribute to expression and each has

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a unique function. Whether these introns can function independently of the *LeSUT1* promoter remains to be determined.

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