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ORIGINAL ARTICLE

# The *sucrose synthase-1* promoter from *Citrus sinensis* directs expression of the *β-glucuronidase* reporter gene in phloem tissue and in response to wounding in transgenic plants

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**Abstract** Interest in phloem-specific promoters for the engineering of transgenic plants has been increasing in recent years. In this study we isolated two similar, but distinct, alleles of the *Citrus sinensis sucrose synthase-1* promoter (*CsSUS1p*) and inserted them upstream of the *β-glucuronidase* (*GUS*) gene to test their ability to drive expression in the phloem of transgenic *Arabidopsis thaliana* and *Nicotiana tabacum*. Although both promoter variants were capable of conferring localized *GUS* expression in the phloem, the *CsSUS1p-2* allele also generated a significant level of expression in non-target tissues. Unexpectedly, *GUS* expression was also instigated in a minority of *CsSUS1p::GUS* lines in response to wounding in the leaves of transgenic *Arabidopsis*. Deletion analysis of the *CsSUS1p* suggested that a fragment comprising nucleotides −410 to −268 relative to the translational start site contained elements required for phloem-specific expression while nucleotides −268 to −103 contained elements necessary for wound-specific expression. Interestingly, the main difference between the two *CsSUS1p* alleles was the presence of a 94-bp insertion in allele 2. Fusion of this indel to a minimal promoter and *GUS* reporter gene indicated that it contained stamen and carpel-specific enhancer elements. This finding of highly specific and separable regulatory units within the *CsSUS1p* suggests that this promoter may have a potential application in the generation of constructs for the use in the development of transgenic plants resistant to a wide variety of target pests.

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**Abbreviations**

| | |
|---|---|
| BA | Benzyl adenine |
| CaMV | Cauliflower mosaic virus |
| *CsSUS1p* | *SUS1* upstream region from *Citrus sinensis* |
| GUS | *β*-Glucuronidase |
| MS | Murashige and Skoog |
| MU | Methylumbelliferone |
| PCR | Polymerase chain reaction |
| 5′ RACE | 5′ Rapid amplification of cDNA ends |
| 5′ RAGE | 5′ Rapid amplification of genomic DNA ends |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SUS | Sucrose synthase |
| UDP | Uridine diphosphate |

## Introduction

The challenge of preventing damage induced by plant pathogens and insect pests has been escalating worldwide for some time (Moffat 2001). A variety of tactics have been developed to cope with this in recent years through the engineering of crops with improved resistance to particular pathogens and such approaches involve the expression of particular gene products that are either toxic to a given pest, augment the natural defenses of the plant, or impede virus replication within the host plant. To date, the expression of transgenes has generally been under the control of strong, constitutive promoters. However, the

high level of transgene expression provided throughout the plant by this type of promoter is often undesirable as it may impose an increased metabolic load on the plant. This could result in detrimental consequences to plant development, growth and crop yield, and has in some cases been found to cause unanticipated negative effects in non-target organisms, including humans (reviewed by Shelton et al. 2002). Consequently, there is a growing interest in promoters that drive foreign gene expression in a highly defined spatial-, temporal- and developmental-specific manner in transgenic plants. For example, several stamen- and/or carpel-specific promoters have been applied to the engineering of sterility via their fusion to cytotoxic genes for the containment of transgene flow in numerous plant species (Block et al. 1997; Luo et al. 2006; Roque et al. 2007; Liu and Liu 2008). Phloem-specific promoters are also becoming essential for use in a range of applications, such as conferring resistance to phloem-limited pathogens and phloem-feeding insects (for example Graham et al. 1997; Nagadhara et al. 2003; Saha et al. 2006; Sadeghi et al. 2007).

Currently, one of the most commonly used types of phloem-specific promoters in plant biotechnology are those derived from homologs of genes encoding sucrose synthase (SUS), such as *AtSUS1* from *Arabidopsis* (Martin et al. 1993), *Shrunken-1* (*Sh1*) from maize (Yang and Russell 1990) and *RSs1* from rice (Wang et al. 1992). In plants, the majority of organic carbon generated during photosynthesis is utilized for the synthesis of sucrose, which is the main form of assimilates transported from photosynthetic organs (source) to energy-consuming organs (sink) through the phloem. Before its utilization in sink tissues, the glycosidic bond of sucrose must initially be cleaved by either invertase, which hydrolyzes sucrose into fructose and glucose, or sucrose synthase, which catalyzes the reversible conversion of sucrose and uridine diphosphate (UDP) into fructose and UDP-glucose (reviewed by Sturm and Tang 1999). Sucrose synthase is ubiquitous in higher plants and carries out a variety of important functions, such as providing substrate for starch synthesis in storage organs (for example Sun et al. 1992; Wang et al. 1994) and cell wall biosynthesis (for example Ricard et al. 1991; Martin et al. 1993). In addition, it has been suggested to play a central role in phloem loading and unloading by providing substrate for respiration (Daie 1989; Martin et al. 1993; Nolte and Koch 1993) and in meeting increased glycolytic demands during anaerobic and cold stress (for example Maraña et al. 1990; Ricard et al. 1998; Zeng et al. 1998).

*SUS* genes typically comprise small gene families, with most plant species possessing at least two homologs that tend to exhibit distinct differences in their spatial and temporal expression patterns (for example Maraña et al. 1990; Fu and Park 1995; Sturm et al. 1999; Komatsu et al. 2002; Chiu et al. 2006; Bieniawska et al. 2007). Phylogenetic analyses have shown that these genes can be classified into at least four major branches, including a monocot group, as well the *SUSA* and *SUS1* dicot groups (Fu and Park 1995; Sturm et al. 1999; Komatsu et al. 2002; Baud et al. 2004). In *Citrus unshiu*, three sucrose synthase genes belonging to both the dicot *SUS1* (*CitSUS1* and *CitSUS2*) and *SUSA* (*CitSUSA*) groups have been identified (Komatsu et al. 2002). Although *CitSUS2* was expressed only very weakly in citrus plants, *CitSUS1* and *CitSUSA* appeared to exhibit differential expression in leaves, fruit development, and in response to sugars.

In an attempt to characterize the *SUS1* promoter from *C. sinensis*, which belongs to the same phylogenetic group as the phloem-specific *AtSUS1* gene from *Arabidopsis*, we cloned two distinct *CsSUS1* promoter (*CsSUS1p*) alleles and fused them to the *GUS* reporter gene. The resulting constructs were transformed into both *Arabidopsis* and tobacco, and GUS activity was analyzed to determine whether they were capable of driving phloem-limited expression. In addition, deletions of the *CsSUS1* promoters were carried out to identify regions containing putative *cis*-elements required for their proper function. We found that both promoter alleles were able to drive relatively high levels of expression in the phloem of both plant species tested and unexpectedly, a minority of lines exhibiting phloem-limited expression in the leaves also appeared to demonstrate expression in response to wounding. This highly specific pattern of phloem- and wound-restricted expression in the leaves of transgenic plants suggests that the *CsSUS1* promoter may be of potential use for directing the expression of particular proteins, such as lectins, or double-stranded RNA expression for the generation of RNA interference, as a measure to combat a variety of pests in genetically engineered crops.

## Materials and methods

### Cloning of *SUS1* 5′ upstream regions from *Arabidopsis* and *C. sinensis*

Primer sequences can be found in Table 1. Genomic DNA was extracted from *A. thaliana* col-0 and *C. sinensis* cv. valencia (Four Winds Growers, Winters, CA) using the E.Z.N.A. Plant DNA extraction kit according to the manufacturer's instructions (Omega Bio-Tek, Norcross, GA). To amplify the region upstream of the *SUS1* gene from *Arabidopsis*, which has been shown previously to drive phloem-specific expression (Martin et al. 1993; Bieniawska et al. 2007), primers AtSUS1F1XbaI and AtSUS1R1BamHI were designed based on those utilized by Sadeghi et al. (2007) and included restriction sites at

**Table 1** Primer sequences

| Primer name | Primer sequence (5′–3′) |
|---|---|
| AtSUS1F1XbaI | *TCTAGA*GATATCATTTCATATCATCA |
| AtSUS1R1BamHI | *GGATCC*AAAAGAGACGCAGAAAACAG |
| SUSdegF1 | AGGAAGCVATWGTTYTGCCTCC |
| SUSdegR1 | TGAATYCKRTCATTCARCATCA |
| CsSUS1F1AscI | *GGCGCGCC*GAGGAAGCAACACTAATAC |
| CsSUS1R1BamHI | *GGATCC*TGATTCTCAGACAAACAAAAACTTC |
| CsSUS1F2AscI | *GGCGCGCC*AAATGAGAGAGGTCCCAC |
| CsSUS1F3AscI | *GGCGCGCC*ACCTCTAGAAACTCAGC |
| CsSUS1F4AscI | *GGCGCGCC*TATCCCTGGTAGCCACACA |
| CsSUS1F5AscI | *GGCGCGCC*TAAGACGGTAATATAAGTTG |
| CsSUS1F6AscI | *GGCGCGCC*ATTCGGATTATCCATAATCC |
| CsSUS1R4AscI | *GGCGCGCC*GAGAAGAATGGATTATGGATA |
| CsSUS1R5AscI | *GGCGCGCC*TAAATTTTGGATCAAAGCTTTTC |
| CsSUS1R6AscI | *GGCGCGCC*AAAATTAACATTGCGTCTTAAG |
| CsSUS1R7AscI | *GGCGCGCC*GGATCCTGATTCTCAGAC |
| CsSUS1R2AscI | *GGCGCGCC*AGCTAATCGGATAAGCT |
| CsSUS1F11AscI | *GGCGCGCC*AACAGTACCAGTCGCCATC |
| CsSUS1-1R1AscI | *GGCGCGCC*GAATGGGAATGCAAGCTAGA |
| GUSintF1 | CGTTGGTGGAAAGCGCGTTAC |
| GUSintR3 | CTGCGATGGATTCCGGCATAG |
| GUSRTR1 | CACCACCTGCCAGTCAACAGACGC |
| GUSR6 | GACATCGGCTTCAAATGGCGTATAGC |
| GUSR2 | GCAATTGCCCGGCTTTCTTG |

Italicized regions indicate restriction sites inserted at the 5′ ends of the respective primer

their 5′ ends to facilitate its subsequent cloning into a promoterless *GUS* vector. A 1,515-bp fragment of the *AtSUS1* promoter with its 3′ terminus immediately upstream of the start codon was amplified using Platinum High Fidelity Supermix (Invitrogen, Carlsbad, CA) and primers at a final concentration of 200 nM. Cycling parameters were as follows: 94°C for 2 min, 35 cycles of 94°C for 30 s, 50°C for 30 s, 68°C for 2 min, and a final elongation step of 68°C for 5 min.

Because no *SUS1* sequence had been previously published from *C. sinensis*, a genomic region including a 5′ portion of the coding sequence was cloned initially using degenerate primers designed based on CLUSTAL alignments made with known *SUS* mRNA sequences from *C. unshiu* (GenBank accession AB022092.1), *Pyrus pyricola* (GenBank accession AB045710.1), *Populus tremuloides* (GenBank accession AY341026.1), *Arabidopsis* (GenBank accession NM_001036838.1) and *Zea mays* (GenBank accession NM_001111941.1). The approximately 600-bp fragment was amplified from *C. sinensis* genomic DNA using primers SUSdegF1 and SUSdegR1 at a 1 μM final concentration and GoTaq Hot Start Mastermix (Promega, Madison, WI). Cycling parameters were 95°C for 2 min, 35 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final elongation of 72°C for 5 min.

5′ Rapid amplification of genomic ends (RAGE) was carried out according to Liu and Baird (2001) to clone the region upstream of the *CsSUS1* coding sequence. In brief, genomic DNA was extracted from *C. sinensis* cv. valencia leaves using the OMEGA SP Plant DNA midi kit according to the manufacturer's instructions (Omega Bio-Tek) and PCRs were performed using primers that were initially designed to anneal near the 5′ end of the *CsSUS1* coding sequence. Progressively upstream 5′ fragments were obtained using a gene-walking strategy (primer sequences can be obtained upon request). All PCR products were cloned into pGEM-T easy (Promega) and subsequently sequenced.

## Plasmid constructs

Diagrammatic representations of the transformation vectors utilized in this study can be found in Fig. 1. All constructs were generated using standard protocols and are present in a pBINplus (van Engelen et al. 1995) background. PCR amplifications were conducted using Platinum High Fidelity Supermix. Vectors containing *CsSUS1* promoter::*GUS* fusions were generated by first producing constructs in which the *GUSAint* sequence (Ohta et al. 1990) and *nopaline synthase* transcriptional terminator cassette was inserted into the PZP–RSC1 multiple cloning site (Hajdukiewicz et al. 1994) that had been introduced into the pBINPLUS vector. Primers CsSUS1F1AscI and CsSUS1R1BamHI were utilized to amplify 1620-bp (allele 1; SS152) and 1704-bp (allele 2; SS153) fragments immediately upstream of the *CsSUS1* start codon. Various 5′ deletion fragments were also generated using primers CsSUS1F2AscI and CsSUS1R1BamHI to amplify 1060-bp (allele 1; SS155) and 1145-bp (allele 2; SS156) fragments, primers CsSUS1F3AscI and CsSUS1R1BamHI to amplify a 548-bp fragment (SS157), primers CsSUS1F4AscI and CsSUS1R1BamHI to amplify a 410-bp fragment (SS230), primers CsSUS1F5AscI and CsSUS1R1BamHI to amplify a 268-bp fragment (SS231), and primers CsSUS1F6AscI and CsSUS1R1BamHI to amplify a 134-bp fragment (SS232). The resulting products were subsequently introduced into the *Asc*I/*Bam*HI site of the promoterless *GUSAint* vector (SS80), which resulted in the insertion of the putative promoter sequences upstream of the *GUS* sequence. A positive control vector (SS149) was obtained by introducing the partially duplicated, constitutive, CaMV *35S* promoter (Kay et al. 1987) upstream of the *GUSAint* sequence.

To test sequences for enhancer function, the minimal CaMV *35S* promoter (position −60; Kay et al. 1987) was fused directly upstream of the promoterless *GUSAint* sequence (SS106). PCR amplifications were carried out using primers CsSUS1F3AscI and CsSUS1R5AscI to

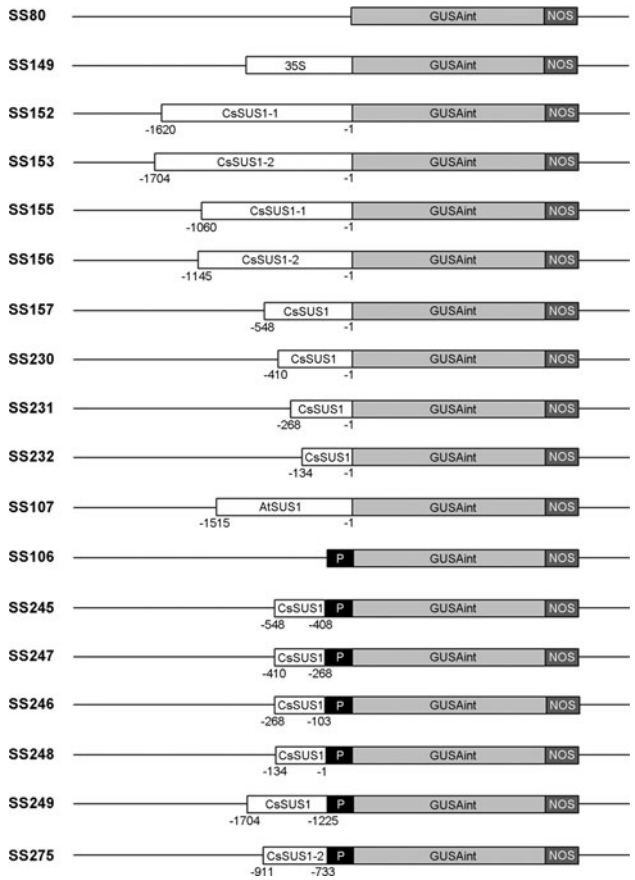


**Fig. 1** Schematic representation (not to scale) of constructs utilized in this study. All constructs shown are in a pBINPLUS background. Vectors SS80 and SS106 are negative control *GUS* plasmids lacking promoter and enhancer sequences, respectively. Construct SS107 is a positive control vector for phloem-specific expression bearing the *AtSUS1* upstream region from *Arabidopsis* fused to *GUS*, while SS149 is a constitutive positive control vector bearing the partially duplicated *35S* promoter fused to *GUS*. Constructs SS152 and SS153 contain 'full-length' *CsSUS1p-1* and *CsSUS1p-2* sequences, respectively, fused to the *GUS* reporter gene. Vectors SS155, SS156, SS157, SS230, SS231 and SS232 contain various progressive 5′ deletions of the *CsSUS1p* sequences fused to *GUS*. Vectors SS245, SS247, SS246, SS248, SS249 and SS275 include various 5′ and 3′ deletion fragments of the *CsSUS1p* fused to the minimal *35S* promoter and downstream *GUS* gene to identify any putative *cis*-acting enhancer elements. *CsSUS1-1* allele 1 of the *CsSUS1* upstream region, *CsSUS1-2* allele 2 of the *CsSUS1* upstream region, *CsSUS1* upstream region of the *CsSUS1* gene not encompassing the 94-bp indel, *35S* partially duplicated CaMV *35S* promoter, *P* CaMV *35S* minimal promoter, *GUSAint β-glucuronidase* coding sequence with inserted intron, *NOS nopaline synthase* transcriptional terminator

amplify nucleotides −548 to −408 (where +1 is the first nucleotide of the translational start codon; SS245), primers CsSUS1F4AscI and CsSUS1R6AscI to amplify nucleotides −410 to −268 (SS247), primers CsSUS1F5AscI and CsSUS1R4AscI to amplify nucleotides −268 to −103 (SS246), primers CsSUS1F6AscI and CsSUS1R7AscI to amplify nucleotides −134 to −1 (SS248), primers CsSUS1F1AscI and CsSUS1R2AscI to amplify nucleotides −1704 to −1,225 (SS249), and primers CsSUS1F11AscI and CsSUS1R1-1AscI to amplify nucleotides −911 to −733 of allele 2 (SS275). The resulting products were then inserted into the *Asc*I site upstream of the minimal *35S* promoter::*GUS* fusion.

## Transformation of *Arabidopsis* and tobacco

Vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation and the resulting recombinant bacteria were used for the transformation of *Arabidopsis* ecotype col-0 and *N. tabacum* cv. NC95 (provided by Dr. Georg Jander, Boyce Thompson Institute for Plant Research, Cornell University). *Arabidopsis* transformation was carried out via the floral dip method (Clough and Bent 1998), which has been demonstrated previously to result in the introduction of a single T-DNA insert in over 50% of transgenic lines (Alonso et al. 2003; Rosso et al. 2003). First generation transformants were selected by plating surface-sterilized seeds on standard Murashige and Skoog (MS) media (Murashige and Skoog 1962) containing 60 mg/l kanamycin. Conversely, tobacco leaves were transformed in vitro (Horsch et al. 1985) and transformed shoots were selected on standard MS media containing 1 mg/l benzyl adenine (BA), 300 mg/l timentin, and 100 mg/l kanamycin and rooted on the same medium lacking BA. Transgenic plants utilized in this study were phenotypically normal primary transformants, and their identities were confirmed by PCR.

## Histochemical staining and fluorometric assays of GUS activity

Histochemical staining for GUS activity was conducted as described by Jefferson et al. (1987). Various tissues from a selection of transgenic *Arabidopsis* and tobacco lines bearing each vector were utilized for histochemical assays. In the case of transgenic *Arabidopsis*, these included leaves that were unmarred, as well as those that were inadvertently wounded either through mechanical means during sample processing or from exposure to insect pests. All tissues were incubated in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc; in 100 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferrocyanide and 0.1% Triton X-100) at 37°C for 24 h. Stained tissue was subsequently de-pigmented in a series of 70% ethanol washes and images were obtained using an Olympus BX50 light microscope (Olympus America Inc., Center Valley, PA) with attached SPOT Idea digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

Fluorometric assays of GUS activity were conducted as described by Hily et al. (2009) to quantify levels of GUS

protein from three 4-week-old leaves from a minimum of 12 independent *Arabidopsis* lines containing each vector found by histochemical analyses to drive expression in the phloem, as well as an untransformed control. Concentrations of methylumbelliferone (MU) produced were determined from the linear regression slopes of fluorescence emitted by a series of MU standards. Protein concentrations were established using the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard. Each sample was assayed in duplicate and GUS activities were expressed as the mean value of pmol MU generated per minute per mg protein. Statistical analyses were executed using the Mann–Whitney test for non-parametric data and differences were considered significant at $P \leq 0.05$.

### RT-PCR analysis of *GUS* expression

Total RNA was extracted from leaf tissues using the E.Z.N.A. Plant RNA Mini Kit (Omega Bio-tek) and contaminating DNA was removed using the TURBO DNA-free system (Ambion, Austin, TX). RT-PCR analyses were carried out using the Superscript III first-strand cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). In each case, 450 ng RNA was reverse transcribed with an oligo(dT) primer in a final volume of 10 µl and 1 µl of the resulting cDNA mixture was used as template in PCR assays using HotStart GoTaq polymerase (Promega) in a final volume of 25 µl. Primers GUSintF1 and GUSintR3, which anneal on either side of the intronic region, were utilized to amplify a 614-bp *GUS*-specific product. A 630-bp *EF1α*-specific product was also amplified as an internal control using primers EF1αF and EF1αR (Hily and Liu 2009). The conditions for PCR amplification of *GUS*-specific cDNA were 95°C for 2 min, followed by 28 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min, with a final elongation step of 72°C for 5 min. The same general parameters were utilized to amplify *EF1α*-specific fragments with the exception of the extension time, which was 1 min, the annealing temperature, which was 58°C, and the utilization of 22 cycles. PCR products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

### 5′ rapid amplification of cDNA ends (RACE)

The 5′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) was utilized to map the 5′ ends of *GUS* transcripts from SS152 and SS153 lines. First-strand cDNA synthesis reactions were performed using 1 µg DNase-treated total RNA (prepared as described in the previous section) with primer GUSRTR1. The resulting cDNAs were C-tailed and utilized in subsequent PCRs using Platinum High Fidelity Supermix (Invitrogen) with primers GUSR6 and AAP (Invitrogen), which anneals to the 5′ polyC tail. Nested PCRs were subsequently carried out to improve specificity and increase the amount of product generated using 1 µl of PCR product (1/10 dilution) as template and primers GUSR2 and AUAP (Invitrogen). In each case, purified products were cloned into pGEM-T easy (Promega) and at least seven separate clones were sequenced in each instance.

### Bioinformatic analyses

Sequences were scanned for potential promoter regions and transcription initiation sites using the Berkeley Drosophila Genome Project's Neuronal Network Promoter Prediction program (http://www.fruitfly.org/seq_tools/promoter.html) while putative TATA boxes and other *cis*-acting promoter regulatory elements were identified using the PlantCARE database (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html; Lescot et al. 2002) and the plant *cis*-acting regulatory DNA elements (PLACE) database (Higo et al. 1999). Nucleotide sequences were aligned using CLUSTAL W (Thompson et al. 1994) and were compared with previously reported sequences using BLAST (Altschul et al. 1990).

## Results

### Sequence analysis of *SUS1* promoter regions

The 1,515-bp At*SUS1* promoter sequence isolated from *A. thaliana* col-0 in this study exhibited 95% identity at the nucleotide level with a previously published sequence (GenBank accession ×70990). Two separate genomic sequences of 607 and 600 bp, which displayed homology to *SUS1* genes previously identified in other plant species, were amplified from *C. sinensis* cv. valencia, respectively. The first fragment exhibited 99% nucleotide identity with the *C. unshiu CitSUSA-2* gene (GenBank accession AB025778.1) while the second displayed 99% nucleotide identity with the *C. unshiu CitSUS1-2* gene (GenBank accession AB029401.1). In addition, these two sequences exhibited 64% identity with one another at the nucleotide level. Since the *CitSUS1-2* sequence has been found to be more closely related to the phloem-specific *SUS1* genes from other plants than the *CitSUSA-2* sequence (Komatsu et al. 2002), we focused the remainder of this study on the *CsSUS1* sequence.

5′ RAGE was utilized to clone the region upstream of the *CsSUS1* coding sequence, including the putative promoter. Two distinct regions of 1620 bp (*CsSUS1p-1*; GenBank accession JF522372) and 1704 bp (*CsSUS1p-2*;

GenBank accession JF522373) were isolated which exhibited 96% nucleotide identity with one another but displayed no significant homology to known *SUS1* upstream regions from other plant species. The two citrus upstream regions displayed several nucleotide substitutions (44) between them, as well as a small number of insertion/deletion events of 1–7 nucleotides (9) along the length of the sequences. Interestingly, the main difference between the two sequences was a 94-bp insertion in *CsSUS1p-2* as compared to *CsSUS1p-1*, with its 5′ terminus located 902 bp upstream of the translational start codon. This indel bore no significant homology to any other known sequences.

To ascertain that *CsSUS1p-1* and *CsSUS1p-2* were derived from separate alleles of the same gene and were not promoters of distinct members of the *SUS* gene family, PCRs were carried out using forward primers that annealed to regions of *CsSUS1p-1* and *CsSUS1p-2* upstream of the 94-bp indel and reverse primers that annealed to citrus *SUS1*, *SUSA* and *SUS2* coding regions, respectively. While no amplification products were obtained when using *SUSA*- and *SUS2*-specific primers, two amplification products corresponding to both alleles of the *CsSUS1* promoter (confirmed via sequencing) were obtained when using a *SUS1*-specific reverse primer (data not shown).

### The *CsSUS1* upstream regions drive the expression of a reporter gene in the phloem of both transgenic *Arabidopsis* and tobacco

Vectors were generated in which the 1620-bp *CsSUS1p-1* (SS152) and 1704-bp *CsSUS1p-2* (SS153) upstream regions, as well as that of *AtSUS1* (utilized as a phloem-specific positive control; SS107), were fused to a downstream *GUS* gene and subsequently transformed into both *Arabidopsis* and tobacco. Leaf, flower and stem tissues from numerous independent *Arabidopsis* transformants, as well as petiole sections from transgenic tobacco, were then stained histochemically for visualization of *GUS* expression.

Although only 50% of the SS107 *Arabidopsis* lines containing the *AtSUS1::GUS* cassette exhibited relatively weak vascular-specific expression and the remainder exhibited patchy expression or no GUS staining, the majority of *CsSUS1p-1* (SS152) and *CsSUS1p-2* (SS153) lines displayed strong vascular-specific expression in leaf tissues (88 and 56%, respectively). Stem cross-sections indicated that this expression was limited to the phloem of the vascular bundle. However, in the case of *CsSUS1p-2* lines (SS153), there appeared to be an increased tendency for constitutive or leaky *GUS* expression (Table 2; Fig. 2a). Furthermore, a small number (approximately 5%) of SS152 and SS153 lines were also found to express *GUS* in cells surrounding sites of wounding. This pattern of wound-limited expression appeared to be identical regardless of whether the wound site was the result of insect feeding or a mechanical injury sustained during sample processing (Table 2; Fig. 2c). This wound-responsive *GUS* expression was never observed in any line bearing the control vectors, including the *AtSUS1::GUS* cassette (SS107). Both *CsSUS1* promoter regions were also found to drive *GUS* expression in the sepals and filaments of stamens in transgenic *Arabidopsis* flowers, while lines containing the *AtSUS1p::GUS* cassette displayed GUS staining in all floral organs. As expected, positive control *Arabidopsis* lines bearing the *35S::GUS* cassette (SS149) exhibited constitutive *GUS* expression in all tissues tested while negative control lines carrying the promoterless*::GUS* reporter gene (SS80) displayed no GUS staining (Fig. 2a).

In transgenic tobacco leaves, 75% ($n = 12$) of SS107 lines containing the *AtSUS1::GUS* cassette exhibited phloem-specific expression, while the remainder did not generate any GUS staining. In the case of *CsSUS1p::GUS* lines, 67% ($n = 12$) of SS152 lines displayed phloem-specific expression and 70% ($n = 10$) of SS153 lines exhibited phloem-specific expression, and in both cases a single line displayed constitutive *GUS* expression. Positive control lines bearing the *35S::GUS* cassette (SS149) exhibited constitutive *GUS* expression throughout petiole cross-sections, while negative control lines containing the promoterless *::GUS* cassette (SS80) displayed no GUS staining (Fig. 3).

A series of vectors carrying various 5' deletions of the *CsSUS1p-1* and *CsSUS1p-2* upstream regions fused directly to the *GUS* reporter gene were also generated and transformed into *Arabidopsis* to narrow down the sequence required for phloem-specific expression (Fig. 1). Since very few differences were noted between *CsSUS1p-1* and *CsSUS1p-2* other than the presence of the 94-bp indel in *CsSUS1p-2*, we have simply termed any deletion of the *CsSUS1* promoter with a 5′ terminus downstream of the indel (and thus not including the insert sequence) *CsSUS1p*. Histochemical analyses of leaf tissues indicated that 44% of lines containing a vector harboring nucleotides −1060 to −1 of the *CsSUS1p-1* (SS155) and 81% of lines bearing a vector including nucleotides −1145 to −1 of the *CsSUS1p-2* (SS156) exhibited phloem-specific *GUS* expression in the leaves without the apparent leakiness observed in SS153 lines. Similarly, 56 and 82% of lines containing vectors with nucleotides −548 to −1 (SS157) and −410 to −1 (SS230) of the *CsSUS1p*, respectively, were found to drive phloem-specific expression in leaf tissues. Interestingly, as was the case for SS152 and SS153 lines, a small percentage (approximately 7%) of lines containing each of the *CsSUS1p* deletion vectors SS155, SS156, SS157 and SS230 were also found to express *GUS* at wound sites (Table 2; Fig. 2c). Furthermore, transgenic lines bearing a vector including nucleotides −268 to −1 of

**Table 2** *GUS* expression in *Arabidopsis* lines bearing *AtSUS1 p::GUSAint* and *CsSUS1p::GUSAint* constructs

| Line | $n$[d] | Phloem[e] | Constitutive | Other | No *GUS* |
|---|---|---|---|---|---|
| SS80[a] | 15 | 0 (0)[f] | 0 | 0 | 15 |
| SS107 | 12 | 6 (0) | 0 | 4 | 2 |
| SS152 | 17 | 15 (1) | 1 | 0 | 1 |
| SS153 | 18 | 10 (1) | 5 | 1 | 2 |
| SS155[b] | 27 | 12 (2) | 0 | 4 | 11 |
| SS156 | 31 | 25 (2) | 0 | 4 | 2 |
| SS157 | 23 | 13 (3) | 0 | 3 | 7 |
| SS230 | 22 | 18 (1) | 0 | 0 | 4 |
| SS231 | 17 | 0 (4) | 1 | 0 | 12 |
| SS232 | 12 | 0 (0) | 0 | 0 | 12 |
| SS106[c] | 13 | 0 (0) | 0 | 0 | 13 |
| SS245 | 16 | 0 (0) | 0 | 0 | 16 |
| SS247 | 32 | 29 (0) | 0 | 2 | 1 |
| SS246 | 13 | 0 (2) | 0 | 0 | 11 |
| SS248 | 17 | 0 (0) | 0 | 0 | 17 |
| SS249 | 15 | 0 (0) | 0 | 0 | 15 |

[a] Lines utilized in the analysis of 'full-length' *SUS1* promoter::*GUS* fusions

[b] Lines bearing progressively larger 5′ deletions of the *CsSUS1p* fused to *GUS*

[c] Lines bearing 5′ and 3′ deletions of the *CsSUS1p* fused to *GUS* for the identification of putative *cis*-acting enhancer elements

[d] The number of independent transgenic lines analyzed histochemically for GUS staining in each case

[e] Headings indicate tissue specificity of GUS staining in the leaves of lines containing each construct

[f] Numbers outside the parentheses indicate the number of independent transgenic lines exhibiting *GUS* expression in a given tissue. Numbers in parentheses indicate the number of lines in which *GUS* expression was observed in tissue immediately surrounding wound sites, sometimes in conjunction with phloem-limited expression

the *CsSUS1p* (SS231) did not exhibit *GUS* expression in phloem tissues, but often (24%) displayed expression specifically at the sites of wounding (Table 2; Fig. 2c). In every case, the wound-responsive pattern of expression was identical to that observed in lines containing the full-length *CsSUS1p::GUS* constructs. Transgenic lines containing vectors with nucleotides −134 to −1 of the *CsSUS1p* (SS232) did not drive *GUS* expression in any tissue analyzed (Table 2; Fig. 2b). RT-PCR analyses of *GUS* expression from leaf tissues of the aforementioned transgenic *Arabidopsis* lines were consistent with GUS staining results in every case (Fig. 2d).

## Fluorometric analyses of GUS protein levels in 'full-length' and 5′ deletion lines

To quantify the levels of GUS protein in the leaves of transgenic *Arabidopsis* lines carrying 'full-length' and 5′ deletion *CsSUS1p* vectors capable of driving phloem-specific expression of a downstream reporter gene, GUS fluorometric assays were carried out. Lines containing the control *AtSUS1::GUS* vector (SS107) generated relatively

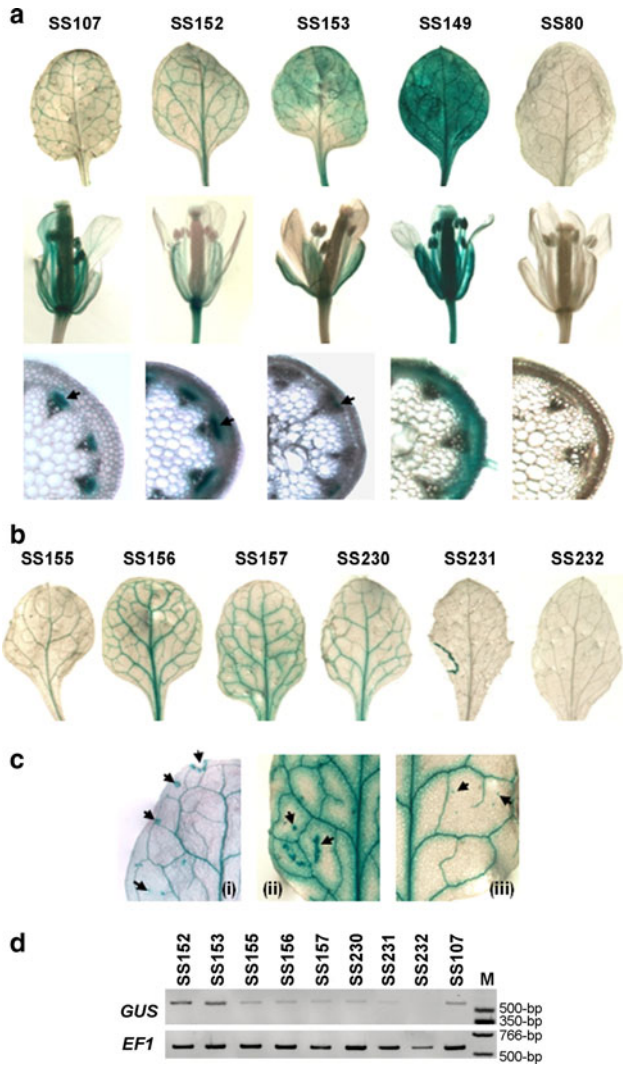


**Fig. 2** Analysis of *GUS* expression in various transgenic lines of *Arabidopsis*. Leaves, flowers and stem cross-sections from *Arabidopsis* lines transformed with constructs bearing 'full-length' *AtSUS1* (SS107), *CsSUS1p-1* (SS152) and *CsSUS1p-2* (SS153) fragments fused to the *GUS* reporter gene, as well as a *35S::GUS* positive control (SS149) and promoterless *GUS* negative control (SS80), were analyzed histochemically for GUS staining (**a**). GUS staining in the leaves of *Arabidopsis* lines bearing progressive 5′ deletions of the *CsSUS1p* sequence are shown in (**b**). In every case, *images* depict GUS staining from representative lines transformed with each construct. Leaves from representative (SS152 **(i)**, SS156 **(ii)** and SS157 **(iii)**) *CsSUS1p::GUS* lines displaying GUS staining in both phloem tissue and in response to wounding as observed in a minority of all *CsSUS1p*-containing lines are displayed in (**c**). Semi-quantitative RT-PCR analysis of *GUS* expression in leaf tissue from transgenic *Arabidopsis* lines containing 'full-length' and 5′ deletions of the *CsSUS1p* fused to *GUS*, along with the positive control *AtSUS1p::GUS* (**d**). The expression of *EF1α* was utilized as an internal control. *GUS β-glucuronidase*, *EF1 EF1α*

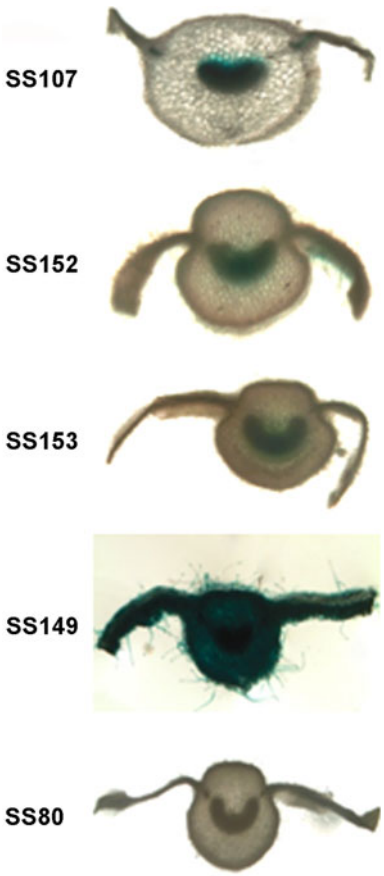


**Fig. 3** Analysis of *GUS* expression in various transgenic lines of tobacco. Petiole cross-sections of transgenic tobacco lines bearing 'full-length' *AtSUS1* (SS107), *CsSUS1p-1* (SS152) and *CsSUS1p-2* (SS153) fragments fused to the *GUS* reporter gene, as well as a *35S::GUS* positive control (SS149) and promoterless *GUS* negative control (SS80), were assayed histochemically for GUS activity

low levels of GUS in leaf tissues ($n = 13$; 142.79 ± 42.34 pmol MU/min mg protein), which was significantly higher than the background fluorescence observed in untransformed controls ($n = 8$; 31.74 ± 2.84 pmol MU/min mg protein). Interestingly, those lines containing 'full-length' *CsSUS1p::GUS* cassettes (SS152 and SS153), as well as the SS155 and SS156 deletion vectors, generated significantly higher levels of GUS protein than *AtSUS1*-derived lines. Lines harboring the 'full-length' *CsSUS1p-2::GUS* cassette (SS153) produced the highest levels of GUS protein ($n = 12$; 2083.78 ± 690.19 pmol MU/min mg protein), while lines containing the 'full-length' *CsSUS1p-1::GUS* cassette (SS152) produced significantly lower levels of GUS ($n = 14$; 573.20 ± 197.24 pmol MU/min mg protein). Lines containing the 5′ *CsSUS1p-1* deletion vector SS155 ($n = 15$; 259.16 ± 49.88 pmol MU/min mg protein) generated similar levels of GUS protein as the 'full-length' *CsSUS1p-1* promoter. Conversely, lines containing the 5′ *CsSUS1p-2* deletion vector SS156 ($n = 15$; 510.66 ± 201.18 pmol MU/min mg protein) produced significantly less GUS protein than its 'full-length' counterpart, SS153. Lines containing SS157 ($n = 14$; 252.17 ± 94.75 pmol MU/min mg protein) and SS230 ($n = 16$; 265.4 ± 46.7 pmol MU/min mg protein) vectors generated levels of GUS comparable to SS152, SS155 and SS156 lines (Fig. 4).

## Characterization of enhancer regions within the *CsSUS1* upstream sequences

In an attempt to specify putative enhancer regions within the *CsSUS1* upstream sequences, vectors were generated in which various fragments of the promoters were fused to the minimal *35S* promoter and *GUS* reporter gene, and the resulting constructs were transformed into *Arabidopsis*. Histochemical analysis of GUS staining in leaf tissues indicated that lines containing the enhancerless minimal promoter fused to *GUS* (SS106), as well as those bearing nucleotides −548 to −408 (SS245), −134 to −1 (SS248) and −1704 to −1225 (SS249) of the *CsSUS1p* fused to the minimal *35S* promoter did not drive *GUS* expression. Conversely, 91% of lines containing nucleotides −410 to −268 of the *CsSUS1p* fused to a minimal promoter (SS247) exhibited highly specific phloem-limited expression while 15% of lines containing nucleotides −268 to −103 of the *CsSUS1p* fused to a minimal promoter (SS246) exhibited wound-specific *GUS* expression (Table 2; Fig. 5a). *GUS* expression was never observed in any other tissue, including the phloem, of these latter lines.

To characterize the function of the 94-bp insert in the *CsSUS1p-2* upstream region, which exhibited no significant homology to other previously published sequences, a vector was generated in which this region (nucleotides −911 to −733) was fused to the minimal *35S* promoter and *GUS* reporter gene (SS275). Histochemical analyses indicated that *GUS* was not expressed in leaf tissues. Instead, flower-specific *GUS* expression was observed in very young buds, as well as stamens and carpels of young flowers from 50% of the lines tested ($n = 6$). This expression became restricted to the ovules and filaments in mature flowers (Fig. 5b).

## Characterization of *CsSUS1* transcriptional start sites in a transgenic system

Bioinformatic analyses of the 'full-length' *CsSUS1p-1* upstream sequence indicated that it contained three putative promoter regions. The most upstream potential promoter was situated between nucleotides −1573 and −1524 with a transcriptional start site at −1533 (score of 0.99), another was located between −819 and −770 with a transcriptional start site at −779 (score of 0.94), and the third was found between −539 and −490 with a

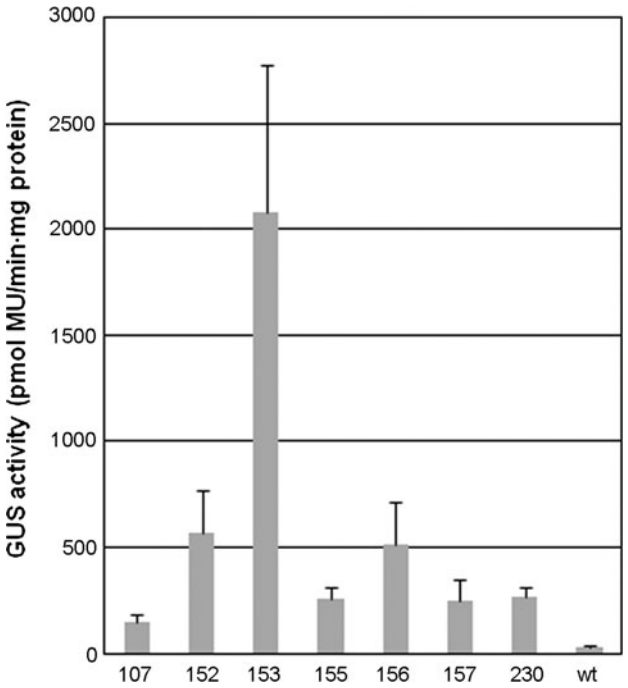


**Fig. 4** Fluorometric analysis of GUS activity in various transgenic lines of *Arabidopsis*. The *graph* displays the mean GUS activities, along with standard errors, in pmol MU $min^{-1}$ $mg^{-1}$ of protein from 3 leaves of at least 12 independent transgenic lines bearing constructs found by histochemical analyses to induce phloem-specific *GUS* expression, respectively, or 8 untransformed controls

transcriptional start site at −499 (score of 0.98). In the case of the *CsSUS1p-2* upstream sequence, four putative promoters were predicted with the first between nucleotides −1657 and −1608 with a transcriptional start site at −1617 (score of 0.99; corresponds to first putative promoter in *CsSUS1p-1*), the second between −807 and −758 with a transcriptional start site at −767 (score 0.94; corresponds to the second putative promoter in *CsSUS1p-1*), the third between −786 and −737 with a transcriptional start site at −746 (score 0.82), and the fourth between −537 and −488 with a transcriptional start site at −497 (score 1.00; corresponds to the third putative promoter in *CsSUS1p-1*).

In an effort to elucidate the actual transcriptional start sites in *CsSUS1p-1* and *CsSUS1p-2* upstream regions when fused to a downstream *GUS* gene in transgenic lines, total RNA from SS152 and SS153 leaf tissue was subjected to 5′ RACE analysis. In both cases, several alternative start sites were found (Fig. 6). One set of transcriptional start sites appeared to initiate between −103 and −53 nucleotides upstream of the ATG in both alleles (type I). Interestingly, a second set of transcriptional start sites (type II) initiated at least 727 nucleotides upstream of the ATG. This second type of initiation site contained a spliced intron that possessed a variety of 5′ splice sites and a 3′ splice site at nucleotide −27. Although the majority of SS152 clones possessed type I initiation sites, the majority of SS153 clones possessed type II initiation sites.

## Discussion

The need for a broad spectrum of promoters that differ in their ability to control spatiotemporal expression patterns for transgenic research has become increasingly clear in recent years. Indeed, the use of such tightly regulated promoters to drive transgene expression can be of vital importance to the perceived safety of transgenic crops due to the minimization of potential adverse side effects of engineered plants on non-target organisms (reviewed by Potenza et al. 2004). In this study, we endeavored to isolate the promoter sequence of the *SUS1* homolog from *C. sinensis* and subsequently determine whether it, like many of its counterparts from other plant species, such as *Arabidopsis* (Martin et al. 1993), maize (Yang and Russell 1990) and rice (Shi et al. 1994), was capable of driving the expression of a reporter gene predominantly in the phloem tissue of transgenic plants.

Two distinct alleles of *SUS1* upstream regions were identified in *C. sinensis* of 1620 bp (*CsSUS1p-1*) and 1704 bp (*CsSUS1p-2*). The two regions exhibited 96% nucleotide identity, with a relatively small number of nucleotide substitutions and minor insertion/deletion events between the two sequences. Neither sequence exhibited significant homology to known *SUS* promoter sequences from other plant species, which is not all that surprising given that while the coding regions of *SUS* genes tend to be relatively highly conserved between species, the promoter regions do not, even though they often display functional homologies.

When the 'full-length' *CsSUS1p-1* and *CsSUS1p-2* promoter alleles were fused to the *GUS* reporter gene (SS152 and SS153, respectively; Fig. 1) and subsequently transformed into *Arabidopsis* and tobacco, both alleles were capable of driving a relatively high level of *GUS* expression in the phloem of leaves and stems in the majority of independent transgenic lines analyzed (Figs. 2, 3; Table 2), which indicates that the *CsSUS1* promoters are functional in multiple plant species. The level of expression induced by each of the citrus promoter alleles was significantly higher than that induced by the *AtSUS1* upstream region in *Arabidopsis*, which was utilized as a phloem-specific control (Figs. 2a, 4). However, lines containing the *CsSUS1p-2::GUS* cassette (SS153) appeared to demonstrate an increased tendency for leaky *GUS* expression in other tissues (Figs. 2a, 4; Table 2). This tendency for constitutive expression was lost in *Arabidopsis* lines containing reporter cassettes carrying a 5′ deletion of the *CsSUS1p-2* sequence (nucleotides −1145 to −1 relative to

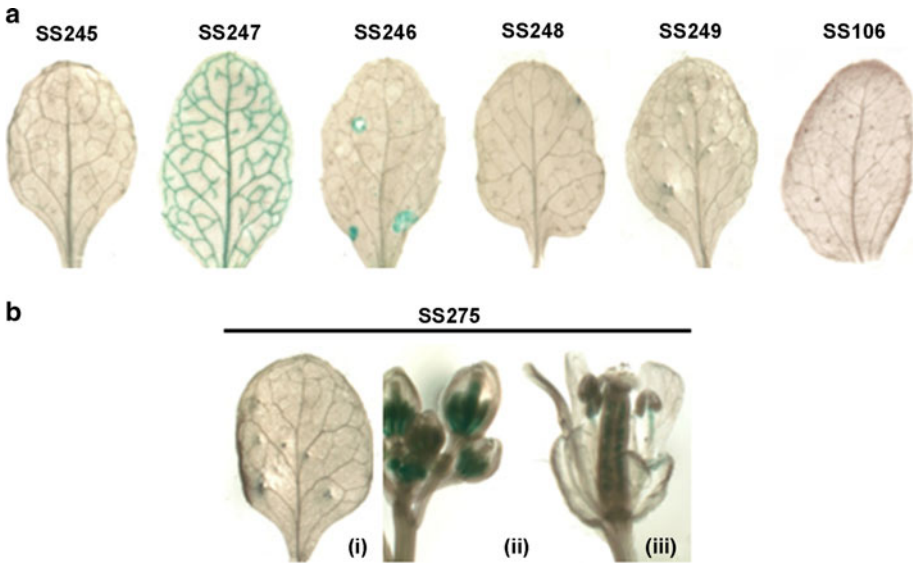


**Fig. 5** Analysis of *GUS* expression conferred by putative enhancer regions in various transgenic lines of *Arabidopsis*. Leaves from *Arabidopsis* lines transformed with constructs bearing various 5′ and 3′ deletions of the *CsSUS1p* were fused to the *35S* minimal promoter and *GUS* reporter gene, as well as an enhancerless *GUS*-negative control (SS106), were analyzed histochemically for GUS staining **(a)**. Images depict GUS staining from representative lines transformed with each construct. Representative leaves **(i)**, young flowers **(ii)** and mature flowers **(iii)** of *Arabidopsis* lines bearing a fragment comprising the 94-bp indel from *CsSUS1p-2* fused to the *35S* minimal promoter and *GUS* reporter gene stained histochemically for GUS activity are displayed in (**b**)

the translational start codon; SS156) and expression in the leaves was limited to phloem tissue at a level similar to 'full-length' *CsSUS1p-1::GUS* lines (SS152; Figs. 2, 3, 4).

Interestingly, the most noteworthy difference between the two promoter alleles was the presence of a 94-bp insertion 902-bp upstream of the translational start codon in *CsSUS1p-2* compared with *CsSUSp-1*. This is not the first time polymorphisms have been detected in a *SUS* promoter. Lingle and Dyer (2004) found several polymorphisms in two distinct promoter alleles of the *Sus2* gene from sugarcane, the majority of which occurred upstream of the transcription start site. In this case, seven indels of 233 to 247 bp were found to differ between the two genotypes, which exhibited 62–70% homology to the Candystripe (*Cs*1) transposon from *Sorghum* (Chopra et al. 1999) suggesting that the indels may be derived from a transposon. Conversely, the 94-bp *CsSUS1p-2* indel discovered in this study was not found to show any significant similarities to known transposons.

When fused to a minimal promoter and *GUS* reporter gene, this small indel was found to drive expression specifically in floral tissues (Fig. 5b), suggesting that it contains a tissue-specific enhancer element. Although relatively strong GUS staining was observed in very young buds, developing young flowers displayed no GUS staining. However, once young flowers reached a stage with expanded petals, they exhibited stamen-specific expression, while those at a slightly more mature stage exhibited both stamen- and carpel-specific expression. In mature flowers, *GUS* expression appeared to be limited to the filaments of stamens and the ovules of carpels (Fig. 5b). Intriguingly, histochemical assays for *GUS* expression of *CsSUS1p-1::GUS* (SS152; which lack the indel) and *CsSUS1p-2::GUS* (SS153; which bear the indel) lines did not reveal any noticeable differences in GUS staining patterns in floral tissues. In both cases, *GUS* expression was noted in the sepals, along with weak expression in the filaments of anthers, in mature flowers (Fig. 2a). However, it cannot be ruled out that variations in floral-specific expression were present in the earlier stages of floral development.

For *SUS* genes to successfully carry out their essential roles in the partitioning of assimilate from source to sink (Sturm and Tang 1999; Gibson 2000), their expression must be tightly regulated at both the transcriptional and post-transcriptional levels in an environmental- and/or tissue-specific manner (Koch et al. 1992; Winter and Huber 2000). One way in which numerous genes achieve a certain level of control over their expression at the transcriptional level is through the presence of enhancer elements within intronic sequences (for example Goto et al. 1990; McElroy et al. 1990; Oshima et al. 1990). Indeed, one of the most robust enhancers of gene expression identified to date in plants is the leader intron of the *Sh1* gene from maize, which has been found to be remarkably effective when included in the 5′ region of transgenic cassettes (Vasil et al. 1990; Maas et al. 1991; Clancy et al. 1994). Similarly, the leader introns from potato *SUS* genes have also been found to be involved in the control of both the level and pattern of

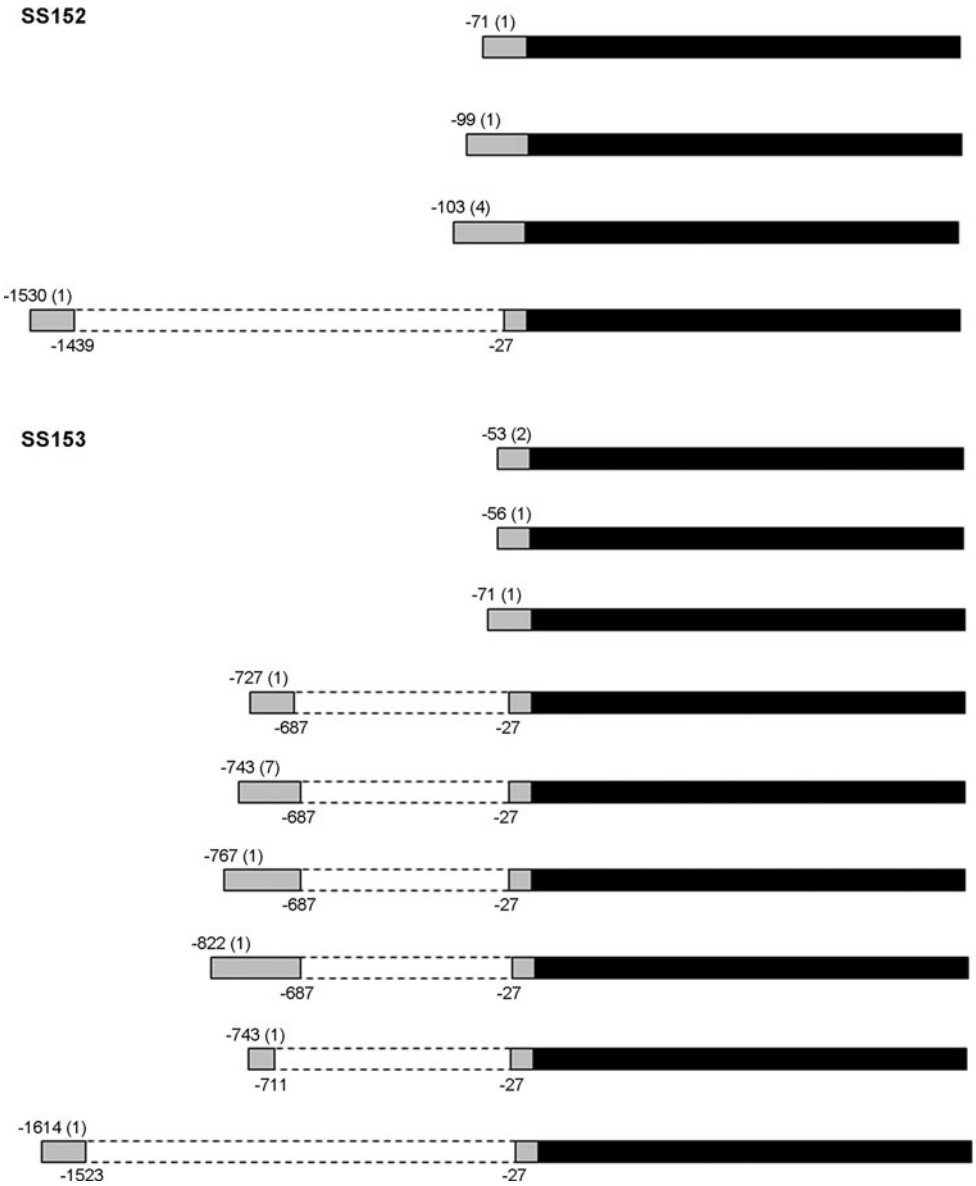


**Fig. 6** Schematic diagram of the transcriptional start sites of *GUS* transcripts in the leaves of SS152 and SS153 *Arabidopsis* lines. 5′ RACE was utilized to determine transcriptional start sites of *GUS* transcripts from the leaves of *Arabidopsis* lines containing 'full-length' *CsSUS1p-1* and *CsSUS1p-2* fused to the *GUS* reporter gene (SS152 and SS153, respectively). *Black boxes* denote *GUS*-specific sequence while *gray boxes* indicate *CsSUS1p*-specific sequence. *Dashed boxes* represent spliced leader intronic sequence. *Numbers* indicate nucleotides of start sites, as well as intronic splice sites, relative to the translational start codon. *Numbers* in *parentheses* denote the number of clones with the given transcript characteristics in each case. Two classes of transcriptional start sites were observed in both cases: type I initiated near the 3′ end of the *CsSUS1p* sequence with no spliced intron, whereas type II transcripts initiated upstream of a variable spliced leader intron

expression they confer in transgenic plants (Fu et al. 1995b), which suggests that the long leader introns of *SUS* genes may provide a functional role.

As is the case for the *SUS* genes from the majority of plant species, including *Sus3* and *Sus4* from *Solanum tuberosum* (Fu et al. 1995a), *Sus2* from *Saccharum* spp. (Lingle and Dyer 2004), *Susy*Dc1* from *Daucus carota* (Sturm et al. 1999), *MtSucS1* from *Medicago truncatula* (Hohnjec et al. 1999), *AtSUS2* from *Arabidopsis* (Chopra et al. 1992) and *Sh1* from *Zea mays* (Clancy et al. 1994; Clancy and Hannah 2002), the *CsSUS1* promoter contains an intron in the 5′ untranslated region of the pre-mRNA. Unexpectedly, 5′ RACE analysis indicated that whereas the 3′ splice site did not vary at its position of nucleotide-27 relative to the translational start codon, alternative 5′ splice sites existed for the leader intron, at least in *CsSUS1p-2* lines. Although the majority of transcripts appeared to possess a 660-bp leader intron, a small minority included a 684- or 1496-bp intron (Fig. 6). This is not the first account of alternative splice sites in a *SUS* leader intron, as they have also been noted in the leader intron of the potato *Sus3* gene; however, in this case it is the 3′ splice site that differs by 26 bp (Fu et al. 1995a) rather than the 5′ splice site as was the case in our system.

Progressive 5′ deletion analysis of the *CsSUS1p* revealed that a fragment as small as the 3′ 410 bp of the promoter (−410 to −1 relative to the translational start site; SS230) was sufficient to confer highly specific *GUS* expression in phloem tissue from the leaves of transgenic *Arabidopsis* at a level comparable to the 'full-length' *CsSUS1p-1* sequence (SS152; Figs. 2b, d, 4), which bears the entire 1412-bp leader intron, and −1145 to

−1 5′ deletion fragment of *CsSUS1p-2* (SS156), which includes the smaller intron variant. This suggests that the full-length leader intron is not required for enhancement of phloem-specific expression in the leaves and is reminiscent of the *Sh1* promoter wherein a 145-bp fragment of the 1028-bp intron (including 20 bp at its 5′ end and 125 bp at its 3′ end to ensure efficient splicing) was found to induce gene expression by 20- to 50-fold (Clancy and Hannah 2002). However, it has been found that splicing of the *Sh1* leader intron is important for its function, even when large internal deletions of the intron were present (Clancy and Hannah 2002). This does not appear to be the case for the *CsSUS1* gene, because no 5′ splice site was present in our SS230 construct (including nucleotides −410 to −1 of the *CsSUS1p*). This suggests that the *CsSUS1* leader intron may be more functionally similar to the leader intron of the *PAT1* gene from *Arabidopsis*, which does not require splicing for it to enable enhancement of gene expression (Rose and Beliakoff 2000). Alternatively, it is also feasible that a cryptic 5′ splice site was present in the 383 bp of leader intron fragment in SS230 lines, which would have allowed efficient splicing of the intronic fragment.

Interestingly, transcription start sites were observed both upstream and downstream of the −410 site (Fig. 6), which suggests that only those transcripts derived from transcription initiating downstream of this site are of absolute necessity for phloem-specific expression in leaf tissue. At least, three putative promoter sequences were predicted in silico in both *CsSUS1p-1* and *CsSUS1p-2*, with the most proximal to the translational start site located between nucleotides −539 and −490 (*CsSUS1p-1*) or −537 and −488 (*CsSUS1p-2*). All of these promoter-like sequences may be functional in the *CsSUS1* upstream sequences as actual alternative transcription initiation sites were found within and downstream of each of them using 5′ RACE. This is especially true for the two most upstream sequences, in which genuine transcription start sites were detected that were very near to, if not identical to, the predicted transcription start sites (Fig. 6). However, it is possible that there is an additional promoter even more proximal to the translation start site, as none of the predicted promoters are included between nucleotides −410 to −1 of the *CsSUS1p*; a fragment which was found to be capable of driving both phloem-specific and wound-inducible expression of a downstream *GUS* gene. In addition, numerous transcripts were identified via 5′ RACE with transcriptional start sites between nucleotides −103 and −53, and several putative TATA boxes were identified between nucleotides −260 and −97, which hints at the possibility of a nearby promoter region.

To further corroborate the *CsSUS1* 5′ deletion results and identify any phloem-specific enhancer containing regions, small 5′ and 3′ deletion fragments were inserted upstream of a minimal promoter and *GUS* reporter gene (Fig. 1). Interestingly, only the fragment comprising nucleotides −410 to −268 (SS247) was found to confer phloem-specific *GUS* expression in the leaves (Fig. 5a). Therefore, it seems that this 142-bp region within the leader intron contains putative *cis*-acting enhancer elements required for phloem-limited expression. Previously, the highly conserved motif ‘ATAAGAACGAATC’ was found to play a role in the strength and specificity of expression in various phloem-specific promoters, such as the Coconut Foliar Decay Virus (CFDV) promoter, rolC promoter from *Agrobacterium rhizogenes*, Commelina Yellow Mottle Virus (CoYMV) promoter, Rice Tungro Bacilliform Virus (RTBV) promoter and the pea *GS3A* promoter (Hehn and Rohde 1998). In addition, sequences required for high levels of phloem-specific expression exhibiting significant homology to this 13-bp motif were identified in the promoter of the phloem-specific *PP2* genes from *Cucurbita maxima* and *C. moschata* (Guo et al. 2004). However, no sequence bearing significant homology to this motif was detected in the *CsSUS1p* −410 to −268 fragment, which suggests that other, as of yet unidentified, sequences must be involved in the phloem-specific enhancement of *CsSUS1* promoter activity.

Unexpectedly, further 5′ deletion of the *CsSUS1p* to a 268-bp fragment (nucleotides −268 to −1; SS231) resulted in *GUS* expression in response to wounding incurred through insect feeding or mechanical injury in the leaves of transgenic *Arabidopsis* lines (Table 2; Fig. 2b). Correspondingly, when a *CsSUS1p* fragment comprising nucleotides −268 to −103 was fused to a minimal promoter and *GUS* reporter gene (SS246), expression was restricted to cells directly surrounding wounds (Fig. 5a) suggesting that this region bears putative *cis*-acting element(s) necessary for wound-responsive expression. Because the −268 to −1 *CsSUS1p* fragment is contained within the −410 to −1 *CsSUS1p* fragment (along with each of the longer 5′ *CsSUS1p* deletion cassettes), it is reasonable to expect that wound-inducible expression would be noted, along with phloem-specific expression, in these lines. This was indeed the case, as *GUS* expression at wound sites was observed in a small minority of all *GUS*-expressing 5′ deletion lines (Table 2), and the rarity of such expression may simply have resulted from a paucity of pest-elicited or inadvertent mechanical wounding in the tissues analyzed in this study.

The observation of stress-induced expression by a sucrose synthase gene implies that this gene may play a role in providing a supply of carbohydrates to cells involved in the wound response, which is not wholly unexpected as wounded tissues behave as sinks (reviewed by Ibraheem et al. 2008). Bioinformatic analysis of the wound-responsive −268 to −103 *CsSUS1p* fragment indicated the presence of several pathogen-induced

*cis*-acting elements. In particular, a "W-box" (TGACY), which is the binding site of WRKY transcription factors (Rushton et al. 1996) and has been suggested to be involved in the activation of expression as a result of wounding (Rushton et al. 2002; Nishiuchi et al. 2004) was found at nucleotide −139 relative to the translational start site. However, further research will be required to determine whether this site plays a similar role in the wound-responsiveness of the *CsSUS1p*.

In summary, we have identified two separate alleles of the upstream *SUS1* region from *C. sinensis* that are capable of driving phloem-specific expression of a reporter gene in the leaves of multiple plant species. A 410-bp 3′ fragment of this sequence was able to effectively elicit this same pattern of expression, while the region between nucleotides −410 and −268 was found to be capable of conferring phloem specificity when fused to a minimal promoter. Furthermore, the region comprising nucleotides −268 to −103 of the *CsSUS1p* was found to be responsible for eliciting *GUS* expression in cells immediately surrounding wound sites in the leaves of *Arabidopsis*. Interestingly, the main difference between the two *CsSUS1p* alleles was the presence of a 94-bp indel, which was found to confer floral organ-specific expression of a reporter gene in *Arabidopsis* when fused to a minimal promoter. Although we have identified three discrete regions of a single promoter which drive phloem-, wound-, and floral-specific expression, respectively, further study will be necessary to characterize the specific enhancer elements that direct each of these expression patterns. The use of promoters that drive localized transgene expression specifically in tissues targeted by particular pests or in response to wounding is of tremendous importance in the future generation of resistant plants as it would restrict any introduced gene product(s) to sites of attempted pathogen invasion. Therefore, the highly specific, efficient and separable expression directed by various fragments of the *CsSUS1p* could be of great value for the targeting of a range of different pathogens, including those that limit themselves to the phloem.

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