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ORIGINAL PAPER

GUS expression in sweet oranges (*Citrus sinensis* L. Osbeck) driven by three different phloem-specific promoters

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Abstract Huanglongbing (HLB) is associated with *Candidatus* Liberibacter spp., endogenous, sieve tube-restricted bacteria that are transmitted by citrus psyllid insect vectors. Transgenic expression in the phloem of specific genes that might affect *Ca.* Liberibacter spp. growth and development may be an adequate strategy to improve citrus resistance to HLB. To study specific phloem gene expression in citrus, we developed three different binary vector constructs with expression cassettes bearing the β-glucuronidase (GUS) reporter gene (*uid*A) under the control of one of the three different promoters: *Citrus phloem protein 2* (CsPP2), *Arabidopsis thaliana phloem protein 2* (AtPP2), and *Arabidopsis thaliana sucrose transporter 2* (AtSUC2). Transgenic lines of 'Hamlin', 'Pera', and 'Valencia' sweet oranges [*Citrus sinensis* (L.) Osbeck] were produced via *Agrobacterium tumefaciens* transformation. The epicotyl segments collected from in vitro germinated seedlings were used as explants. The gene *npt*II, which confers resistance to the antibiotic kanamycin, was used for selection. The transformation efficiency was expressed as the number of GUS-positive shoots over the total number of explants and varied from 1.54 to 6.08 % among the three cultivars and three constructs studied. Several lines of the three sweet orange cultivars analyzed using PCR and Southern blot analysis were genetically transformed with the three constructs evaluated. The histological GUS activity in the leaves indicates that the *uid*A gene was preferentially expressed in the phloem, which suggests that the use of the three promoters might be adequate for producing HLB-resistant transgenic sweet oranges. The results reported here conclusively demonstrate the preferential expression of GUS in the phloem driven by two heterologous and one homologous gene promoters.

Key message The results reported here conclusively demonstrate the preferential expression of GUS in the phloem driven by two heterologous and one homologous gene promoters.

Keywords Disease resistance · Genetic transformation · Histological analysis · Huanglongbing

Introduction

Huanglongbing (HLB) is currently considered to be the most destructive disease in citrus because no resistance genes have been found in the *Citrus* genome. HLB is associated with endogenous sieve tube-restricted bacteria (*Candidatus* Liberibacter spp.) that are transmitted by citrus psyllid insect vectors (*Diaphorina citri* in Asia and America and *Trioza erytreae* in Africa) (Bové 2006).

The eradication of affected trees, vector population control, and establishment of new groves with pathogen-

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free nursery trees are among the recommendations for HLB management. However, genetic resistance to HLB in the sweet orange must be pursued through cultivar improvement.

Among the several tools that can be integrated into citrus breeding programs, genetic transformation is a promising option in which several strategies to obtain resistance may be applied, including the use of RNA-mediated gene silencing, major resistant (*R*) genes, genes that code for antimicrobial peptides, and genes that code for pathogenesis-related (PR) proteins.

The majority of the research concerning disease resistance through genetic transformation in citrus crops has involved gene constructs bearing strong, constitutive promoters, especially the 35S promoter (derived from the *Cauliflower mosaic virus*). However, relatively recent studies have indicated that this approach in plant biotechnology might lead to undesirable effects (Singer et al. 2011).

We have hypothesized that strategies for resistance to HLB in citrus mediated through genetic transformation must include the use of phloem-specific promoters to deliver the coding sequence directly to the phloem, where the bacteria colonize the plant and new infections occur as the result of psyllid feeding. Therefore, to study specific phloem gene expression in citrus, three different binary vector constructs with expression cassettes bearing the β-glucuronidase (GUS) reporter gene (*uid*A) under the control of the promoters *Citrus phloem protein 2* (CsPP2), *Arabidopsis thaliana phloem protein 2* (AtPP2), and *Arabidopsis thaliana sucrose transporter 2* (AtSUC2) were developed and utilized in the genetic transformation of three different sweet orange cultivars. Although the transformation efficiency varied among gene constructs and citrus cultivars, transgenic transformation was confirmed in all studied cases. Histological analyses of the confirmed transgenic plants demonstrated that the *uid*A gene was preferentially expressed in the phloem. Therefore, we concluded that the use of the three promoters might be adequate for producing HLB-resistant transgenic sweet oranges.

Materials and methods

Plant material

Seeds were extracted from ripe fruits of *Citrus sinensis* cvs. 'Hamlin', 'Valencia', and 'Pera' and dried at room temperature (24 h). The seed coat was removed, and surface sterilization was performed with sodium hypochlorite solution (0.5 %) for 15 min, followed by three rinses in sterile distilled water. The seeds were cultured in test tubes (150 × 25 mm) containing MS culture medium (10 mL; Murashige and Skoog 1962) supplemented with sucrose (25 g L^{-1}), solidified with phytagel (2 g L^{-1}; Sigma), and with the pH adjusted to 5.8 before autoclaving. The cultures were incubated at 27 °C in the dark for 30 days followed by 10–15 days under a 16-h photoperiod (40 μmol m^{-2} s^{-1}). Subsequently, the seedlings were used as the explant source for genetic transformation experiments.

Plasmid constructs

Three different binary vector constructs were developed that contained expression cassettes bearing the β-glucuronidase (GUS) reporter gene (*uid*A) under the control of one of three different promoters: *Arabidopsis thaliana phloem protein 2* (AtPP2), *Citrus phloem protein 2* (CsPP2) and *Arabidopsis thaliana sucrose transporter 2* (AtSUC2). A 980-bp segment of the promoter region of the *Arabidopsis thaliana* AtPP2-A1 gene (At4g19849) was amplified by PCR using the following pair of primers: AtPP2-F (5′AAGCTTTATGCAA CACATGATTG3′) and AtPP2-R (5′CCATGGACCAGT ATGATGTATTTATTT3′). This gene is orthologous to the *Cucurbita maxima* phloem protein 2 gene (L31550), which has been shown to be specifically expressed in phloem companion cells (Bostwick et al. 1992). The *Citrus sinensis* EST sequence (CB292710), which encodes the N-terminus of a protein with similarity to the *Cucurbita maxima* phloem protein 2, was found in GenBank using tblastn. A 560-bp fragment of the *Citrus sinensis* genomic sequence upstream from this EST was cloned by genome walking using the GenomeWalker Universal kit (Clontech). A 1,009-bp fragment of the promoter region of the *A. thaliana* sucrose transporter (AtSUC2) gene was amplified by PCR using the primer pair AtSUC2-F (5′AAGCTTCACACCACATTTAA ATAGTTTA3′) and AtSUC2-R (5′CCATGGTTGACAAA CCAAGAAAGTAAG3′). These promoters were cloned first into the *Hin*dIII and *Nco*I restriction sites of pCAMBIA1201 to replace the CaMV35S promoter that controls the *uid*A gene, and subsequently, the promoter-*uid*A cassette was transferred to pCAMBIA2201 using the *Hin*dIII and *Bst*EII restriction sites. The gene *npt*II, which confers resistance to the antibiotic kanamycin, was used as the selection marker during plant transformation (Fig. 1).

Genetic transformation and plant regeneration

Epicotyl segments (0.8–1 cm) collected from in vitro germinated seedlings were incubated with an *Agrobacterium* suspension (5 × 10^8 cell mL^{-1}; 10 min) containing AtPP2, AtSUC2, or CsPP2 expression cassettes. After inoculation, the explants were blotted dry and transferred to MT culture medium (Murashige and Tucker 1969) supplemented with benzylaminopurine (BAP 1.0 mg L^{-1}) for a 2-day

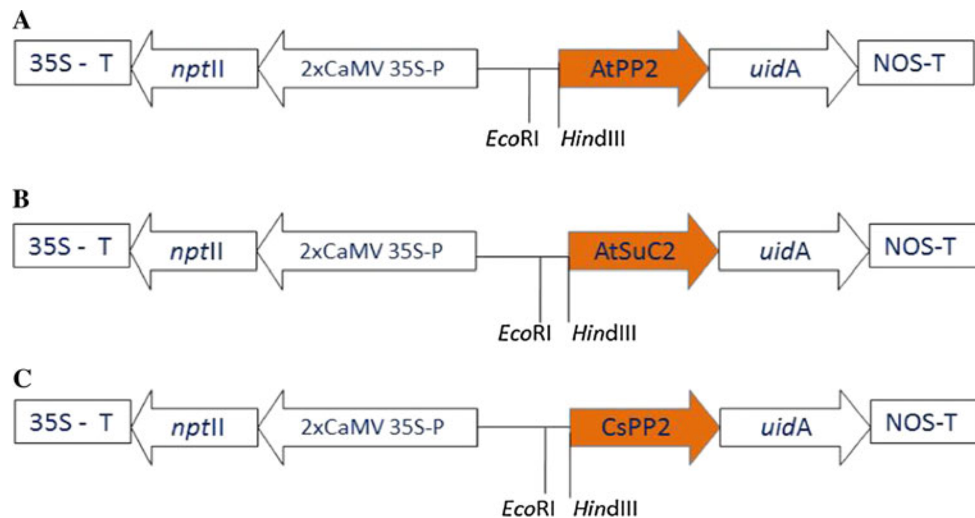

Fig. 1 Schematic representation of constructs utilized in the study. All constructs were prepared in a pCAMBIA2201 background with a *npt*II gene selection agent under the control of the 35S promoter. **a** Expression cassette AtPP2 containing the *uid*A gene driven by the AtPP2 (*Arabidopsis thaliana phloem protein*) promoter; **b** expression cassette AtSUC2 containing the *uid*A gene driven by the AtSUC2 (*Arabidopsis thaliana sucrose transporter* 2) promoter; **c** expression cassette CsPP2 containing the *uid*A gene driven by the CsPP2 (*Citrus phloem protein* 2) promoter

co-cultivation period (24 °C in the dark). After co-cultivation, the explants were transferred to a selection medium consisting of MT medium supplemented with BAP (1 mg L^{-1}), kanamycin (50 mg L^{-1}), and cefotaxime (500 mg L^{-1}). Cultures were incubated in the dark (27 °C) for 4 weeks and then kept under a 16-h photoperiod (40 µmol m^{-2} s^{-1}) at 27 ± 1 °C. The developed shoots (0.5–0.8 cm long) were carefully excised from the explant for in vitro grafting. These shoots were carefully prepared into graft tips to be top grafted onto Carrizo citrange [*Citrus sinensis* × *Poncirus trifoliata* (L.) Raf] seedlings grown in test tubes (150 × 25 mm) containing MS culture medium. Plants were then cultivated at 27 °C under a 16-h photoperiod (40 µmol m^{-2} s^{-1}) for about 30 days. Developed in vitro grafted plants were further transferred and acclimatized in plastic pots (0.4 L) containing a commercial potting mix for development and further analysis.

Molecular confirmation of transgenic citrus lines

Genomic DNA was extracted (Doyle and Doyle 1990) from the leaves of developed plants after in vitro grafting. Putative transgenic plants were identified using PCR analysis. Plants containing the gene construct AtPP2 were analyzed with the pair of primers 5′-CGATAGTTGCTGC CAAAAC-3′ and 5′-ACGAGTCGTCGGTTCGT-3′, which amplify a 504-bp fragment corresponding to the AtPP2 promoter and part of the *uid*A gene. Plants regenerated from experiments with the gene construct AtSUC2 were analyzed with the pair of primers 5′-CTATCCGAATTTCT CGCTTC-3′ and 5′-ACGAGTCGTCGGTTCGT-3′, which amplify a 694-bp fragment corresponding to part of the AtSUC2 promoter and part of the *uid*A gene. The reactions were performed under the following conditions: 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C, with a final extension at 4 min for 72 °C. Plants containing the gene construct CsPP2 were analyzed with the primer pair 5′-CAACGAACTGAACTG GCA-3′ and 5′-CATCACCACGCTTGGGTG-3′, which amplify a 817-bp fragment of the *uid*A gene. PCR-positive plants were acclimatized to greenhouse conditions.

Southern blot analyses were performed on the PCR-positive plants to confirm the genetic transformation. DNA was extracted from the fully expanded leaves of acclimatized plants. A non-transgenic plant was used as a negative control, and the fragment of the promoters and part of the *uid*A gene were used as positive controls. DNA was digested with *Eco*RI (16 h; 37 °C), separated on an agarose gel (0.8 %) using electrophoresis (30 V cm^{-1}; 16 h), transferred to a positively charged nylon membrane (Hybond-N^{+}, GE Healthcare) and subsequently fixed at 80 °C. A PCR-amplified alkaline phosphatase-labeled (AlkPhos Direct Labeling and Detection System, GE Healthcare) fragment from the coding region of the *uid*A gene (817 bp) was used as a probe. Pre-hybridization, hybridization (60 °C), washing and detection were performed with a Gene Images CDP-Star Detection Kit (GE Healthcare) according to the supplier's instructions.

Histochemical GUS assay and histological analyses

The GUS activity in regenerated plants was determined using the histochemical method. Young leaves were incubated in X-GLUC solution (5-bromo-4-chloro-3-indolyl-beta-D-glucoronide) at 37 °C overnight. The leaves were then cleared by incubation in 70 % ethanol and examined using a stereomicroscope (Jefferson 1987). Leaves from a transgenic sweet orange plant bearing a gene construct including the *uid*A gene controlled by 35S promoter were used as the positive control. A negative control (non-transgenic sweet orange plant) was also prepared. Gene expression in the phloem region was determined by microscopic analysis of the petiole segments. For the histological analyses, the petiole segments (0.5–1.0 mm) were sampled from acclimatized plants. The samples were incubated in X-GLUC solution (5-bromo-4-chloro-3-indolyl-beta-D-glucoronide) at 37 °C for 3–5 h, washed with ethanol (70 %) to remove the chlorophyll, dehydrated in ethanol (80, 90 and 100 %), and embedded in plastic resin (Leica Historesin®, Heidelberg, Germany). Serial cross sections (5 µm thickness) were cut and mounted on Entellan® synthetic resin (Merck, Darmstadt, Germany). The sections were observed, and images were captured digitally with a Leica DM LB microscope with a video camera attached to a PC using the IM50 image analysis software.

Results and Discussion

Genetic transformation was accomplished for the three sweet orange cultivars and for the three gene constructs involved in the study. The preliminary identification of the putative transgenic plants was conducted using PCR in GUS-positive regenerated shoots. The expected fragments of lengths 504, 694, and 817 bp corresponding to the AtPP2, AtSUC2, and CsPP2 genetic constructs, respectively, were amplified in several lines for the three sweet orange cultivars (Fig. 2).

The confirmation of the genetic transformation was verified in PCR-positive plants through Southern blot analysis. Digestion of the genomic DNA by the *Eco*RI enzyme indicated that most of the evaluated plants were derived from independent transformation events. However, similar fragment lengths were observed in ‘Hamlin’ sweet orange transgenic lines for the AtSUC2 constructs (Fig. 3). Most of the transgenic lines had one transgene insertion event, as determined using Southern blot analysis, except for one line of the ‘Pera’ sweet orange, which was transformed with the AtPP2 construct (Fig. 3). No hybridization was observed in the DNA samples of the control (non-transgenic) plants. The transformation efficiency, expressed as the number of GUS-positive shoots per total explant, varied from 1.54 to 6.08 % (Table 1).

The GUS activity in young leaves collected from several transgenic lines expressing the three studied promoters indicated that the expression was concentrated in the vascular bundle for the three sweet orange cultivars studied (Fig. 4).

Histological analyses of the petiole segments confirmed preferential GUS expression in the phloem tissue and some weak expression in the parenchymal radial cells of the xylem (Fig. 5). Moreover, relatively strong GUS expression was detected in the companion and radial parenchyma cells of the phloem (Fig. 5).

Most citrus genetic transformation studies for disease resistance have used the strong and constitutive 35S *Cauliflower mosaic virus* gene promoter (Gutiérrez-E et al. 1997; Peña et al. 1997, 2008; Yang et al. 2000; Domínguez et al. 2000; Fagoaga et al. 2001; Febres et al. 2003, 2008; Kayim et al. 2004; Boscariol et al. 2006; Fagoaga et al. 2006; Mitani et al. 2006; Gentile et al. 2007; Distefano et al. 2008; Zanek et al. 2008). However, a few studies have demonstrated the effectiveness of citrus genetic transformation to control spatial and temporal gene expression. Thus, Valencia sweet orange transgenic lines that contain the *cecropin*MB39 gene that codes for an antimicrobial peptide were produced under the control of a phenylalanine ammonia-lyase gene promoter from citrus in an attempt to target *Xylella fastidiosa* bacteria, which are the causal agent of citrus variegated chlorosis (CVC) and colonize xylem vessels (Paoli et al. 2007). In another study, transgenic ‘Hamlin’ sweet orange plants expressing the *hrpN* gene, which is a harpin (plant elicitor) derived from *Erwinia amylovora* (Barbosa-Mendes et al. 2009) were produced. To prevent the production of abnormal, and sometimes lethal, phenotypes in plants that constitutively express plant elicitors, the construct included the promoter from the potato defense *gst1* gene that encodes glutathione S transferase, which is activated by several pathogens in the potato. Some of the sweet orange transgenic lines displayed gene expression after *Xanthomonas axonopodis* pv. *citri* inoculation and demonstrated an increased resistance to this pathogen.

The goal of our study was to identify potential gene promoters that could be associated with gene expression in the sieve elements of phloem in an attempt to introduce specific genes to inhibit the growth and development of *Ca.* Liberibacter spp., the causal agent of HLB, which colonizes this tissue in sweet oranges. Furthermore, we hypothesized that constructs containing these promoters, including the *uid*A gene, could provide preferential expression, thereby permitting the development of specific constructs for HLB control by genetic transformation.

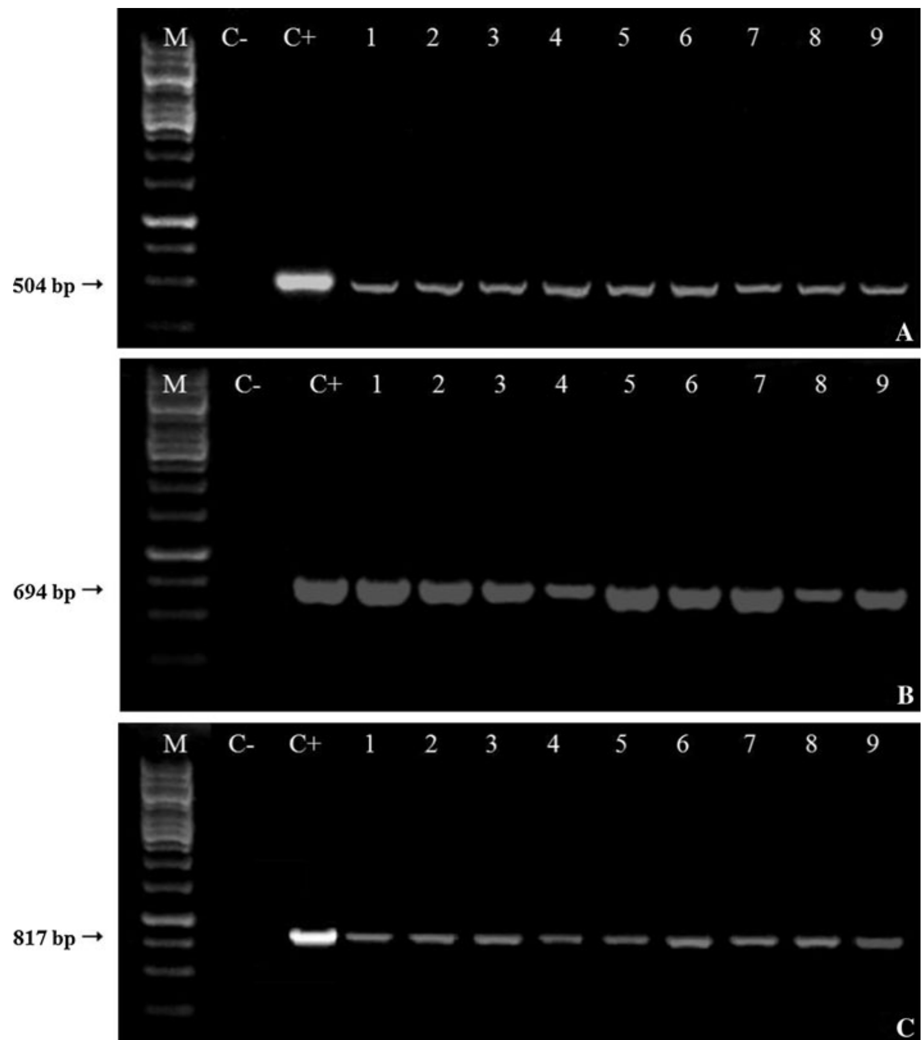

Fig. 2 PCR analyses for detection of AtPP2 (**a**), AtSUC2 (**b**), and GUS from CsPP2 gene construct (**c**). *M* 1 kb ladder (Fermentas), *C−* negative control (water), *lanes 1–3* plants of 'Hamlin' sweet orange, *lanes 4–6* plants of 'Valencia' sweet orange, *lanes 7–9* plants of 'Pera' sweet orange

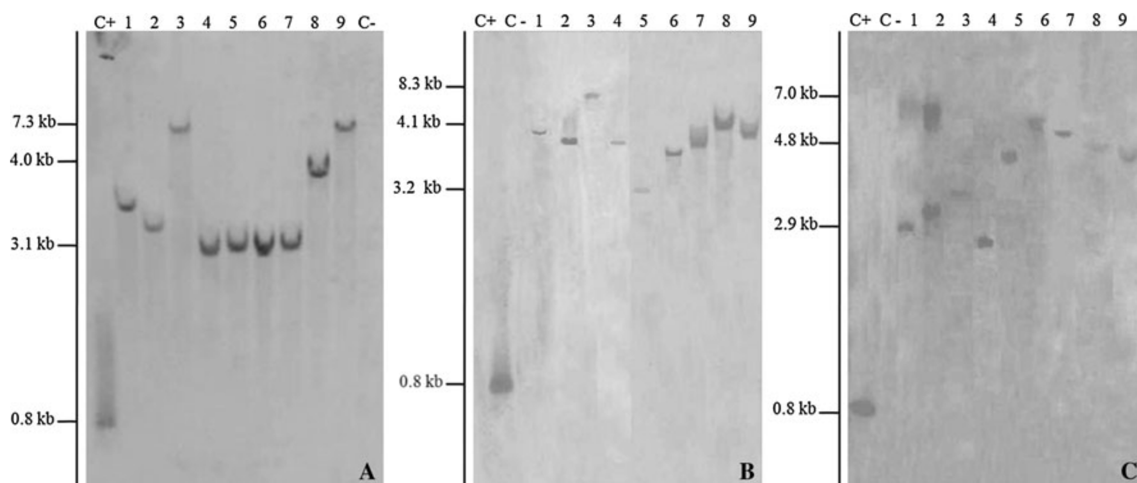

Fig. 3 Southern blot analyses of *Citrus sinensis* cvs. 'Hamlin' (**a**), 'Valencia' (**b**), and 'Pera' (**c**). DNA (40–60 μg) was digested with *Eco*RI and hybridized with a probe corresponding to an 817-bp fragment of the *uid*A gene. *C+* positive control (817 bp *uid*A gene fragment), *C−* negative control (DNA from a non-transgenic plant), *lanes 1–3* AtPP2 gene construct transgenic plants, *lanes 4–6* AtSUC2 gene construct transgenic plants, *lanes 7–9* CsPP2 gene construct transgenic plants

Table 1 *Citrus sinensis* cvs. 'Hamlin', 'Valencia', and 'Pera' genetic transformation with gene constructs AtPP2, AtSUC2, and CsPP2

Gene construct	Responsive explants/ total explants	GUS+ shoots/ evaluated shoots	Genetic transformation efficiency (%)[a]
'Hamlin'			
AtPP2	33/1950	34/47	1.79
AtSUC2	81/1730	77/108	4.40
CsPP2	115/2020	123/157	6.08
'Valencia'			
AtPP2	394/2318	95/523	3.68
AtSUC2	170/1770	62/205	3.59
CsPP2	153/2360	17/167	2.87
'Pera'			
AtPP2	127/2210	34/174	1.54
AtSUC2	111/2480	36/145	1.59
CsPP2	134/2900	31/154	1.70

[a] Number of GUS+ shoots/ total explants

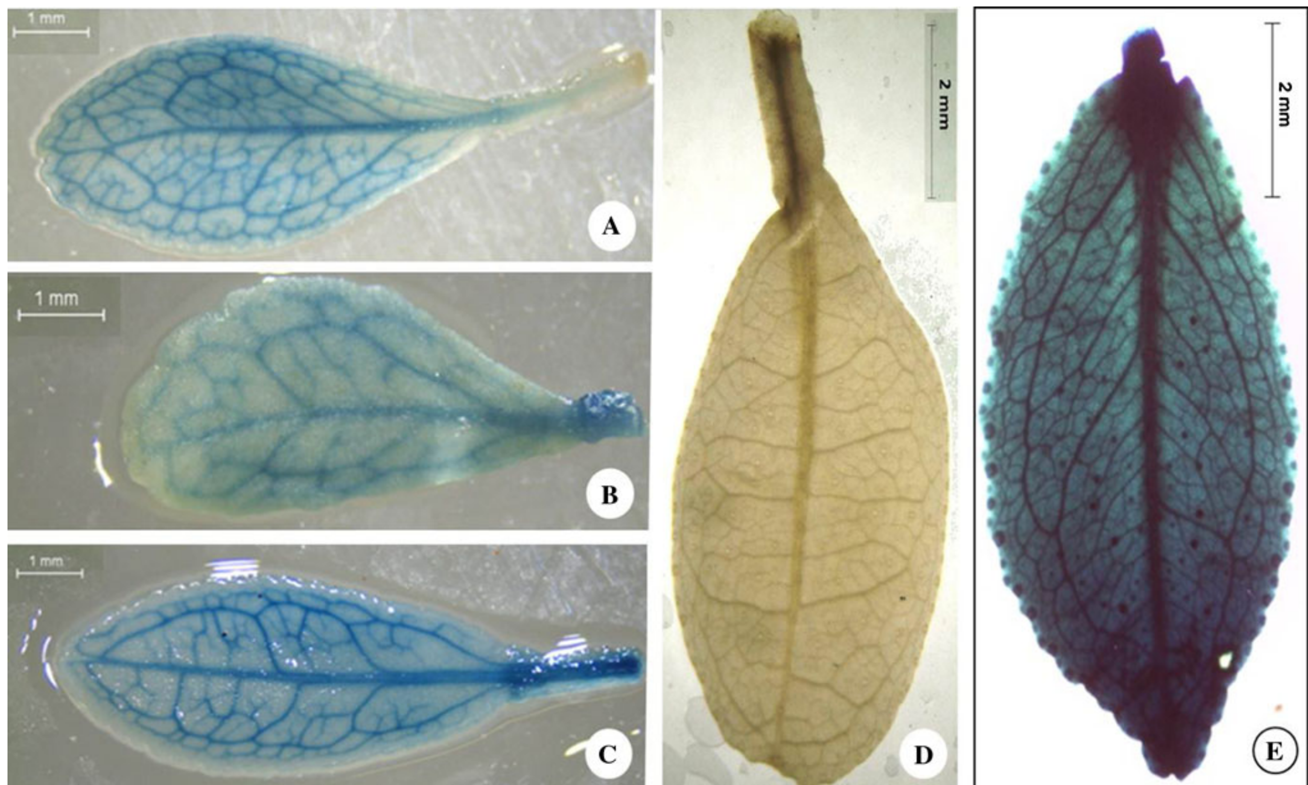

Fig. 4 Leaves of *Citrus sinensis* cv. 'Hamlin' containing the gene construct AtPP2 (**a**), AtSUC2 (**b**), CsPP2 (**c**) showing GUS activity concentrated in the vascular tissue, **d** negative control (non-transgenic plant), and **e** positive control (transgenic plant bearing a gene construct including the *uid*A gene controlled by 35S promoter)

In our study, we decided to evaluate two heterologous gene promoters and one homologous gene promoter involved in gene expression in the phloem via the transformation of three different and commercially important sweet orange varieties. The results indicated adequate transformation efficiency with the expected variation among cultivars. Previous studies (Mendes et al. 2002) have shown that, in general, 'Hamlin' sweet oranges might show higher transformation efficiencies than other sweet orange cultivars.

Leaf preparations from sweet orange cultivars indicated that the GUS activity was concentrated in the phloem in the transgenic lines expressing the three studied promoters. Histological analyses of the petiole segments confirmed

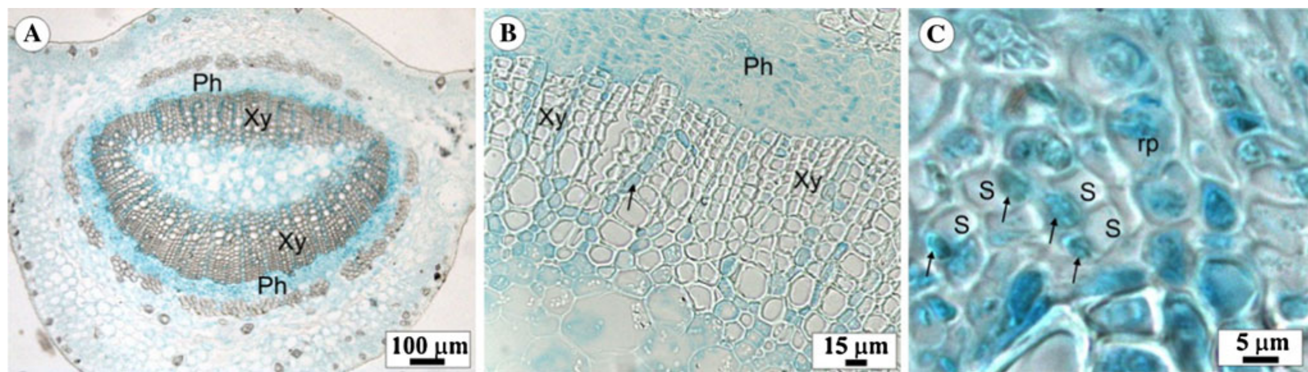

Fig. 5 Histological analyses of the petiole segments from *Citrus sinensis* cv. 'Hamlin' transgenic plants containing the gene construct pCAMBIA2201-CsPhP2-GUS. **a** Transverse section of a leaf petiole showing preferential GUS expression in the phloem tissue. **b** Detailed view of the vascular system of the leaf petiole. The *arrow* indicates GUS activity in the radial parenchyma. Note the absence of GUS activity in the tracheary elements. **c** Companion cells (*arrowed*) and radial parenchyma cells (*rp*) of the phloem stained for GUS activity and sieve elements (*s*) without staining. *Ph* phloem tissue, *Xy* xylem tissue

preferential GUS expression in the phloem tissue as well as some weak expression in the radial parenchyma cells of the xylem. Moreover, consistent GUS expression was specifically observed in the radial parenchyma and companion cells. These results indicate that the genes associated with these promoters might play an important role in the active transport of sucrose from the apoplast into the companion cells, as previously reported (Sauer 2007).

Relatively few differentially expressed promoters have been studied in plants. A few studies related to the promoter of the gene that encodes sucrose synthase have been reported, including those in *Arabidopsis* (Martin et al. 1993; Dutt et al. 2012), maize (Yang and Russel 1990), rice (Wang et al. 1992), and even citrus (Singer et al. 2011).

However, few other studies have addressed the promoters utilized in the present study. One of these promoters drives the expression of an important gene involved in the long-distance transport of sucrose, AtSUC2, from *A. thaliana*. Along with AtSUC1, AtSUC2 acts as a primary phloem-loading transporter via a sucrose-H^+ symporter (Sauer and Stolz 1994). In *Arabidopsis*, the AtSUC2 carrier catalyzes phloem loading to transport sucrose from the apoplast into the companion cells and into the conducting sieve tubes (Stadler and Sauer 1996; Gottwald et al. 2000). The evidence of phloem loading and unloading through AtSUC2 was also demonstrated by GUS reporter activity in transgenic plants for constructs containing the β-glucuronidase (*uid*A) gene fused with the SUC2 promoter in *Arabidopsis* (Truernit and Sauer 1995), in strawberry (Zhao et al. 2004), and 'Mexican' lime (*Citrus aurantifolia* Swingle) (Dutt et al. 2012).

Another promoter utilized in the present study drives the expression of the phloem protein 2 gene. More than 100 phloem proteins have been described for cucurbitaceous plants. However, PP1 and PP2 are predominant (Bostwick et al. 1992; Guo et al. 2004). The PP2 proteins are dimeric lectins with a size of approximately 49 kDa, are linked to filaments by bisulfide bridges (Read and Northcote 1983) and might be associated with different plant functions, including plant defense and plant–microbe interactions (Read and Northcote 1983; Gómez and Pallás 2001; Owens et al. 2001). Transgenic tobacco plants with a gene construct bearing the *uid*A gene fused with a PP2 promoter from *Cucurbita moschata* indicated preferential activity in the phloem tissue (Guo et al. 2004). In our study, the promoters from orthologs of the PP2 gene from *A. thaliana* and *Citrus sinensis* were transformed into *Citrus sinensis*, which showed preferential expression in the phloem tissue. Interestingly, a gene encoding a PP2-like protein was found to be upregulated in citrus plants infected with the huanglongbing bacterium *Ca.* Liberibacter asiaticus but it was distinct from the member of the gene family from which we cloned the promoter (Albrecht and Bowman 2008; Kim et al. 2009). Genetic transformation for disease resistance in woody crops has been reported with consistent results for virus resistance, especially the plum pox model in *Prunus* (Ravelonandro et al. 2000; Scorza et al. 2001; Collinge et al. 2008) and papaya (Tennant et al. 1994). However, partial resistance to fungal and bacterial infections has also been reported, especially in apple and citrus (Bolar et al. 2000; Fagoaga et al. 2001; Faize et al. 2004; Azevedo et al. 2006; Boscariol et al. 2006; Cardoso et al. 2010; Mendes et al. 2010). Therefore, the use of differentially expressed promoters in specific plant tissues might increase the chances of producing more bacterial disease-resistant transgenic cultivars.

To our knowledge, this is the first report of the genetic transformation of three different commercially important sweet orange cultivars with gene constructs involving the fusion of the *uid*A coding sequence with three different

phloem-specific promoters. The results reported here conclusively demonstrate the preferential expression of GUS in the phloem driven by two heterologous and one homologous gene promoters. Future studies concerning sweet orange genetic transformation for HLB resistance might involve constructs that target genes against causal agents using any of these promoters to enhance tissue-specific expression where the bacteria are located and where plants may be subjected to new infections through vector feeding.

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