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Genetics and Plant breeding

How to drive phloem gene expression? A case study with preferentially expressed citrus gene promoters

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Abstract – New approaches for developing disease-resistant genetically modified organisms have included specific targets for gene expression to enhance the chances for pathogen control. Gene expression driven by phloem-derived *Citrus sinensis* gene promoters could be evaluated and compared with the expression induced by a strong constitutive promoter in the same tissue, leading to the production of transgenic sweet oranges potentially more resistant to diseases caused by phloem-limited bacteria. 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] was transformed, via *Agrobacterium tumefaciens*, with the binary vector pCAMBIA2301 bearing the *uid*A gene (β-glucuronidase) driven by the CaMV35S constitutive promoter (CaMV35S::*uid*A) or by the CsPP2.B1 (CsPP2.B1::*uid*A) or by the CsVTE2 (CsVTE2::*uid*A) citrus promoters. *In vitro* regenerated shoots were grafted onto 'Rangpur' lime (*C. limonia* Osbeck). The genetic transformation was confirmed by *Southern blot* analyses. *uid*A gene expression was evaluated by RT-qPCR, and gene histolocalization controlled by these three promoters was accessed by X-GLUC treated stem sections. *uid*A gene expression exhibited by tissue-specific promoters was overall lower than from the constitutive promoter CaMV35; however, constructs driven by tissue-specific promoters may lead to expression in restricted tissues. CsPP2.B1 and CsVTE2 promoters can be considered adequate for the utilization in gene constructs aiming disease resistance.
Index Terms: *Candidatus* Liberibacter spp.; disease resistance; genetic transformation; GUS; histolocalization.

Como regular a expressão de genes no floema? Um estudo de caso com promotores de genes de citros preferencialmente expressos

Resumo – Novas abordagens para o desenvolvimento de organismos geneticamente modificados, resistentes a doenças, incluem alvos específicos de expressão gênica, visando a aumentar as chances de controle do patógeno. A expressão gênica, induzida por promotores de genes de *Citrus sinensis,* derivados do floema, poderia ser avaliada e comparada com aquela induzida por um promotor constitutivo no mesmo tecido, levando à produção de laranjas-doces transgênicas, potencialmente mais resistentes a doenças causadas por bactérias de floema. Plantas de citrange 'Carrizo' [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] foram transformadas, via *Agrobacterium tumefaciens*, com o vetor binário pCAMBIA2301 contendo o gene *uid*A (β-glucuronidase), dirigido pelo promotor constitutivo CaMV35S (CaMV35S :: uidA) ou pelos promotores de citros *Cs*PP2.B1 (CsPP2 .B1 :: *uid*A) ou CsVTE2 (CsVTE2 :: *uid*A). Brotos regenerados *in vitro* foram enxertados em limão 'Cravo' (*C. limonia* Osbeck). A transformação genética foi confirmada por análises de *Southern blot*. A expressão do gene *uid*A foi avaliada por RT-qPCR, e a histolocalização do gene, dirigida por esses três promotores, foi acessada por seções de ramos tratadas com X-GLUC. A expressão do gene *uid*A, exibida por promotores específicos de tecido, foi, em geral, menor do que a do promotor constitutivo CaMV35; no entanto, as construções dirigidas por promotores específicos de tecido podem levar à expressão em tecidos restritos. Os promotores CsPP2.B1 e CsVTE2 podem ser considerados adequados para utilização em construções gênicas, visando à resistência a doenças.
Termos para indexação: *Candidatus* Liberibacter spp.; resistência a doenças; transformação genética; GUS; histolocalização.

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Introduction

The production of disease-resistant genetically modified organisms (GMOs) has been significantly increased within the last three decades. In the case of citrus, the first generation of GMOs included the production of transgenic plants expressing reporter genes, normally controlled by strong constitutive promoters. After the optimization of the transformation protocol, GM plants have been produced expressing large spectrum antimicrobial peptides (AMPs) or other candidate genes frequently driven by constitutive promoters. The most recent generation of GMOs for disease resistance has now been produced, including strategies to target gene expression within specific tissues where the potential pathogen is hosted, and also aiming to avoid the unnecessary gene expression in all plant tissues. The idea is to encode specific proteins using tissue promoters to activate the expression of a certain gene in a particular cell type without causing any changes in the whole plant tissues (ALI; KIM 2019). Moreover, the DNA sequencing results from different species have allowed the identification of tissue-specific expressed genes and their respective promoters, leading to a broad possibility of their use (DUTT et al. 2012; DONMEZ et al. 2013). Therefore, the search and evaluation of new tissue-specific promoters, derived from their own plant species, that could directly or preferentially target gene expression within the pathogen preferential site of colonization, could be very beneficial for applying these new strategies on the production of potentially disease-resistant GMOs (SCHAART et al. 2016).

In citrus, huanglongbing (HLB) is considered one of the most devastating diseases that threaten the citrus industry worldwide. HLB-associated bacteria (*Candidatus* Liberibacter spp.) can grow inside the sieve tube elements of the phloem (STEs), leading to significant changes in the metabolism of the infected plants (BOVÉ 2006). Soon after the HLB report in Brazil, two species were found associated with this disease, *Ca*. L. americanus (*C*Lam), and *Ca*. L. asiaticus (*C*Las), both transmitted through contaminated tissues or by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (TEIXEIRA et al. 2005). Surveys conducted in the following years revealed that *C*Las has been the predominant species in citrus groves in Brazil (SILVA et al. 2019), in Florida, USA, and worldwide (FOLIMONOVA et al. 2009).

Hypertrophy and hyperplasia in parenchyma cells of HLB infected plants have been reported for decades (SCHNEIDER, 1968). Recent ultrastructural studies revealed that early stages of *C*Las infection in sweet orange plants include a significant swelling of the middle lamella between cell walls, around the sieve elements. Plants with advanced symptoms of HLB infection show necrotic phloem cell parenchyma pocket formations, leading to an interruption in the flow of photoassimilates, and, consequently, to a sequence of events that result in the disease symptoms, including leaf yellowing (leaf mottling), plant stunting, small, irregular, and acid fruits, aborted seeds, and, consequently, yield reduction leading to grove economical unviability (FOLIMONOVA; ACHOR 2010).

Sieve tube elements colonization by the HLB-associated bacteria causes anatomical modifications of the phloem tissues, triggers plant defense mechanisms that involve the action of structural proteins (P-proteins), and induces callose deposition in injured STEs, preventing the transport of photoassimilates to the root system (FOLIMONOVA; ACHOR 2010; ETXEBERRIA; NARCISO 2012). Thus, P-proteins have a fundamental role in the plant defense mechanism against sap loss caused by injuries, as well as other functions during pathogen and pest defenses (READ; NORTHCOTE 1983; VAN BEL 2003). In general, two types of P-proteins are very abundant in the sap of angiosperms: Phloem Protein 1 (PP1) and Phloem Protein 2 (PP2) (EVERT 2006). In the peripheral cytoplasm of mature STEs, the function of P-proteins is analogous to an endoskeleton, keeping the cytoplasm of these specialized cells in a parietal position, i.e., close to the cell walls (MACHADO; CARMELLO-GUERREIRO 2012). The production site of these proteins is attributed to the companion cells of the sieve tube/companion cell complexes, where they are synthesized and then transported to the STEs through the plasmodesmata (READ; NORTHCOTE 1983). In citrus, the induction of PP2 expression was reported in the presence of *C*Las and *C*Lam (ACHOR et al. 2010; ALBRECHT; BOWMAN 2012; MAFRA et al. 2013; BOAVA et al. 2017).

In addition to PP2 genes, other genes involved in the phloem functionality have also been identified and are induced by the presence of the HLB-associated bacteria (MAFRA et al. 2013). Among those identified transcripts, a homogentisate fitiltransferase (VTE2), encodes an enzyme involved in tocopherol biosynthesis. Tocopherols are important antioxidants in the protection and adaptation of plants to the environment, and are responsible for loading substances into the phloem (MAEDA et al. 2006; MAFRA et al. 2013; MAEDA et al. 2014).

Information about the role of proteins related to the phloem tissue can create new possibilities of use in genetic engineering. Knowing the site of the protein expression driven by their promoters with reporter genes will reveal the strength and specificity of their expression. Those promoters can be used in gene construct aiming at vascular disease control, such as HLB, along with other strategies for disease control, e.g., plant defensins (LAY; ANDERSON 2005).

In citrus, there are few studies aiming to evaluate gene expression in constructs driven by endogenous, pathogen-induced or tissue-specific promoters (DUTT et al. 2012, ZOU et al. 2014), including some studies from our group (MIYATA et al. 2012; MIYATA et al. 2017). Considering these facts, we evaluated other gene constructs bearing different citrus promoters potentially expressed in the phloem tissue driving the *uid*A reporter gene expression (encoding the β-glucuronidase (GUS) protein), through more detailed molecular and histological analyses, in an attempt to select tissue-specific citrus promoters, for further use in gene constructs targeting the control of HLB.

Materials and Methods

Promoter isolation and construction of expression vectors

The sequences promoters VTE2 (*Homogentisate phytyltransferase* – accession number orange1.1g015340m.g) and PP2.B1 (*F-Box Phloem protein 2 – B1 – related* – accession number orange1.1g024543m.g) were identified from *Citrus sinensis* gene in database Phytozome (https//:phytozome.org). The promoter fragments CsVTE2 and CsPP2.B1 were PCR amplified from *C. sinensis* genomic DNA, using specific primers designed to amplify 904 bp and 475 bp, respectively. In spite of having a relatively short sequence, specially for the CsPPs.B1, the constructions bear all the necessary regulatory elements for adequate *uid*A gene expression. The promoters were cloned into pCAMBIA 2301 binary vector, replacing the CaMV35S promoter that controls the *uid*A gene (Figure 1). The original pCAMBIA2301 containing CaMV35S::*uid*A was used as control. All expression vectors were introduced into the *Agrobacterium tumefaciens* strain EHA105.

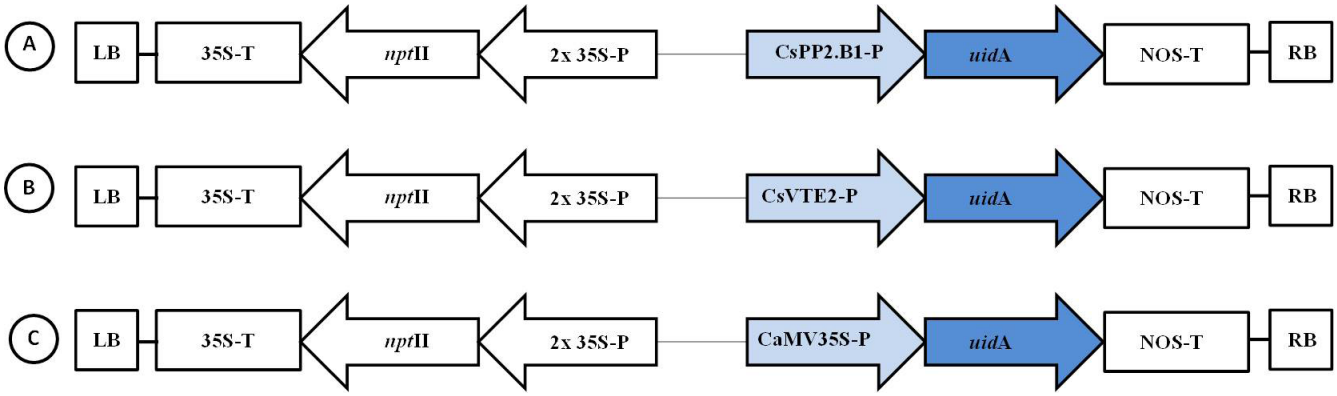

Figure 1. Schematic representation of the transfer region (T-DNA) in the three gene constructions, bearing the *uid*A gene, driven by three different promoters, and utilized to produce and evaluate different transgenic events. A: pCAMBIA2301/CsPP2.B1::*uid*A binary vector; B: pCAMBIA2301/CsVTE2::uidA binary vector; C: pCAMBIA2301/CaMV35S::*uid*A binary vector. nptII: kanamycin antibiotic resistance gene; 2xCaMV35S: CaMV35S duplicated promoter; 35S-T: 35S-T terminator; NOS-T: nopaline synthase terminator; LB: left border; RB: right border.

Plant transformation and regeneration

Epicotyl segments (0.8 – 1.0 cm) of 'Carrizo' citrange plants [*P. trifoliata* (L.) Raf. x *C. sinensis* (L.) Osbeck] obtained from *in vitro* germinated seedlings were used for genetic transformation. Explants were inoculated with *A. tumefaciens* EHA105, containing expression vectors. After inoculation, the explants were transferred to MT medium supplemented with BAP (1.0 mg L^{-1}) for 2-day co-cultivation period at 24 °C. Explants were transferred selection medium MT supplemented with BAP (1.0 mg L^{-1}), kanamycin (100 mg L^{-1}) and cefotaxime sodium (500 mg L^{-1}). Cultures were incubated in the dark for 4 weeks at 27 °C, and then transferred to a 16-h photoperiod at 27 °C. The developed shoots were *ex vitro* grafted onto 'Rangpur' lime (*C. limonia* Osbeck) (MIYATA et al., 2012). Acclimated transgenic plants were then cultivated in a greenhouse in order to be later evaluated. Six-year-old transgenic plants were used in the experiments. Plants were grown in 4.8-liter plastic bags with a commercial potting mix based on pine-bark and vermiculite (Multiplant® -Terra do Paraíso, Holambra, Brazil), and cultivated with weekly nutrient applications through fertigation.

Southern blot

The *Southern blot* analysis was carried out following standard procedures in all experimental plants to confirm the genetic transformation and to verify the number of insertion events in the citrus genome. Genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (DOYLE; DOYLE, 1990), from the young well-developed leaves of acclimatized plants. DNA samples were quantified by the Qubit™ Fluorometer using QuantiT™ dsDNA BR assay kit (Invitrogen, Carlsbad, USA). The DNA (10 to 30 µg) was digested for 16 h with the EcoRI restriction enzyme (5 U μg^{-1} – Invitrogen, Carlsbad, USA), which cuts the T-DNA in only one site. The digested DNA was separated by electrophoresis on a 0.8% agarose gel (1.3 V cm^{-1}, 16 h), transferred to a nylon membrane (Amersham Hybond-N+™ - GE Healthcare, Buckinghamshire, UK) by capillarity and the DNA was fixed at 80 °C, for 2 h. The 416 bp PCR product of the coding sequence of the *uid*A gene was labelled with alkaline phosphatase (Amersham AlkPhos Direct™ Labelling Reagents; GE Healthcare, Buckinghamshire, UK). Pre-hybridization, hybridization (60 °C) and membrane washings were performed following the supplier's instructions. The detection was performed with Amersham CDP - Star™ Detection Reagent (GE Healthcare, Buckinghamshire, UK) and the membranes were analyzed by autoradiography (Amersham Hyperfilm™ ECL, GE Healthcare, Buckinghamshire, UK).

Analysis of transgene expression by quantitative real-time PCR (qPCR)

Total RNA was extracted from young well-developed leaves by the Lithium Chloride method (CHANG et al. 1993), modified to 5% CTAB. Extracted RNA was treated with DNase I – Rnase-free (Thermo Scientific, Vilnius, Lithuania), and was quantified and 260/280 ratios were estimated using NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). The RNA integrity was evaluated in a 1.5% agarose gel. cDNA synthesis, corresponding to each sample were generated using Platus Transcriber RNase H- cDNA First Strand kit (Sinapse Inc, Hollywood, USA) and following the manufacturer's protocol, using 1 µg of RNA. PCR was performed for three biological replicates, with three technical replicates from each transgenic plant, non-transgenic plant and water as control. The qPCR was performed using GoTaq™ qPCR Master Mix (Promega, Madison, USA) on 7500 Fast™ Real-Time PCR System (Applied Biosystems, Foster City, USA). The reaction was accomplished using 3.0 µL cDNA and 300 nM of each gene-specific primer in a final volume of 15.5 µL.

Quantification cycle (*C*q) values and primer pairs efficiencies were determined for each individual reaction using Real-time PCR Miner (ZHAO; FERNALD 2005). Gene expression analyses were performed according to the Δ*C*q model using *F-box and Ef1-α* reference genes (MAFRA et al. 2013) (Table 1) to verify *uidA* gene expression in the citrus transgenic plants.

The efficiency of qPCR amplification and threshold cycle (Ct) for each gene were obtained using the Miner web-based tool (http://ewindup.info/miner/) from raw data of the kinetics of individual qPCR assay, according to Zhao and Fernald (2005). The relative quantification of gene expression level was calculated using the GenEx v2.6.4 software (MultiD analysis, Exiqon, USA).

Table 1. Primer sequences utilized for *f-box, ef1*-α and *uid*A genes amplifications in qPCR analyses, and estimated sizes of the respective amplicons.

Gene	Sequence 5'-3'*F*	Sequence 5'-3'*R*	Amplicon (bp)
f-box	TTGGAAACTCTTTCGCCACT	CAGCAACAAAATACCCGTCT	112
ef1-α	TCAGGCAAGGAGCTTGAGAAG	GGCTTGGTGGGAATCATCTTAA	80
*uid*A	TGTGGAGTATTGCCAACGAA	GAGCGTCGCAGAACATTACA	135

Histolocalization of *uid*A gene expression

Free-hand longitudinal and cross-sections were made in 3-5 cm portions of young shoots, 15 cm from the shoot apical meristem. Stem sections were immersed in X-GLUC solution (1 mM) (JEFFERSON et al. 1987) and incubated for 30 to 120 minutes at 37 ºC in darkness. The time of incubation varied according to the necessity for the tissue to react with the X-GLUC solution. After the incubation period, the samples were washed according to the methodology of Jefferson et al. (1987) with ethanol:acetic acid solution (3:1) to stop the enzymatic reaction and tissue diaphanization. To visualize the *uid*A gene expression, the microscope slides were mounted in ethanol (70%). To detect the nucleus of the STEs companion cells, the sections were hydrated in decreasing ethylic series (70, 50, 30, 10%), and then washed in distilled water and stained with DAPI fluorescent dye (0.1 µg mL^{-1} in phosphate buffer) (RUZIN 1999). In order to obtain an overview of *uid*A gene expression, low magnification images were captured in a Leica M205 C stereomicroscope coupled to a Leica DFC295 video camera, LAS 4.0 software. For the high magnification, the slides were observed under the Leica DM5500B epifluorescence microscope with a coupled Leica DFC295 video camera. For the DAPI-stained sections analysis, the DAPI filter (Ex: 340-380 nm; Em: 425 nm) was used.

Results and Discussion

Southern blot

The transgene insertion was confirmed in 16 plants for the CsPP2.B1::*uid*A construct, in eight plants for the CsVTE2 *uid*A construct, and in three plants for the CaMV35S::*uid*A construct. No signs of hybridization were observed in DNA samples from non-transgenic plants, used as negative controls. The *Southern blot* positive plants were then used in further analyses. The number of copies of the transgenes varied from one to two, with most plants exhibiting a single copy. The T-DNA insertions occurred in different genome positions within the evaluated plants bearing the different gene constructs (Figure 2).

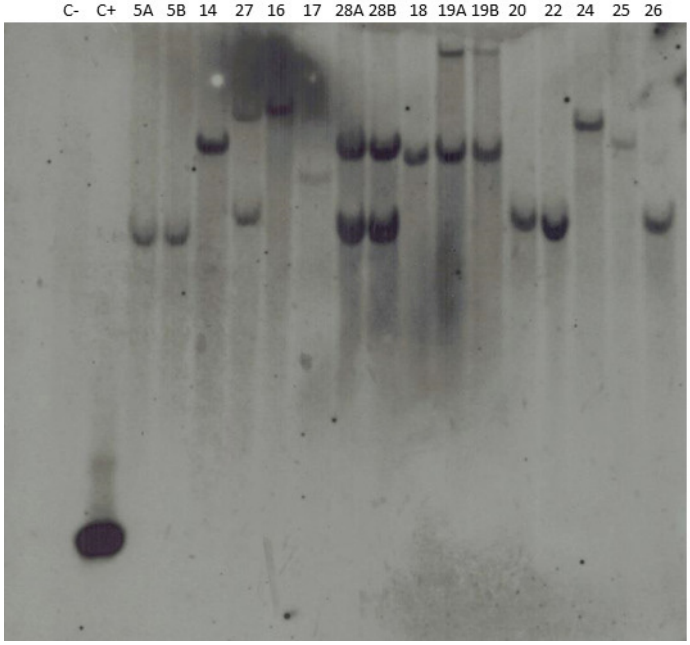

Figure 2. Southern blot analysis in 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] transgenic plants C-: Negative control (DNA from non-transgenic plant); C+: Positive control (fragment of the *uid*A gene). 5A, 5B, 14 and 27: CsPP2.B1::*uid*A gene construct; 28A, 28B, 18, 19A, 19B, 20 and 22: CsVTE2::*uid*A gene construct; 24-26: CaMV35S::*uid*A gene construct. The DNA of all samples was digested with *Eco*RI restriction enzyme and hybridized with a probe containing the *uid*A gene amplicon.

Analysis of transgene expression by quantitative real-time PCR (qPCR)

The qPCR analysis was carried out in transgenic plants that were confirmed by *Southern blot* analysis. One transgenic plant bearing the CsPP2.B1::*uid*A construct was used to calculate the relative expression in other transgenic plants, since it exhibited the lowest gene expression of the target gene (*uid*A).

The *uid*A gene expression had great variation among plants and among different promoters, ranging from 2.5 to 21.3 times for CsPP2.B1, from 7.3 to 107.5 times for CsVTE2, and from 46.1 to 258 times for CaMV35S gene constructs (Figure 3).

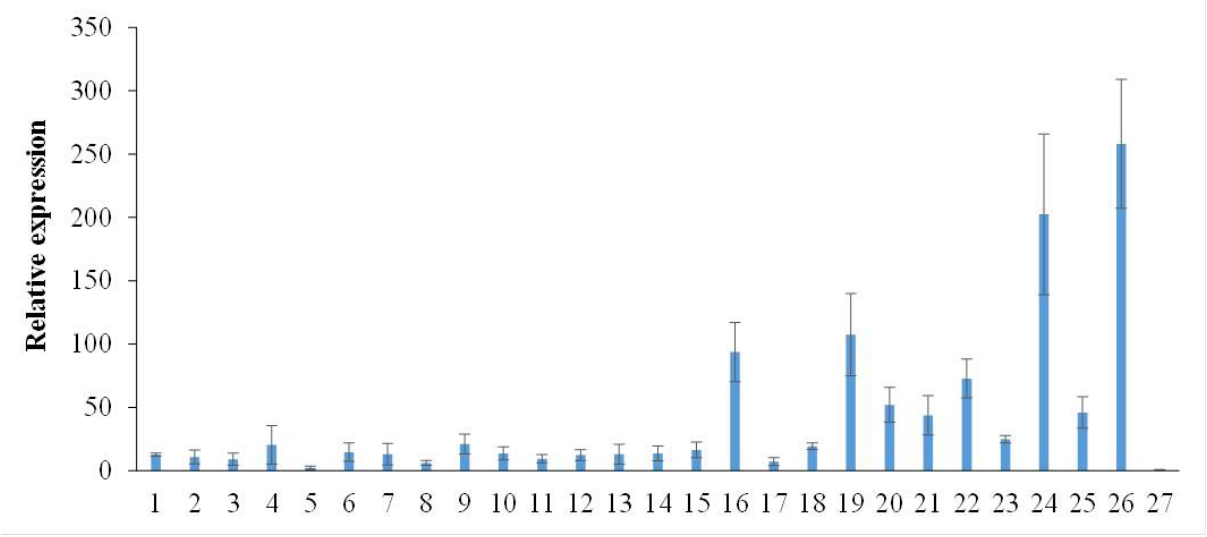

Figure 3. Quantification of the *uid*A gene expression driven by different promoters in 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck] transgenic plants. 1-15; 27: CsPP2.B1 promoter. 16-23: CsVTE2 promoter. 24-26: CaMV35S promoter. Plant #27 was used as the calibrator.

The CaMV35S promoter was chosen as a gold standard to compare the expression levels of this well-known strong and constitutive promoter with the gene expression levels induced by the new promoters evaluated in this work. The results from the qPCR analyses indicated that the average gene expression induced by the CsPP2.B1 and CsVTE2 promoters was approximately 7% and 31% of the expression level induced by CaMV35S promoter, respectively.

Histolocalization of *uid*A gene expression

The histological analysis allowed the characterization of the expression sites of the GUS reporter gene (*uid*A) driven by the different promoters (Figures 4; 5; 6). The control treatment (non-transgenic plant) showed no reaction to the X-GLUC treatment (Figures 4A; E). In the transgenic plants, the product of the GUS reaction could be seen in different tissues. For the gene construct in which *uid*A expression was driven by the CaMV35S constitutive promoter, the blue staining was detected in all the stem tissues (Figures 4 B; F).

Plants containing the *uid*A gene driven by the CsPP2.B1 or CsVTE2 promoters showed β-glucuronidase expression in specific tissues. In tissue samples from plants with the gene construct driven by the CsPP2.B1 promoter, the blue staining resulting from the enzymatic activity was observed, preferentially, in the phloem tissue (Figures 4C; G, 5B; C). On the other hand, β-glucuronidase activity was not observed in the cortex and in the epidermis (Figure 5A). In a more detailed examination, it was possible to identify that the product of the GUS reaction occurred within the sieve elements. The β-glucuronidase reaction product was observed in the sieve plate or adjacent to the walls of the sieve tubes elements (Figures 5D; E). The use of DAPI in the longitudinal stem sections from plants containing the gene construct under the control of the CsPP2.B1 promoter showed cells with nuclei that characterize companion cells adjacent to sieve tube elements (Figure 5F). Moreover, high amounts of the β-glucuronidase reaction product were observed in the companion cells (Figures 5E; F).

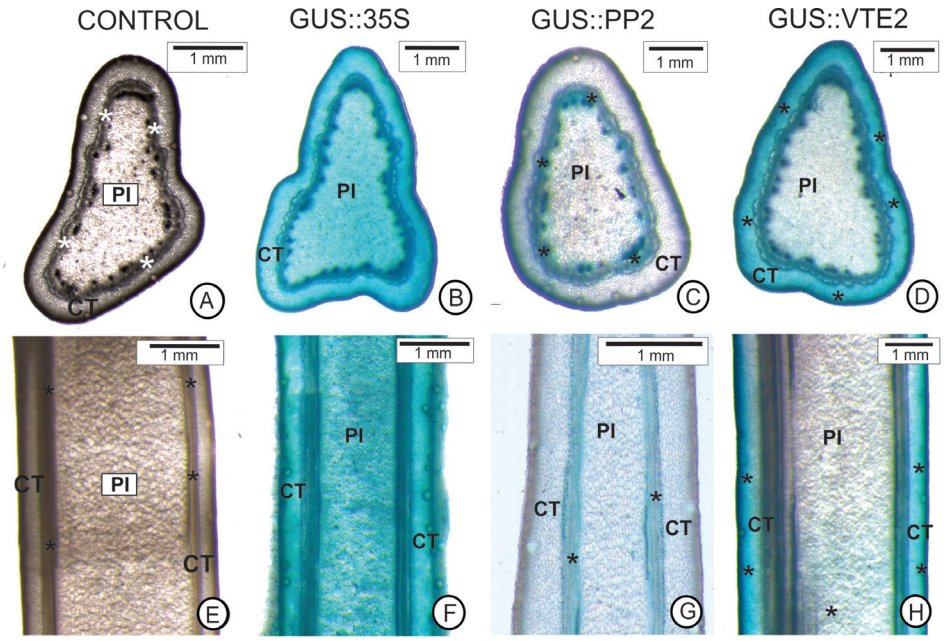

Figure 4. Histochemical assay for the detection of *uid*A (GUS) gene activity under the control of CaMV35S, CsPP2.B1, and CsVTE2 promoters. Cross (A-D) and longitudinal (E-H) sections of young branches of 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck]. Images were taken using a stereomicroscope. A, E: Negative control (non-transformed plants). B, F: CaMV35S::*uid*A. The transgenic expression is seen in all tissues of the stem. C, G: CsPP2.B1::*uid*A. Expression of *uid*A gene mainly in the phloem region (*). D, H: CsVTE2::*uid*A. Expression of *uid*A gene mainly in the cortical region (*). CT: Cortex; PI: Pith.

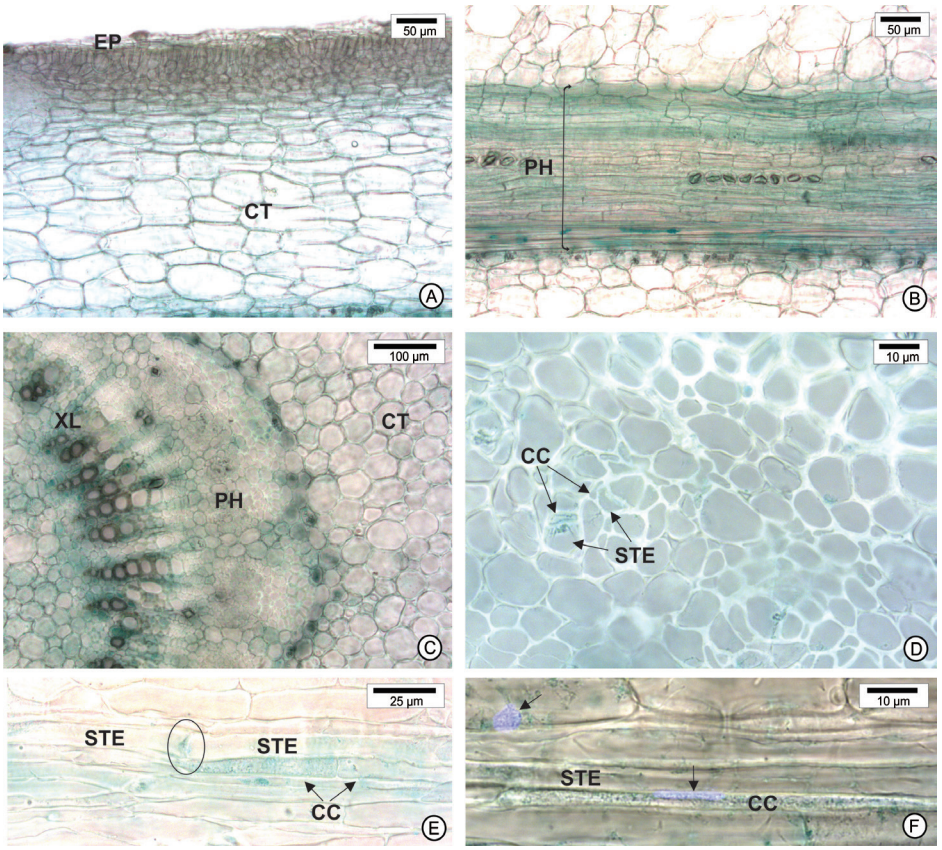

Figure 5. The expression of the *uid*A (GUS) gene under the control of CsPP2.B1 promoter, detected by light microscopy in young branches of 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck]. Longitudinal (A, B, E, F) and cross (C-D) sections. A: Cortical region. No β-glucuronidase enzyme reaction was seen. B: Phloem showing cells with a positive reaction. C: Cross-section pointing to the phloem. D: Detailed region of the phloem. Sieve tube elements (STE) and companion cells (CC) indicating the *uid*A gene expression at the cell wall that separates STE and CC (arrows in E). E: longitudinal section indicating the deposition of the β-glucuronidase reaction product on the sieve plate (circle) and into the STEs. F: Merged image with bright field and DAPI filter. The cell nucleus showed blue fluorescence (arrows). Companion cells showed a marked nucleus and deposition of the β-glucuronidase reaction product. EP: Epidermis; CT: Cortex; PH: Phloem; XL: Xylem.

The analysis of transgenic plants containing the gene construct under the control of the CsVTE2 promoter showed a strong presence of the β-glucuronidase reaction product in the cortical parenchyma, especially in the regions close to the epidermis (Figures 4D; H). The blue staining was also detected near the phloem, indicating that, despite probably being non-phloem-specific, this promoter drives the expression to or nearby the phloem tissue (Figure 6A). The presence of β-glucuronidase reaction was observed in the outer layers of the cortical parenchyma that contains chloroplasts, that is, the chlorenchyma (Figures 6B; C). This intrinsic relationship with chloroplasts was also observed in the epidermis, where only the cells with chloroplasts, the guard cells, also had the blue staining derived from the β-glucuronidase activity, unlike the ordinary cells of the epidermis (Figure 6D).

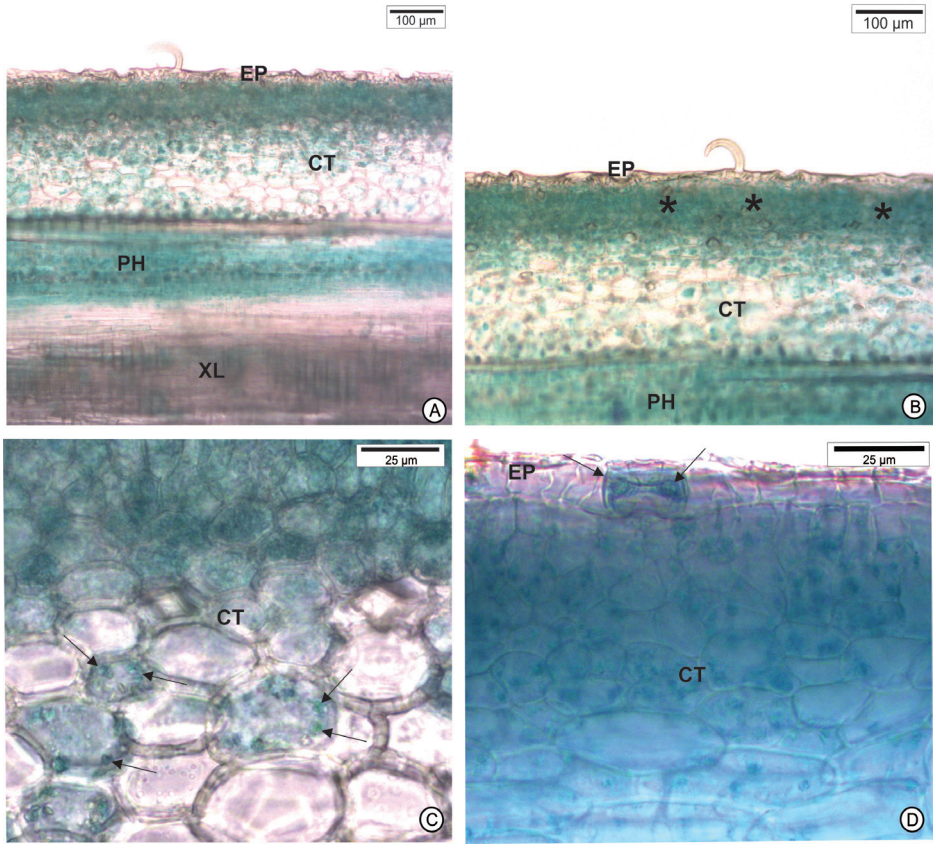

Figure 6. The expression of the *uid*A (GUS) gene under control of CsVTE2 promoter detected by light microscopy in young branches of 'Carrizo' citrange [*Poncirus trifoliata* (L.) Raf. x *Citrus sinensis* (L.) Osbeck]. Cross (C) and longitudinal (A, B; D) sections. A: Overview of vascular tissue, cortical parenchyma and epidermis. No β-glucuronidase enzyme reaction was seen at xylem and epidermis. B: Cortical parenchyma (*) showing cells with a positive reaction. C: Detailed cortex. A considerable expression is seen in cells with chloroplasts (arrows). D: Detailed view of the epidermis. The arrow points to a stoma and with its guard cells, the only epidermal element with GUS positive reaction. EP: Epidermis; CT: Cortex; PH: Phloem; XL: Xylem.

Genetic engineering has been widely used to obtain genotypes of agronomic interest with potentially accurate results (DUTT et al. 2015). However, in most cases, the transgenes are driven under the control of constitutive promoters. Tissue-specific promoters have been reported in the literature for different species, including citrus (WANG et al. 1992; TRUERNIT; SAUER 1995; IMLAU et al. 1999; ZHAO et al. 2004; DUTT et al. 2012; MIYATA et al. 2012; DUTT et al. 2014). However, most of these promoters are derived from genetically distant organisms of the species of interest, which may hinder the acceptance of these GMOs (LASSEN et al. 2002). Therefore, the use of cisgenes may bring several benefits (JACOBSEN; SCHOUTEN 2009).

Here, we report the expression levels of two new tissue-specific citrus promoters and compare these results with the expression of the strong, constitutive and heterologous CaMV35S promoter. Our results indicate differences among *uid*A gene expressions driven by these promoters, showing a variation in relative expression. The expression of the gene *uid*A when controlled by the CaMV35S promoter was higher than the expression driven by citrus tissue-specific promoters. These results were expected because tissue-specific promoters are restricted to the expression site, whereas the CaMV35S promoter is expressed in all tissues. On the other hand, specific citrus constitutive promoters CYP, GAPC2, and EF1 were recently described by our group, demonstrating

its potential of reduction in inter-individual variation of transgene expression (ERPEN et al. 2018).

The CsPP2.B1 promoter showed expression in phloem tissue, which can be verified by the presence of the blue staining as a result of the reaction of the *uidA* gene with the X-GLUC substrate. These results corroborate those reported by Miyata et al. (2012), who demonstrated the importance of the *uid*A gene in the phloem tissue of transgenic sweet orange plants of different cultivars containing an *Arabidopsis thaliana* (*At*PP2) PP2 derived promoter. The β-glucuronidase reaction product was observed in phloem tissue, especially in companion cells (CC) and sieve tube elements (STEs). When mature, the complex CC-STEs is a functional unit of the phloem and is separated from the neighbor cells through symplastic isolation (OPARKA; TURGEON 1999). Companion cells are the only nucleated phloem cells and, therefore, is where both mRNA expression and translation of proteins and small RNAs occur in cells (LOUGH; LUCAS 2006). Regarding the PP2 mRNA, it was demonstrated that it is expressed in the CC, however, the protein is also observed in the STE (DANNENHOFFER et al. 1997; DINANT et al. 2003). Based on the information that *uidA* gene expression is driven by CsPP2.B1 promoter in the CC and the β-glucuronidase reaction product was observed in both CC and STE cells, we cannot affirm whether the *uid*A protein or the mRNA is exported from the companion cells to the STEs. Additional immunohistochemical and *in situ* hybridization assays need to be conducted to shed light on the CsPP2.B1 promoter functional role. CsPP2.B1 promoter showed expression in phloem tissue, which can be verified by the presence of the blue staining as a result of the reaction of the *uid*A gene.

The expression pattern of the reporter gene in transgenic plants bearing the construct driven by the CsVTE2 promoter suggests that the expression targeted to the cortical parenchyma, mainly close to the epidermis, as well as to the phloem tissue. Because VTE genes are involved in the tocopherol biosynthesis and these substances have an important role in the oxidative stress and in loading of photoassimilates, this can explain the expression sites of the reporter gene. Tocopherols are synthesized in photosynthetically active cells, what could explain the directed expression of the reporter gene in the cortical parenchyma, where there is a high chloroplast concentration (YABUTA et al. 2013). Besides the strong expression in the cortical parenchyma, the β-glucuronidase reaction product was detected in the stomatal guard cells in the epidermis. In the analyzed samples, guard cells are the only cells in the epidermis that have chloroplasts, increasing, therefore, the evidence of the strong relationship between this promoter to the plastids (EVERT 2006; YABUTA et al. 2013). Also, immunocytochemical assays identify the presence of VTE inside the plastid plastoglobule (AUSTIN et al. 2006).

In general, *uid*A gene expression driven by CsVTE2 promoter was higher than that driven by CsPP2.B1 promoter (Figures 4C; D) but similar to the expression levels driven by the *At*SUC2 promoter reported by Benyon et al. (2013) and Tavano et al. (2015). Though the *At*SUC2 promoter drives the expression to specific phloem tissues with good expression levels, this promoter is derived from *A. thaliana*, a phylogenetic distant organism from citrus. Based on the results here presented, we suggest that the CsVTE2 promoter is potentially interesting to be used in citrus genetic transformation, once it is derived from citrus.

Research studies carried out with biosynthetic VTE mutants in *Arabidopsis*, have demonstrated the importance of the tocopherols, not only in photoprotection against oxidative stress but also in the loading of photoassimilates and in plant acclimation to low temperatures. Tocopherol deficient *VTE2* mutants presented an inhibition in photoassimilate transport, followed by an increase in sugar levels and starch accumulation in the leaves, coinciding with callose deposition in the phloem parenchyma, which, in turn, was later also accumulated in the cells of the sieve tube element complex and companion cells (MAEDA et al. 2006). The damage caused to the flow of photoassimilates is one of the main factors that negatively affect the Liberibacter-infected plants, so this sequence of events is similar to what occurs in infected plants with the HLB-associated bacteria, in citrus (BOVÉ 2006).

The levels of expression exhibited by tissue-specific promoters were lower than those driven by the constitutive promoter CaMV35S; however, constructs that have tissue-specific promoters may lead to expressions in restricted and relevant tissues.

Conclusions

CsPP2.B1 and CsVTE2 citrus promoters can be considered adequate for their utilization in gene constructs aiming disease resistance, as an alternative to the use of constitutive and/or tissue-specific promoters isolated from organisms phylogenetically distant from citrus.

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