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PHLOEM PROMOTERS IN TRANSGENIC SWEET ORANGE ARE DIFFERENTIALLY TRIGGERED BY *Candidatus* Liberibacter asiaticus[1]

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ABSTRACT – The use of promoters preferentially expressed in specific plant tissues is a desirable strategy to search for resistance for pathogens that colonize these tissues. The bacterium *Candidatus* Liberibacter asiaticus (Las), associated with huanglongbing disease (HLB) of citrus, colonizes phloem vessels. Some promoters, besides conferring tissue-specific expression, can also respond to the presence of the pathogen. The objective of the present study was to verify if the presence of Las could modulate the activation of the phloem-specific promoters AtPP2 (*Arabidopsis thaliana* phloem protein 2), AtSUC2 (*A. thaliana* sucrose transporter 2) and CsPP2 (*Citrus* phloem protein 2), known to be expressed in *Citrus sinensis* phloem. 'Hamlin' sweet orange plants (*Citrus sinensis* L. Osbeck) transformed with the *uid*A (GUS) reporter gene under the control of AtPP2, AtSUC2 and CsPP2 promoters were infected to evaluate the interdependence between transgene expression and the concentration of Las. Plants were inoculated with Las by *Diaphorina citri* and eighteen months later, bacterial concentration and *uid*A expression were determined by qPCR and RT-qPCR, respectively. Reporter gene expression driven by AtSUC2 promoter was strongly and positively correlated with Las concentration. Therefore, this promoter combines desirable features of both tissue-specificity and pathogen-inducibility for the production of transgenic plants tolerant to Las.
Index terms: *Citrus sinensis*, *Diaphorina citri*, genetic transformation, GUS, huanglongbing, quantitative real-time PCR.

PROMOTORES DE FLOEMA SÃO DIFERENTEMENTE ATIVADOS POR *Candidatus* Liberibacter asiaticus EM LARANJEIRAS DOCES TRANSGÊNICAS

RESUMO – A utilização de promotores preferencialmente expressos em tecidos vegetais específicos é uma estratégia desejável na busca por resistência a patógenos que colonizam tais tecidos. A bacteria *Candidatus* Liberibacter asiaticus (Las), associada à doença huanglongbing (HLB) em citros, coloniza o tecido floemático. Alguns promotores, além de conferir expressão específica em certos tecidos, podem também responder diferencialmente à presença de patógenos. O objetivo deste estudo foi verificar se a presença de Las poderia modular a ativação dos promotores de floema AtPP2 (*Arabidopsis thaliana* phloem protein 2), AtSUC2 (*A. thaliana* sucrose transporter 2) e CsPP2 (*Citrus* phloem protein 2), conhecidos por expressão específica em floema de *Citrus sinensis*. Plantas de laranja 'Hamlin' (*Citrus sinensis* L. Osbeck) transformadas com o gene reporter *uid*A (GUS) sob controle dos promotores AtPP2, AtSUC2 e CsPP2 foram infectadas para avaliar a interdependência entre a expressão dos transgenes e concentração de Las. Plantas foram inoculadas com Las por meio de *Diaphorina citri* e dezoito meses após, a concentração de bactéria e expressão do gene *uid*A foram determinadas por qPCR e RT-qPCR, respectivamente. A expressão do gene reporter dirigida pelo promotor AtSUC2 foi forte e positivamente correlacionada com a concentração de Las. Portanto, este promotor contém características desejáveis quanto à especificidade de expressão e indução específica por patógeno para a produção de plantas transgênicas tolerantes a Las.
Termos para indexação: *Citrus sinensis*, *Diaphorina citri*, transformação genética, GUS, huanglongbing, PCR quantitativo em tempo real.

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INTRODUCTION

Plant genetic engineering strategies may use strong constitutive and non-tissue-specific promoters to drive the expression of transgenes for disease control. However, strong transgene expression without tissue discrimination can result in unnecessary energy expenditure by the plant and possible side effects due to putatively harmful transgene products (ALTPETER et al., 2005; WANG et al., 2005; ZHAO et al., 2004). One method to fine tune transgene expression is to use tissue-specific promoters induced by pathogens or abiotic stresses (BARBOSA-MENDES et al., 2009; DUTT et al., 2012; MIYATA et al., 2012; BENYON et al., 2013; ATTÍLIO et al., 2013; ZOU et al., 2014).

Huanglongbing (HLB), commonly known as greening, is a highly destructive disease in the main citrus growing regions in the world (BOVÉ, 2006). Although three species of Gram negative phloem-limited bacteria *Candidatus* Liberibacter are associated with this disease (*Ca.* Liberibacter asiaticus – Las, *Ca.* Liberibacter africanus – Laf, and *Ca.* Liberibacter americanus – Lam), the Asian species is the most widespread through the citrus regions around the world (BOVÉ, 2006). Las can be naturally transmitted between plants by the psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) (COLETTA FILHO et al., 2014) and by grafting (LOPES et al., 2009). Antibacterial transgene expression at phloem tissues, mainly in young leaves, is therefore a possible strategy for HLB control in citrus plants. However the investigation if the pathogen can modulate the activation of the phloem promoter is imperative to choose the appropriated promoter in transgenic approaches.

We hypothesized that three different promoters, which have been previously demonstrated to drive transgene expression preferentially in citrus phloem (MIYATA et al., 2012), could also show the desirable feature of being pathogen inducible. Therefore, the objective of the present study was to analyze the interdependence between transgene expression of *uid*A (GUS) reporter gene under control of AtPP2, AtSUC2 and CsPP2 phloem-specific promoters and bacterial concentration to correlate the promoter activation with the presence of Las in transgenic 'Hamlin' sweet orange plants (*Citrus sinensis* L. Osbeck).

MATERIALS AND METHODS

Liberibacter-free transgenic plants were produced by MIYATA (2009) using *Agrobacterium tumefaciens* with three binary vectors containing the *uid*A gene under control of three different phloem-specific promoters: AtPP2 (*Arabidopsis thaliana* phloem protein 2), AtSUC2 (*A. thaliana* sucrose transporter 2) and CsPP2 (*Citrus* phloem protein 2). The *uid*A (GUS) gene expression in the phloem tissue by these three promoters was confirmed in transgenic sweet orange transgenic plants (MIYATA et al., 2012).

Genetic transformation was confirmed by PCR and plants with single transgene insert were selected by Southern blot analysis. Five transgenic lines for each gene construct were multiplied by grafting.

The following transgenic lines of 'Hamlin' sweet orange plants were used: H3, H4, H6, H9 and H12 containing AtPP2/*uid*A; H43, H44, H45, H46 and H55 containing CsPP2/*uid*A; and H71, H72, H73, H75 and H76 containing AtSUC2/*uid*A. A non-transgenic line was used as control. Twenty plants were obtained for each transgenic line, of which 10 were infected with Las by infective *D. citri*, five were subjected to feeding by non-infective *D. citri*, which were used as a control, and five were not subjected to *D. citri* feeding.

Third- and fourth-instar nymphs of *D. citri* were obtained from Las-free colonies reared on orange jessamine plants [*Murraya paniculata* (L.) Jack (Rutaceae)] and used to acquire Las from sweet orange plants positive for the presence of the bacterium. These Las source plants were pruned 3 wks before acquisition, and cages constructed with clear plastic cups (500 ml) and anti-aphid screen were set on 3-4 young shoots of each plant. About 30 nymphs were caged per shoot for a 10-day acquisition access period (AAP). Following the AAP, groups of 10 insects were transferred to a young shoot of each test plant (a healthy plant of each transgenic line), where they were caged for a 10-day inoculation access period (IAP). Ten psyllid adults (about 1-2 wks old) obtained from the same batch reared on orange jessamine, but that did not experience an AAP, were caged for 10 days on a young shoot of each control plant (healthy test plants fed upon by non-infective *D. citri*).

Both the AAP and IAP assays were performed

in three stages at weekly intervals. For each transgenic line, the first stage was composed of two plants, the second by six plants and the third by two plants. In order to obtain uniform young shoots for inoculation, test plants of each stage were pruned 3 wks before the IAP. Following the IAP, cages were removed and the test plants were sprayed with insecticide (Thiamethoxam) to eliminate all remaining insects.

The test plant growth was conducted by keeping a single stem, which was the one caged with the group of 10 psyllids. For molecular analyses, petioles and midveins were sampled from mature leaves above the stem region where the psyllids were caged, at 18 months after inoculation. The same leaves were used for DNA and RNA extractions. For each plant, a 250 mg tissue aliquot was used for DNA extraction and quantification of bacterial concentration, and 500 mg was used for RNA extraction and gene expression analyses.

Plant DNA was extracted according to MURRAY & THOMPSON (1980). Presence and quantification of Las in samples was determined by q-PCR amplifications using sets of 16S-rDNA primers and FAM/MGB label probe (Life Technologies, Carlsbad, CA), as described by Coletta-Filho et al., (2010), but using 4 µl of total DNA at 10 ng.μL^{-1}. Las quantification (copy number-CN of 16S rDNA per gram of citrus tissue) was based on the formula [y= -1.9084x + 36.87 R^2 = 0.99213], where Y is the $\log_{10}$ of CN and X is the Ct mean for the sample. All amplifications were performed in triplicate by ABI 7500 fast thermal cycler (Life Technology), using the default for the cycling conditions.

Based on previous research (AMMAR et al., 2011; HILF, 2011; COLETTA-FILHO et al., 2014), and also on the regression analysis of the dilution curve, the cutoff for the Ct values adopted was 35. Therefore, only plant samples with Ct values <35.0 were considered Las-positives.

Total RNA was extracted using TRIzol (Invitrogen), further purified using RNeasy Plant Mini Kit (Qiagen) and treated with RNase-Free DNase (Qiagen). RNA quality and concentration were determined using a Nanodrop 2000c spectrophotometer (Thermo Scientific). cDNA synthesis was performed using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's recommendations.

Gene expressions were normalized using *FBOX* gene as an internal control (MAFRA et al., 2012). *uid*A gene was amplified using primers 5'-ACCTCGCATTACCCTTACGCTGAA-3' and 3'-GCCGACAGCAGCAGTTTCATCAAT-5'. qPCR reactions were performed in a final volume of 15 µL containing 5 µL of cDNA (30 ng cDNA), 0.3 µL of each primer pair (200 nM final concentration), 7.5 µL of Fast SYBR Green Master Mix and 1.9 µL of nuclease free water. All reactions were performed in triplicate. *uid*A gene expression was calculated by the ΔΔCt method using non-inoculated transgenic plants as calibrators (LIVAK & SCHMITTGEN, 2001).

Interdependence between Las concentration and transgene expression was verified by Pearson product-moment correlation coefficient. Correlations were qualitatively classified as weak, moderate, strong or very strong according to CALLEGARI-JACQUES (2003).

RESULTS AND DISCUSSION

Overall average infection frequency with Las was around 30%, what is in accordance with previous studies regarding *D. citri* transmission efficiency under controlled conditions (PELZ-STELINSKI et al., 2010; COLETTA-FILHO et al., 2014). In spite of these results, it was possible to carry out further analyses because several transgenic lines had higher infection frequencies. Among the transgenic lines successfully infected, there was a large variation in bacterial concentration among inoculated plants, ranging from 0.98 to 10.49 $\log_{10}$ cells/gram of tissue (Figure 1). This range of bacterial population allowed us to analyze the possible difference in *uid*A gene expression driven by AtPP2, AtSUC2 and CsPP2 in plants carrying different Las titers.

Transgene expression analysis was performed with transgenic lines H9 (AtPP2/*uid*A), H46 (CsPP2/*uid*A) and H71 (AtSCU2/*uid*A) because these lines had more Las-infected plants (Figure 1). High transgene expression variation was observed among plants of the transgenic lines, even when they presented similar bacterial concentrations (e.g., plants H9.1 and H9.9) (Figure 2). It is worth pointing that the calibrator samples were transgenic plants expressing *uid*A gene in the phloem but that were not inoculated, therefore what was measured in the inoculated test plants was an increase or a reduction in the basal expression level conferred by each promoter in presence of Las.

The Pearson product-moment correlation coefficient between Las concentration and gene expression observed for transgenic line H9 (bearing the AtPP2/*uid*A construction) was negative and weak (r=-0.12) according to the scale proposed by CALLEGARI-JACQUES (2003), indicating that the bacterial concentration had little effect on gene expression.

Transgenic line H46 (bearing the CsPP2/*uid*A construction) presented higher correlation value (r=-0.32) than H9. Correlation between gene expression and bacterial concentration was negative and moderate, showing a moderate dependence between the variables.

The highest correlation (r=0.68) was observed for line H71 (AtSUC2/*uid*A), which was strong and positive, i.e., gene expression increased with increasing bacterial concentration.

Las concentration and infection frequency varied independently of the gene construct used, but gene expression was less influenced by bacterial concentration for constructs with PP2 promoters (AtPP2 and CsPP2) than for the construct with the sugar transporter (AtSUC2) because the correlation values were closer to zero (CALLEGARI-JACQUES, 2003).

Considering that there is no curative measurement to its control, current management of the disease includes the scouting and frequent removal of HLB-symptomatic trees to reduce inoculum, and psyllid chemical control to reduce vector population (BELASQUE JR. et al., 2010). Transgenic approaches are considered to have high potential for the development of new strategies to the management of this very destructive disease. This is also the case of citrus, in which a considerable amount of research has been recorded within the last decades, with the production of several transgenic events with potential resistance to biotic and abiotic stresses (GONG & LIU, 2013). Although complete immunity may not be reached, transgenic sweet orange overexpressing genes encoding antimicrobial peptides or genes related to the SAR, may contribute to lower bacteria titers and, consequently, lower infection and epidemiological rates. In our previous work, five different transgenic lines of 'Pera' sweet orange expressing attacin A antimicrobial peptide under control of CaMV35S promoter showed significantly lower CLas titers than non-transgenic plants after bacterial infection through infected budwood (FELIPE et al., 2013).

In the case of HLB in sweet orange, we hypothesized that three different promoters that have been previously demonstrated to drive transgene expression preferentially in the phloem tissue (MIYATA et al., 2012) could also show the desirable feature of being pathogen inducible. Las has a long incubation period and its concentration is timely dependent. Previous research has indicated that the highest concentration of this pathogen is achieved between 150 and 200 days after graf-inoculation (COLETTA-FILHO et al., 2014). Therefore, the incubation period in our study (18 months) was adequate to ensure bacterial population growth in the vector-inoculated plants.

PP2 genes have been shown through microarray analyses to be strongly induced in Las infected citrus plants (ALBRECHT & BOWMAN 2008; KIM et al., 2009; FAN et al., 2011). PP2 genes are encoded by a large gene family with wide distribution in the plant kingdom (DINANT et al., 2003). At least ten PP2-like genes are present in the Affymetrix microarray chip used in those studies, of which the gene represented by probe id Cit.35955.1.S1_at, similar to *Arabidopsis* PP2-B15, is the most strongly induced in HLB affected plants. The citrus and *Arabidopsis* PP2 promoters used in our study were cloned before any information about gene expression in HLB affected plants became available. These promoters were initially selected with the sole purpose of driving transgene expression in phloem tissues and were cloned from citrus and *Arabidopsis* genes most similar to pumpkin (*Cucurbita melo* and *C. maxima*) PP2 cDNAs (BOSTWICK et al., 1992; WANG et al., 1994). These genes are now classified as PP2-A1 which is not the family member induced by Las infection, thus explaining our present results using these gene promoters. Nevertheless, given that the aforementioned microarray studies were performed with plants 4 to 8 months after inoculation, it was still possible that the longer period after inoculation of our study (18 months) could result in some gene induction not detected in the previous studies.

Induction of sucrose-H+ symporter gene (*SUC2*) in citrus plants infected with Las has not been previously reported. Here also, the longer period after inoculation of our study may explain our results, indicating that at least in more advanced stages of the disease, induction of *SUC2* can occur. Accumulation

of sucrose in symptomatic leaves of HLB affected plants (KIM et al., 2009; FAN et al., 2010) could result in the late induction of *SUC2*.

The uneven distribution of Las bacteria in HLB-affected plants (TATINENI et al., 2008; TEIXEIRA et al., 2008) may affect the correlation analysis between bacterial concentration and transgene expression. Even though the bacterial concentration and transgene expression were measured from the same sampled leaves, high bacterial concentration in other parts of the plant could influence, through systemic signaling, transgene expression in a leaf with low bacterial concentration.

Our findings in the present research are relevant because a large group of these plants were evaluated after inoculation with Las by natural infection through *D. citri*. The results reported herein indicate that at least one of these promoters [AtSUC2 (*Arabidopsis thaliana* sucrose transporter 2)] can enhance transgene expression after Las inoculation. As far as we know this is the first report of a research of this kind, involving the evaluation of preferentially expressed phloem promoters after Las infection in transgenic sweet oranges by insect vector, which is what indeed occur in field condition. Transgenic sweet orange plants with the same AtSUC2 promoter of the present study controlling expression of the synthetic antimicrobial peptide *Attacin* A have been developed by our research group (TAVANO et al., 2015), peptide gene expression has been confirmed using RT-qPCR, and the transgenic plants obtained were propagated for evaluation of resistance to Las.

Transgenic citrus transformed with the phloem-specific promoters of this study and new endogenous citrus promoters selected based on inducibility by Las combined with genes encoding proteins with antibacterial activity are promising tools for HLB management.

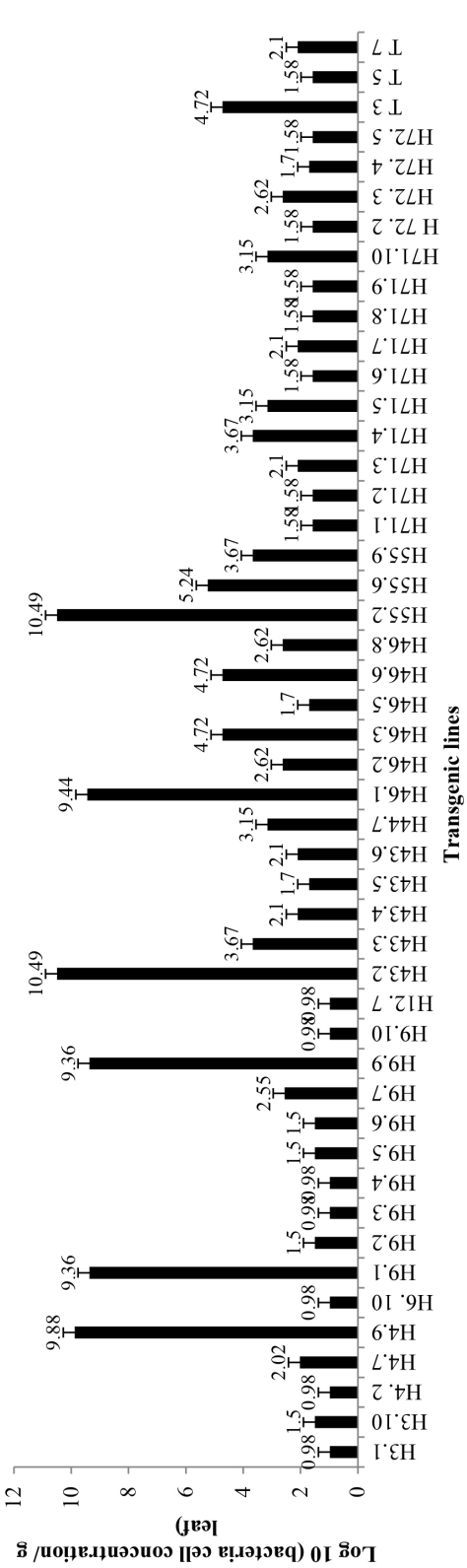

FIGURE 1 -The concentration of *Candidatus* Liberibacter asiaticus quantified using qPCR in plants transformed with gene *uid*A under the control of AtPP2, AtSUC2 and CsPP2 promoters. Plants H3, H4, H6, H9 and H12 with promoter AtPP2. Plants H43, H44, H46 and H55 with promoter CsPP2. Plants H71 and H72 with promoter AtSUC2. T plants are non-transgenic controls. The numbers following the dot correspond to the transgenic line replicate (H3.1=replicate #1 of line H3). Vertical bars represent the standard error (n=3).

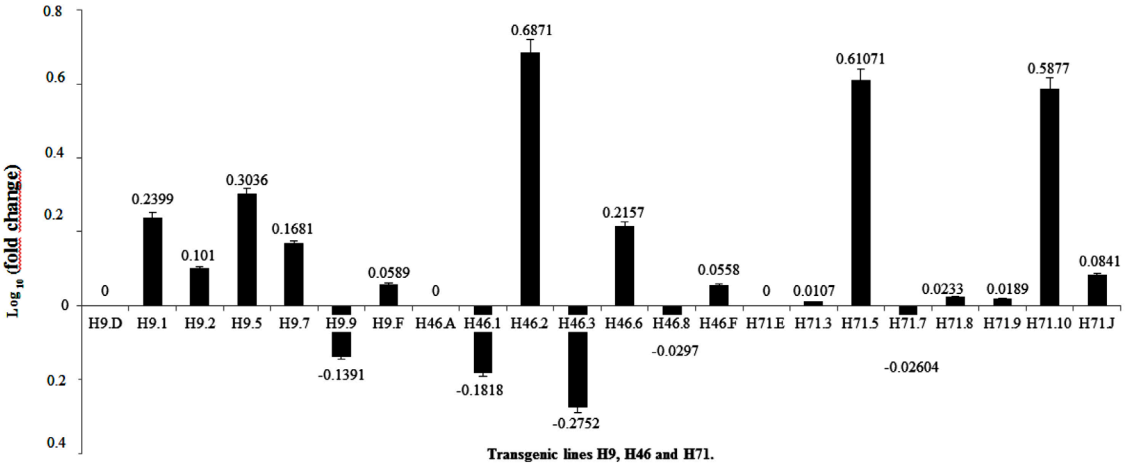

FIGURE 2 - Gene expression, quantified using qPCR, of transgenic lines H9, H46 and H71, which correspond to plants with gene constructs AtPP2/*uid*A, CsPP2/*uid*A and AtSUC2/*uid*A, respectively. Plant H9.D was used as a reference for the remaining H9 plants. Plant H9.F was inoculated with non-infective *Diaphorina citri*. Plant H46.A was used as a reference for the remaining H46 plants. Plant H46.F was inoculated with non-infective *D. citri*. Plant H7.E was used as a reference for the remaining H71 plants. Plant H71.J was inoculated with non-infectious *D. citri*. Vertical bars represent the standard error (n=3).

CONCLUSIONS

No correlation between AtPP2 and CsPP2 promoter modulation is found after Las infection.

AtSUC2 promoter is strongly and positively modulated by Las infection.

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