GUS gene expression driven by a citrus promoter in transgenic tobacco and 'Valencia' sweet orange

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Abstract – The objective of this work was the transformation of tobacco and 'Valencia' sweet orange with the GUS gene driven by the citrus phenylalanine ammonia-lyase (PAL) gene promoter (CsPP). Transformation was accomplished by co-cultivation of tobacco and 'Valência' sweet orange explants with *Agrobacterium tumefaciens* containing the binary vector CsPP-GUS/2201. After plant transformation and regeneration, histochemical analyses using GUS staining revealed that CsPP promoter preferentially, but not exclusively, conferred gene expression in xylem tissues of tobacco. Weaker GUS staining was also detected throughout the petiole region in tobacco and citrus CsPP transgenic plants.

Index terms: *Xylella fastidiosa*, PAL, citrus variegated chlorosis, cultivar resistance.

Expressão do gene *GUS* controlado por promotor de citros em plantas transgênicas de tabaco e laranja 'Valência'

Resumo – O objetivo deste trabalho foi realizar a transformação de plantas de tabaco e laranja 'Valência' com o gene *GUS* controlado pelo promotor do gene da fenilalanina amônia-liase (PAL) de citros (CsPP). Foi realizada transformação genética por meio do co-cultivo de explantes de tabaco e laranja 'Valência' com *Agrobacterium tumefaciens* que continha o vetor binário CsPP-GUS/2201. Após a transformação e a regeneração, a detecção da atividade de GUS por ensaios histoquímicos revelou que o promotor CsPP, preferencialmente, mas não exclusivamente, confere expressão gênica em tecidos do xilema de tabaco. Expressão mais baixa de GUS também foi detectada na região de tecido de pecíolo, em plantas transgênicas (CsPP) de tabaco e laranja 'Valência'.

Termos para indexação: *Xylella fastidiosa*, PAL, clorose variegada dos citros, resistência varietal.

Introduction

Brazil is the leading producer and exporter of frozen concentrated orange juice, with 'Pera', 'Valencia', 'Natal' and 'Hamlin' being the main sweet orange scion cultivars. The restricted use of certain scion or rootstock cultivars has several problems, such as pests and diseases, which results in limitations in plant yield and fruit quality.

Among the main diseases, citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa*, is characterized by colonization and clogging of the xylem vessels.

CVC affects all the major scion cultivars in Brazilian citrus. Infected plants are characterized by general die back, with small yellow internerval spots on the upper leaf face and shortening of the stems internodes, and a trend of fruit in clustering. Also, fruits become hard, very small, and change color prematurely.

Chemical control of plant bacterial diseases, by the use of antibiotics such as streptomycin and tetracycline, is not economically feasible. Specific cultural practices minimize the effects of these diseases, but the use of tolerant or resistant genotypes is a much better alternative, and genetic transformation can be a viable tool in citrus breeding programs.

Agrobacterium tumefaciens-mediated transformation has been shown effective in the regeneration of transgenic citrus plants, including 'Pineapple' (Cervera et al., 1998) and 'Hamlin' sweet orange (Mendes et al., 2002). However, these transgenic plants share a common feature: they were produced using a strong, constitutive, non tissue-specific promoter, the CaMV35S. Future transgenic generations will require the use of suitable promoters, which confer a tissue-specific gene expression, in which the time of activation and the degree of expression can be controlled (Harakava, 2000).

Because *Xylella fastidiosa* is a bacterium that inhabits xylem vessels, development of transgenic resistance against this pathogen requires constructs that cause the expressed protein to accumulate in this part of the plant. Thus, the utilization of promoters from genes that are expressed preferentially in the xylem is necessary. Most of these genes encode enzymes involved in phenylpropanoid metabolism and synthesis of lignin precursors such as phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate-CoA ligase (C4L), cinnamyl-alcohol dehydrogenase (CAD), caffeate 3-O-methyltransferase (COMT), and caffeoyl CoA 3-O-methyltransferase (CCoAOMT).

Transgenic tobacco plants having a *GUS* gene under control of promoter sequences from a *PAL* gene of either *Arabidopsis* sp., common bean, pea, poplar or rice, showed expression of the reporter gene in young cells, below the stem apex, which were differentiating into xylem, as well as in cells of the xylem parenchyma in more developed parts of the plant (Bevan et al., 1989; Liang et al., 1989; Ohl et al., 1990; Zhu et al., 1995; Kawamata et al., 1997; Gray-Mitsumune et al., 1999). *GUS* expression could also be detected in epidermal cells and trichoma, root hairs and root tips, and petals, where production of flavonoids requires PAL activity. Induction of reporter genes by mechanical wounding and bacterial infection has also been demonstrated for *PAL* gene promoters (Huang & McBeath, 1994; Giacomin & Szalay, 1996).

The existence of multiple isoforms of PAL in plants confers flexibility in the expression of this enzyme, each gene being expressed in a particular spatial and temporal manner. This has been demonstrated using the promoters of PAL2 and PAL3 from French bean, fused to GUS and introduced into tobacco, potato, and *Arabidopsis* sp. (Shufflebottom et al., 1993). Among other tissue-specificity differences, a PAL2 promoter-GUS fusion showed the typical xylem-specific expression, while *GUS* expression under the PAL3 promoter was detected only in a single cell layer surrounding the vascular system. The PAL3 promoter also showed an enhanced activity in response to wounding and to treatment with culture filtrates from *Erwinia carotovora*.

Considering that the PAL promoter has been cloned from sweet orange (PAL-Cs) (Harakava, 2000), the objective of this work was the transformation of tobacco and 'Valencia' sweet orange with the *GUS* gene driven by the citrus phenylalanine ammonia-lyase (PAL) promoter (CsPP).

Material and Methods

The promoter of a *PAL* gene from *Citrus sinensis* var. Madam Vinous was cloned by inverse-PCR (Harakava, 2000). Primers were designed based on the cDNA sequence of a *Citrus limon PAL* gene available in the GenBank (U43338) (Seelenfreund et al., 1996). This was the only *PAL* gene sequence available for a citrus species at the time the work was done. Analysis of the CsPP using the transcription factor binding site search program TFSEARCH (Transcriptional Factor Search, 2006) revealed the presence of three binding sites for the maize activator P of flavonoid biosynthetic genes (Grotewold et al., 1994). Two of these sites are also present in the promoter of the *Arabidopsis thaliana*, *PAL*1 gene, which has been demonstrated to drive xylem-specific gene expression (Ohl et al., 1990). Sequences similar to the cis-acting elements P, A, and L, present in PAL and 4CL (4-coumarate:CoA ligase) gene promoters, as described by Logemann et al. (1995), could also be recognized in CsPP.

Tobacco plants (*Nicotiana tabacum* cv. Samsun) were transformed by co-cultivation of leaf disks with *Agrobacterium tumefaciens* GV2260 containing the binary vector CsPP-GUS/2201 (Figure 1). Tobacco seeds were surface-sterilized by treatment with 100% ethanol for 30 s, followed by 0.5% Na hypoclorite in a 1% SDS solution for 5 min, and washing with sterile water four times.

Plantlets were aseptically grown from seeds germinated on R1/2N medium (2.15 g Murashige Skoog salt mixture, 50 mg myoinositol, 18.65 mg Na$_2$EDTA, 13.9 mg FeSO$_4$.7H$_2$O, 10 g sucrose, 6 g agar, for 1 L of medium, pH adjusted to 5.8). Overnight liquid cultures of *A. tumefaciens* were centrifuged and resuspended in 0.5x MS salt solution (2.15 g L^{-1}), to an OD600 of approximately 0.5.

Leaf disks 0.8 cm in diameter were cut, using a cork borer 36, and floated on the *A. tumefaciens* suspension for 10 min. After removing excess bacterial suspension by blotting on sterile filter paper, the leaf disks were placed on solid DBI medium (4.3 g MS salt mixture, 100 mg myoinositol, 4 mg thiamine, 30 g sucrose, 8 g agar, for 1 L of medium, pH adjusted to 5.6), incubated in the dark at room temperature (22–24°C) for 48 hours, then these leaf disks were transferred to DBI medium supplemented with the plant hormones IAA (1 mg mL^{-1}) and kinetin (2 mg mL^{-1}), and the antibiotics kanamycin (50 mg mL^{-1}) and mefoxin (200 mg mL^{-1}). The plates were kept in a growth chamber with cycles of 16-hours light/8-hours dark, at 28°C.

Two to three weeks later, and thereafter, shoots grown from the borders of the leaf disks were transferred to rooting medium R1/2N supplemented with kanamycin (50 mg mL^{-1}) and mefoxin (200 mg mL^{-1}), and then, kept in a growth chamber under the same cultural conditions. Roots usually formed one or two weeks later, and rooted shoots were subsequently transferred to 'baby food' jars containing autoclave-sterilized potting mix. After growing in the growth chamber for about 2–3 weeks, the plantlets were transferred to 20-cm pots and kept in the greenhouse.

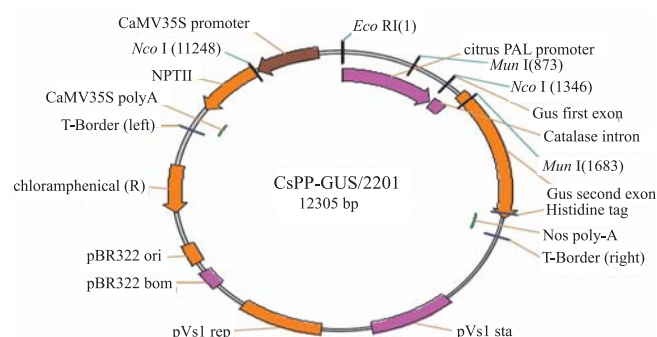**Figure 1.** Citrus PAL promoter fused to GUS in the binary vector pCAMBIA 2201.

'Valencia' orange plantlets, germinated in vitro on Murashige & Tucker culture medium, cultivated for three weeks in the dark followed by 10 to 15 days with a 16-hours photoperiod, were used as explant source. Transformation was accomplished by co-cultivation of epicotyl segments with *A. tumefaciens* GV2260 containing the binary vector CsPP-GUS/2201 (Figure 1). Bacteria were grown in solid YEP medium (15 g L^{-1} agar) for 48 hours. After this period, one colony was transferred to an Erlenmeyer (250 mL) with 50 mL of liquid YEP medium, supplemented with kanamycin (100 mg L^{-1}), and grown under orbital shaking (180 rpm) at 28ºC, for 16 hours. The bacterial suspension was centrifuged at 4,800 rpm (5ºC for 20 min) and resuspended in liquid MT medium.

Epicotyl segments of approximately 1 cm were put into bacterial solution for 20 min, dried on sterile filter paper, then transferred to Petri dishes containing the regeneration medium (MT), and co-cultivated in the absence of light (27ºC), for three days. After that, the explants were transferred to regeneration medium containing the antibiotics kanamycin (100 mg L^{-1}) and cefotaxime (500 mg L^{-1}) and transferred every two weeks. Two experiments were conducted, with 30 explants per plate, in a total of 300 explants.

Initial screening of the transgenic plantlets was done using the GUS assay (Jefferson, 1987). Leaf tips and transverse sections of petioles were incubated in X-gluc solution (1 mg mL^{-1} in 0.1 M phosphate buffer pH 7, 10 mM Na$_2$EDTA) overnight at 37°C. Plants tested negative for GUS were discarded, and positive plants were kept for further GUS assays of more mature tissues.

Leaf petioles from transgenic plants, grown for two months in the greenhouse (30–40 cm tall), were hand-sectioned transversally using a razor blade and incubated in 0.5% formaldehyde in 0.1M sodium phosphate buffer (pH 7) for 30 min at room temperature. The sections were washed four times 10 min with phosphate buffer and stained with X-gluc solution overnight at 37°C.

DNA was extracted from the regenerated sprouts, according to Dellaporta et al. (1983), to confirm the integration of *GUS* by PCR analysis. The amplification of the fragment was carried out using 1 µL of DNA (50–100 ng), 0.2 µL dNTPs (10 mM), 1 µL MgCl$_2$ (10 mM), 2 µL buffer (10x), 0.3 µL *Taq*-DNA polymerase (5 µg µL^{-1}) and 0.3 µL of GUS primers: 5' CAA CGA ACT GAA CTG GCA G 3' and

5' CAT CAC CAC GCT TGG GTG 3'. Reactions were performed using a termocycler (PTC-100, MJ Research, Inc.) with the following conditions: 34 cycles of 1 min at 92ºC, 1 min at 55ºC and 1.5 min at 72ºC.

Sprouts which tested positive for GUS by PCR analysis were in vitro grafted onto Carrizo citrange. After 45 days, plants were acclimated in pots, with plastic cover, containing commercial substrate (Plantimax/Eucatex), and kept for 60 days in a growth chamber under 16-hour photoperiod, with 40 μmol m^{-2} s^{-1} of light intensity, and controlled temperature of 27$\pm$2ºC. After this period, the plants were moved to greenhouse.

At least, ten leaf petiole segments from acclimated plants were collected, prepared by cutting with a surgical blade, and incubated in the absence of light at 37ºC, for 24 hours, in x-gluc solution, for β-glucuronidase (GUS) analysis (Jefferson, 1987). A negative control was also prepared.

Southern blot analysis was performed to confirm the stable integration of the *GUS* gene in the transgenic plants. Total DNA from plants was extracted by DNAzol (Plant DNAzol) method, adding two phenol extraction for a better purification. Approximately 20 μg of DNA were digested with the restriction enzyme *Hind*III, separated in 1% agarose gel through electrophoresis, and transferred to nylon membrane (Hybond-N$^+$).

The probe to the *GUS* gene was prepared by PCR, and the amplified fragment of 750 bp, corresponding to a part of the gene, was purified using the Qiax kit and labeled with fluorescein by the Gene Images Random Primer Labelling Module kit (Amersham Biosciences). Hybridizations were conducted at 60ºC and detected with the CDP-Star kit (Amersham Biosciences).

Results and Discussion

Petioles from CsPP-GUS transgenic plants of tobacco showed GUS staining throughout the transverse section, but a much more intense color was visible in the layer of young xylem cells (Figure 2). Identical patterns were observed in five independent transgenic lines. These results indicate that the PAL promoter cloned from sweet orange preferentially, but not exclusively, confers gene expression in xylem tissues. Weaker GUS staining was also detected throughout the petiole section.

In previous work, in tobacco transformation with PAL promoter cloned from *Arabidopsis* sp. (Harakava, 2000), *GUS* expression was observed exclusively in the xylem parenchyma, in accordance with Ohl et al. (1990). The 1,346 bp fragment from the sweet orange promoter in the CsPP-GUS construct, which is shorter than 1,922 bp from the *Arabidopsis* sp. promoter in the AtPP-GUS construct, was not long enough to confer xylem specific *GUS* expression. It has been demonstrated that the PAL promoter from bean (Leyva et al., 1992), and from *Arabidopsis* sp. (Ohl et al., 1990) are composed of a mosaic of positive and negative cis-elements.

Gray-Mitsumune et al. (1999) transformed *A. thaliana* and tobacco with the *GUS* gene fused with PAL promoter and histochemical analyses of GUS activity in primary transformants, which showed that the activity was consistently localized in vascular system of stems, leaves, roots and flowers.

Tobacco plants with the *GUS* gene driven by *Arabidopsis* sp. (Giacomin & Szalay, 1996) and bean

Figure 2. GUS staining of transverse sections of leaf petioles from CsPP-GUS transgenic tobacco.

(Bevan et al., 1989) PAL promoters demonstrated the expression of the reporter gene in young cells, as well as in xylem parenchyma cells and in developed parts of the plant. *GUS* gene expression could also be realized in epidermal cells and trichomes, radicels, and petals, where the production of flavonoids requires the activity of PAL enzyme.

The transformation and regeneration of 'Valencia' sweet orange CsPP-GUS transgenic plants, via *Agrobacterium tumefaciens* from juvenile epicotyl segments was successful. Fifteen positive plantlets were regenerated out of 155 plantlets produced from 300 explants. Confirmation was initially done by PCR analysis, when the plants were still in vitro (Figure 3 a) and later, after acclimation, by Southern blot analysis, confirming the insertion of at least one copy of the *GUS* gene (Figure 3 b). This is the first report of citrus transformation with a reporter gene under control of a *PAL* gene promoter.

Differently from what was observed in CsPP-GUS transgenic tobacco, the expression of *GUS* in CsPP-GUS transgenic 'Valencia' sweet orange was detected in superficial layers of cells in leaf petioles, but not in the vascular bundle (Figure 3 c). This result suggests that the promoter used in the present study is from a PAL isoform that is not primarily involved in xylem formation. Differential expression of PAL isoforms has been demonstrated in quaking aspen and *A. thaliana* (Kao et al., 2002; Raes et al., 2003). Also, there are reports showing that the expression of genes activated by PAL promoters can be induced by biotic and abiotic factors, including wounding, ultraviolet light, pathogen infection or exposure to heavy metals salts (Kervinen et al., 1998, Levée & Séguin, 2001, Smith et al., 2001).

Currently, there are more than 100 expressed sequence tags (EST) from *Citrus* and *Poncirus* species available at the GenBank, that show similarity to *PAL* genes. Three clusters of 75, 20, and 7 sequences in each could be formed with these EST, indicating that there are, at least, three isoforms of the *PAL* gene in citrus. Verification of the tissues from which these EST derive, does not allow inferring the isoform that is expressed mainly in the xylem. The CsPP used in the present study derives from the isoform corresponding to the cluster with 20 EST. This suggests that it is not the most expressed one, and using the promoter of the isoform correspondent to the cluster of 75 EST would probably provide a stronger expression.

Further investigation is needed, in order to identify the PAL isoform mainly expressed in citrus xylem and to obtain its gene promoter to further improve citrus transformation for CVC resistance.
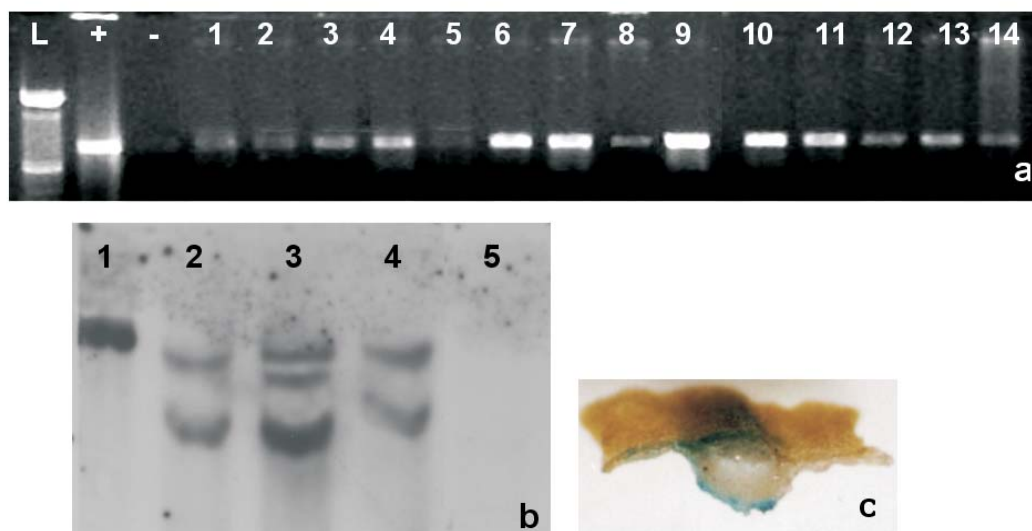
Figure 3. a) PCR analysis: L: 100 pb DNA ladder; + and -: positive and negative controls, respectively; 1–14: GUS + plants; b) southern analysis: 1: positive control (plasmid digested with *Hind*III); 2–4: DNA from CsPP-GUS transgenic 'Valencia' sweet orange plants digested with *Hind*III, after hybridization to probe corresponding *GUS* gene; 5: negative control; c) expression of *GUS* gene, in vascular region of 'Valencia' sweet orange leaf petiole.

Conclusions

1. Phenylalanine ammonia-lyase citrus promoter (CsPP), preferentially, but not exclusively, confers gene expression in xylem tissues of tobacco.

2. GUS staining is detected in superficial layers of cells in leaf petioles of citrus CsPP transgenic plants.

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