

Genetic transformation of sweet oranges with the *D4E1* gene driven by the AtPP2 promoter

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Abstract – The objective of this work was to produce transgenic 'Pêra' and 'Valência' sweet orange plants using the *D4E1* gene driven by the *Arabidopsis thaliana* phloem protein (AtPP2) promoter and to quantify transgene expression in different transformation events. Genetic transformation experiments were carried out with epicotyl segments co-cultivated with *Agrobacterium tumefaciens*. Six plants from 'Pêra' sweet orange and seven plants from 'Valência' sweet orange were confirmed as different transgenic events by means of the polymerase chain reaction (PCR) and the Southern blot techniques. Transgene expression was quantified using real-time quantitative PCR. *D4E1* gene expression levels vary from 5 up to 50 times among different transformation events.

Index terms: *Citrus sinensis*, antimicrobial peptide, tissue-specific promoter.

Transformação genética de laranjas doces com o gene *D4E1* dirigido pelo promotor AtPP2

Resumo – O objetivo deste trabalho foi obter plantas transgênicas de laranja 'Pêra' e 'Valência' por meio do gene *D4E1* dirigido pelo promotor *Arabidopsis thaliana* phloem protein (AtPP2) e quantificar a expressão dos transgenes nos diferentes eventos de transformação. Os experimentos de transformação genética foram realizados com segmentos de epicótilo cocultivados com *Agrobacterium tumefaciens*. Seis plantas de laranja 'Pêra' e sete plantas de laranja 'Valência' foram confirmadas como distintos eventos transgênicos por meio das técnicas de reação em cadeia da polimerase (PCR) e Southern blot. A expressão dos transgenes foi quantificada por PCR quantitativo em tempo real. Os níveis de expressão do gene *D4E1* variam de 5 a 50 vezes entre os diferentes eventos de transformação.

Termos para indexação: *Citrus sinensis*, peptídeo antimicrobiano, promotor tecido-específico.

Introduction

Brazil is the world's largest producer and exporter of frozen concentrated orange juice. It exports 98% of the juice produced in the country, which represents 85% of the juice consumed worldwide. The Brazilian citrus industry is directly and indirectly responsible for more than 230,000 jobs in the country (Neves et al., 2010). The primary scion cultivars used in Brazilian orchards are 'Hamlin', 'Pêra', 'Valência', and 'Natal'; therefore, approximately 90% of the orchards comprise these cultivars, which implies low genetic variability and a contribution to increased vulnerability, especially to diseases (Machado et al., 2010).

Huanglongbing (HLB) is a disease associated with phloem-restricted, Gram-negative bacteria. To

date, three species of HLB-associated bacteria were identified: "*Candidatus Liberibacter africanus*" (CLaf), "*Ca. L. asiaticus*" (CLas), and "*Ca. L. americanus*" (CLam). These bacteria were classified according to the continent where they were first identified. The CLam and CLas species are found in Brazil, and the latter is the most frequently observed in the country. The bacteria are transmitted through propagation by infected budwood or by the *Diaphorina citri* psyllid, which is the disease vector in Brazil (Bové, 2006).

Gene insertion through genetic transformation is an alternative for controlling this disease given the lack of resistance to HLB in the citrus genus, low effective control measures, and rapid disease dissemination (Belasque Júnior et al., 2010). Antimicrobial peptides have been identified in several organisms, from bacteria

to humans, and they are recognized as key components of the defense system. *D4E1* is a synthetic peptide with 17 amino acids and a beta sheet conformation in solution that does not change when interacting with cell membranes (De Lucca et al., 1998); it originates from and operates similarly to cecropins by forming pores in the cell membrane of Gram-positive and Gram-negative bacteria. Tests to evaluate hemolytic activity for *D4E1* in mammalian cells showed that this peptide has low toxicity at the concentrations used for phytopathogen elimination (Stover et al., 2013).

The expression of synthetic peptides in transgenic plants offers advantages over natural analogs, including greater specificity, efficiency at low concentrations and a smaller size compared with natural peptides, without contamination or toxicity for the transgenic plant tissue; furthermore, it better resists degradation compared with natural cecropin (Cary et al., 2000). Transgenic plants from a *Populus tremula* L. x *P. alba* L. hybrid that expressed the *D4E1* peptide showed a decrease in symptom severity for diseases caused by *Agrobacterium tumefaciens* and *Xanthomonas populi* pv. *populi* (Mentag et al., 2003). The genetic transformation of tobacco with a gene encoding the synthetic antimicrobial peptide *D4E1* controlled the growth of *Aspergillus flavus* and *Verticillium dahliae* fungi (Cary et al., 2000); whereas the *D4E1* gene expression in cotton plants reduced disease symptoms caused by *Thielaviopsis basicola* (Rajasekaran et al., 2005). In a recent work, Stover et al. (2013) reported excellent results regarding in vitro control by the *D4E1* peptide of Proteobacteria, which belong to the same family of HLB-associated bacteria (*Ca. Liberibacter* spp.). Presumably, the expression of the *D4E1* peptide in phloem might be effective for controlling HLB-associated bacteria in citrus.

Several promoters have already been identified for tissue-specific expression, including those that preferentially induce expression in the phloem, such as *Citrus sinensis* sucrose synthase-1 promoter (CsSUS1p) (Singer et al., 2011), *Arabidopsis thaliana* phloem protein 2 (AtPP2), *C. sinensis* phloem protein 2 (CsPP2), *A. thaliana* sucrose transporter 2 (AtSUC2) (Miyata et al., 2012), and rice sucrose synthase-1 (RSs-1) (Wang et al., 2005; Dutt et al., 2012). In addition, promoters not originated from plants, such as those from phloem-limited virus genes, including the *Coconut foliar decay virus* (CFDV) (Hehn & Rohde, 1998) and the *Rice tungro bacilliform virus* (RTBV), have also been used as promoters with strong

expression in citrus plants (Dutt et al., 2012). The preferential expression of the β -glucuronidase enzyme in phloem was shown through *uidA* gene expression in transgenic citrus driven by the AtPP2, CsPP2, AtSUC2, RSs-1, and RTVB promoters (Dutt et al., 2012; Miyata et al., 2012).

The expression of an antimicrobial peptide in the citrus plant phloem, particularly in young leaves, can aid in controlling HLB because this disease is associated with bacteria restricted to phloem cells, which are primarily spread by psyllids while feeding on young leaf phloem (Dutt et al., 2012). Another advantage of preferential expression in phloem is that it decreases the expression of the transgene in other tissues (Zhao et al., 2004).

This work is pioneering in Brazil in relation to quantification by quantitative PCR (qPCR) of gene expression driven by phloem specific promoter in citrus, especially in sweet orange. Although these techniques have already been applied in other studies, the genetic transformation of citrus is a complex issue, since this woody species shows different responses depending on the cultivars and gene constructs used.

The objective of this work was to produce transgenic 'Pêra' and 'Valência' sweet orange plants using the *D4E1* gene driven by the AtPP2 promoter and to quantify transgene expression in different transformation events.

Materials and Methods

The study was conducted at the Laboratório de Biotecnologia de Plantas Hortícolas, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, SP, Brazil. For the genetic transformation experiments, the strain of *A. tumefaciens* EHA 105 was used, which expressed the binary vector pCAMBIA 2201/*D4E1* driven by the AtPP2 promoter and the nopaline synthase (NOS) terminator, as well as the *nptII* gene for kanamycin-resistance selection (Figure 1). 'Pêra' and 'Valência' sweet orange cultivars were selected as the genetic material for the transformation experiments. Seed extraction, explant preparation, transformation, and plant acclimation procedures were carried out according to Miyata et al. (2012).

Plant DNA from the regenerated plants was extracted for PCR analysis using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1990). The specific primer sequences from the AtPP2 promoter

to the *D4E1* gene were used (AtPP2/*D4E1*) (Table 1). The samples were amplified in a thermocycler programmed for the following conditions: 94°C for 2 min; 40 cycles at 94°C for 30 s, 58°C for 30 s, and extension at 72°C for 30 s; and the reaction was terminated by maintaining the temperature at 72°C for 4 min. The amplification product size of 353 bp was assessed through electrophoresis on a 1.5% agarose gel stained with ethidium bromide (0.03 µg mL⁻¹) and photographed under UV light.

Southern blot analysis was performed as described by Southern (2006) to assess whether T-DNA was integrated into the plant genome, using a 275 bp probe with the *D4E1* gene and NOS-T terminator (*D4E1*/NOS) (Table 1).

RNA extraction from leaves was performed using TRIzol (Life Technologies, Carlsbad, CA, USA) and the extraction protocol recommended by the manufacturer for performing expression analysis using real-time qPCR. Subsequently, the RNA was purified using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) followed by a DNase treatment using the RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to quantify and assess the RNA. RNA integrity was assessed using 1.5% agarose gel at 60 V for 40 min. Reverse transcription was performed in a total volume of 20 µL, with total RNA (1 µg), oligo (dT) primer (10 µmol L⁻¹), and *Moloney murine*

leukemia virus (M-MLV) enzyme (1 µL). The reaction was carried out according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). The cDNA samples were standardized to 6.6 ng µL⁻¹ upon reaction completion and stored at -20°C. The relative expression was quantified through real-time qPCR analysis upon cDNA synthesis completion.

Two reference genes, cyclophilin (*cyc*) and ubiquitin (*ubq*), were used to analyze gene expression through real-time quantitative PCR and to normalize the *D4E1* gene expression levels via specific primers (Table 1). Different concentrations of primers and samples were used to optimize the reaction efficiency. Real-time qPCR analyses were performed in 15 µL solution with 5 µL of cDNA (30 ng of cDNA), 0.3 µL forward and reverse primers, 7.5 µL Fast Sybr Green Master Mix (Life Technologies, Carlsbad, CA, USA), and 1.9 µL nuclease-free water. Samples were amplified using three replicates per sample, including samples from non-transgenic plants and a control sample without DNA. The qPCR analyses were performed in a 7500 Fast System (Life Technologies, Carlsbad, CA, USA) using the comparative method and the fast mode in the device's default program. The amplicons were assessed at the end of the reaction through melting analysis, which identifies the average melting point of the amplicons formed during the reaction. The detection of a single dissociation peak for the PCR product strands was regarded as evidence for reaction specificity.

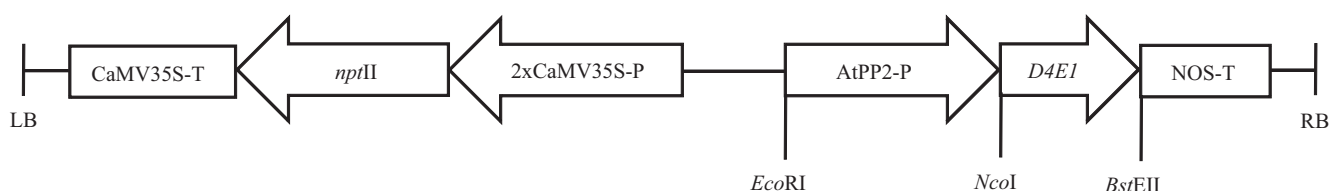


Figure 1. Schematic representation of the T-DNA region in the pCambia2201 vector used for genetic transformation. Gene construct with the *nptII* selection gene controlled by the CaMV35S promoter and the *D4E1* gene controlled by the AtPP2 promoter. LB, left border; RB, right border.

Table 1. Reference and target gene primer sequences and amplicon sizes used in the PCR and qPCR experiments.

Gene	Forward/Reverse 5'-3' sequence	Amplicon (bp)
AtPP2/ <i>D4E1</i>	CGATAGTTGCTGCCAAAAC/GGTCACCTTACAACCTTAATCTTAGC	353
<i>D4E1</i> /NOS	CCATGGGATTTAAGTTGAGAG/TTTGCGCGCTATATTTGTTT	275
<i>cyc</i> ⁽¹⁾	AGAGTATGCAGAGGAATGG/GTCCTTAACAGAAGTCCGT	107
<i>ubq</i> ⁽¹⁾	TTCGTCAGTTGACTAATCCT/GTTGCTGTGTTGACTGTG	95
<i>D4E1</i>	CCATGGGATTTAAGTTGAGAG/GGTCACCTTACAACCTTAATCTTAGC	69

⁽¹⁾Boava et al. (2011).

The quantification cycle (Cq) value was detected for the target gene and for the two reference genes in each transformation event. The data generated during the qPCR analyses were evaluated to assess efficiency using the LinRegPCR software (Ramakers et al., 2003). This software performs a linear regression for each amplification curve and generates an efficiency value for each reaction, as well as an average efficiency for each gene. After this calculation, the Cq data produced through qPCR analysis were entered into the GenEx software, version 5.3.6.170 (Kubista et al., 2006), and the average efficiency calculated from LinRegPCR was used to correct the Cq value for each gene. After correcting the efficiency, the software compared the data using the following relative quantification formula: $RQ = 2^{-\Delta\Delta Cq}$ (Schmittgen & Livak, 2008). The calibrator was the lowest *D4E1*-expressing transformant.

Results and Discussion

The 'Valência' sweet orange explants showed higher bud regeneration and transformation efficiency than those of 'Pêra' sweet orange (Table 2). A similar performance has been observed for 'Pêra' sweet orange in other genetic transformation studies, with lower regeneration and transformation efficiency compared with 'Valência' sweet orange (Boscariol et al., 2003; Fávero et al., 2012). This difference is attributed mainly to the cultivar and to the affinity with *A. tumefaciens* (Mendes et al., 2002; Almeida et al., 2003; Miyata et al., 2011). Citrus species and cultivars show different responses to genetic transformation.

Therefore, specific protocols must be developed and adapted to each genotype (Donmez et al., 2013).

The AtPP2/*D4E1* construct was found in six plants from 'Pêra' sweet orange and in seven plants from 'Valência' sweet orange using PCR analysis (Figure 2). For Southern blot analysis, the transgene copy number in the 'Pêra' sweet orange genome was one for the for the six events, and the integration of two copies in two events and of one copy in the remainder was verified in the 'Valência' sweet orange plants (Figure 3). Noticeably, no event showed gene silencing according to the qPCR analysis results because all transformation events presented gene expression. Gene silencing is typically detected in plants with multiple copies integrated at one or several loci; however, gene silencing was not reported in citrus plants with up to four transgene copies (Dutt et al., 2012; Benyon et al., 2013).

D4E1 gene expression analysis through qPCR showed variable expression, from $\text{Log}_{10}^{0.76}$ to $\text{Log}_{10}^{1.7}$, which corresponds to 5.75 (event 7) and 50 (event 6) times compared with the lowest *D4E1*-expressing transformant (Figure 4). The different expression

Table 2. Number of explants introduced, bud-regenerating explants, PCR-positive plants, and genetic transformation efficiency for 'Pêra' and 'Valência' sweet oranges using the AtPP2/*D4E1* gene construct.

Cultivar	N ^o of cultivated explants	N ^o of explants with buds	PCR-positive plants/EP ⁽¹⁾	Transformation efficiency ⁽²⁾ (%)
'Pêra'	939	73	6/9	0.64
'Valência'	343	44	6/8	1.74

⁽¹⁾EP, evaluated plants. ⁽²⁾Percentage of PCR-positive plants related to total cultivated explants.

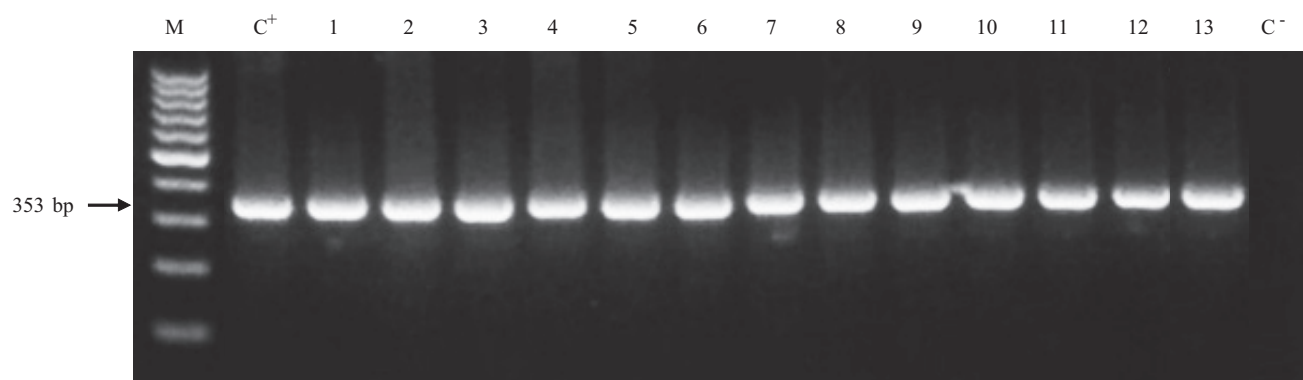


Figure 2. Amplification product from PCR of 'Pêra' (1–6) and 'Valência' (7–13) sweet oranges expressing the AtPP2/*D4E1* gene construct. M, GeneRuler 100 bp DNA Ladder (Fermentas, Vilnius, LTU); C⁺, positive control, reaction with plasmid containing the AtPP2/*D4E1* construct; and C⁻, non-transgenic plant.

levels are related to the number of copies and to the transgene-insertion position in the genome (Elmayan & Vaucheret, 1996). The transgene expression values

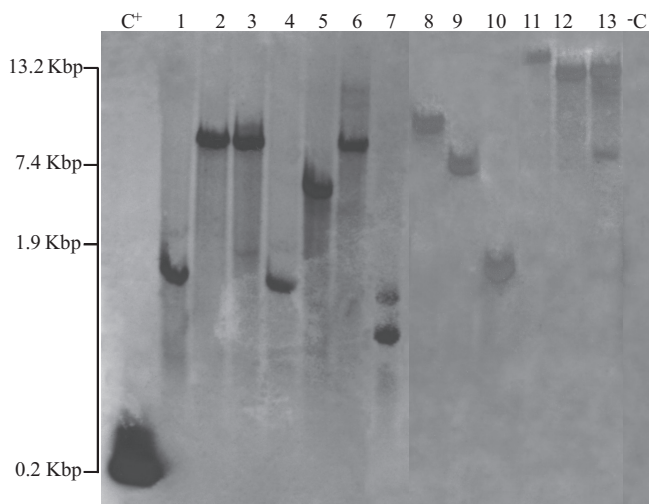


Figure 3. Southern blot analysis for 'Pêra' (1–6) and 'Valência' (7–13) sweet oranges. DNA was digested using the *EcoRI* enzyme and hybridized with a probe containing an amplicon from the *D4E1* gene and NOS terminator. C⁺, positive control, amplicon with *D4E1*-NOS amplified through PCR; C⁻, non-transgenic plant.

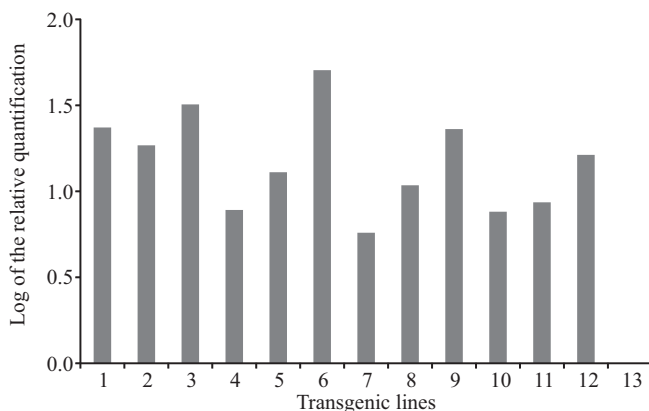


Figure 4. Quantification of relative *D4E1* gene expression driven by the AtPP2 promoter, analyzed through real-time PCR. Columns 1–6, transformation events for 'Pêra' sweet orange plants; columns 7–12, transformation events for 'Valência' sweet orange; and column 13, 'Valência' sweet orange transformation event with lowest *D4E1* expression, used as a calibrator.

are similar to those found by Benyon et al. (2013), who observed values from 4.24 to 118.88, corresponding to $\log_{10}^{0.62}$ to $\log_{10}^{2.07}$ for *uidA* gene expression driven by the AtSUC2 phloem-specific promoter.

Several phloem-specific promoters have been studied for genetic transformation because constitutive transgene expression is unnecessary when localized expression suffices. Miyata et al. (2012) demonstrated specific GUS expression driven by the AtPP2 promoter in phloem tissue of transgenic sweet orange. However, *uidA* gene expression driven by this type of promoter, quantified by qPCR, is relatively lower when compared to genes driven by constitutive promoters (Dutt et al., 2012; Benyon et al., 2013).

Conclusions

1. 'Pêra' and 'Valência' sweet oranges can be transformed with the *D4E1* gene driven by the AtPP2 promoter.
2. *D4E1* gene expression levels vary from 5 up to 50 times among different transformation events.

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