ISSN 0100-2945 DOI: http://dx.doi.org /10.1590/0100-29452019116

Revista Brasileira de Fruticultura

Genetics and plant breeding

In vitro organogenesis and genetic transformation of mandarin cultivars

Leonardo Soriano[1], Eveline Carla da Rocha Tavano[2], Marcelo Favaretto Correa[3], Ricardo Harakava[4], Beatriz Madalena Januzzi Mendes[5], Francisco de Assis Alves Mourão Filho[6]

Abstract – The *in vitro* organogenesis of Fremont (*Citrus clementina* x *Citrus reticulata*), Thomas (*Citrus reticulata*), and Nules (*Citrus clementina*) mandarins was evaluated aiming to optimize a regeneration protocol that could be applied in genetic transformation. The use of epicotyl-derived explants resulted in higher explant responsiveness and number of shoots developed per explant when compared with the use of internodal-derived explants. The highest efficiency in shoot regeneration was observed in the presence of 1 mg L^{-1} of BAP, regardless of the explant type and cultivar. The *in vitro* organogenesis protocol produced transgenic plants from three mandarin cultivars expressing *att*A gene under the control of phloem-specific promoters.

Index Terms: antimicrobial peptide, cultivar improvement, cytokinin, huanglongbing, transgenic plants.

Organogênese *in vitro* e transformação genética de cultivares de tangerina

Resumo – A organogênese *in vitro* das tangerinas Fremont (*Citrus clementina* x *Citrus reticulata*), Thomas (*Citrus reticulata*) e Nules (*Citrus clementina*) foi avaliada visando a otimizar o protocolo de regeneração para aplicação da transformação genética. O uso de explantes derivados de epicótilo resultou em maior número de explantes responsivos e maior número de brotações desenvolvidas por explante, quando comparado ao uso de explantes derivados de segmentos internodais. A maior eficiência na regeneração de brotos foi observada na presença de 1 mg L^{-1} de BAP, independentemente do tipo de explante e da cultivar. O protocolo de organogênese *in vitro* produziu plantas transgênicas das três cultivares de tangerina, expressando o gene *att*A sob o controle de promotores específicos de floema.

Termos para indexação: citocinina, huanglongbing, melhoramento de cultivares, peptídeo antimicrobiano, plantas transgênicas.

Corresponding author:
francisco.mourao@usp.br

Received: September 19, 2018
Accepted: January 04, 2019

[1] Forestry Eng. Dr. Center for Nuclear Energy in Agriculture – Plant Biotechnology Lab.; University of São Paulo/Piracicaba-SP, Brazil. E-mail: soriano84@gmail.com (ORCID 0000-0002-8958-4000)

[2]Biologist, Dr. Center for Nuclear Energy in Agriculture – Plant Biotechnology Lab.; University of São Paulo/Piracicaba-SP, Brazil. E-mail: ectavano@gmail.com (ORCID 0000-0001-5385-3267)

[3]Biologist, Lab. technician – Center for Nuclear Energy in Agriculture – Plant Biotechnology Lab.; University of São Paulo/Piracicaba-SP, Brazil. E-mail: mfcorrea@cena.usp.br (ORCID 0000-0002-9871-9149)

[4]Biologist, Dr – Researcher – State Department of Agriculture and Supply / Biological Institute –Biochemical Plant Pathology Lab.; São Paulo-SP, Brazil. E-mail: harakava@biologico.sp.gov.br (ORCID 0000-0003-1431-2665)

[5]Agr. Eng. – Associate Professor 2 (Retired), Center for Nuclear Energy in Agriculture – Plant Biotechnology Lab.; University of São Paulo – Piracicaba-SP, Brazil. E-mail: bmendes@cena.usp.br (ORCID 0000-0003-0344-3993)

[6]Agr. Eng. – Full Professor – Crop Science Department, University of São Paulo/ Luiz de Queiroz College of Agriculture/ Piracicaba-SP, Brazil. E-mail: francisco.mourao@usp.br (ORCID 0000-0001-8950-9513)

Introduction

In the last three decades, the citrus genetic transformation has been successfully applied to the production of transgenic plants of different citrus species and the introduction of transgenes coding for several characteristics, such as resistance to biotic and abiotic stress (KOBAYASHI; UCHIMYA, 1989; BARBOSA-MENDES et al., 2009; CARDOSO et al., 2010). Although genetic transformation can be accomplished by particle bombardment, PEG, and *Agrobacterium*-mediated, this last method has been the most frequently used in citrus (DONMEZ et al., 2013).

Sweet orange cultivars (BOSCARIOL et al., 2006), Carrizo citrange (DUTT; GROSSER, 2009), and trifoliate hybrids (ZOU et al., 2008) present high genetic transformation efficiency. However, some species, such as sour orange (GUTIÉRREZ et al. 1997; GHORBEL et al., 2000), Rangpur lime (AZEVEDO et al., 2006), and mandarins (CERVERA et al., 2008) are considered recalcitrant to the process. This low genetic transformation efficiency may be influenced by factors related to *Agrobacterium* inoculation procedure, co-cultivation conditions, and *in vitro* regeneration efficiency of explants after infection with *Agrobacterium* (COSTA et al., 2002; RODRIGUEZ et al., 2008; DUTT; GROSSER, 2009; YANG et al., 2016).

In vitro citrus organogenesis is usually induced from juvenile tissue, including epicotyl-derived segments extracted from *in vitro* germinated seedlings (GARCIA-LUIS et al., 2006; SORIANO et al., 2012) or internodal-derived segments extracted from juvenile plants cultivated under greenhouse conditions (TAVANO et al., 2009; MARQUES et al., 2011). In most of citrus tissue culture protocols, the development of adventitious shoots occurred in culture media supplemented with growth regulators (SILVA et al., 2005; OLIVEIRA et al., 2010; BASSAN et al., 2011). However, the requirements for optimal *in vitro* regeneration varies according to the genotype (BORDÓN et al., 2000). In experiments involving transformation of mature tissue of sweet orange (*C. sinensis*), the use of 3 mg L^{-1} of 6-benzylaminopurine (BAP) in the regeneration medium induced an opposite effect in *in vitro* responses, leading to efficient shoot organogenesis in Pineapple, but not in the cultivar Navelina (RODRIGUEZ et al., 2008). Considering these aspects, the *in vitro* organogenesis from closely related mandarin cultivars was studied to adjust the regeneration protocol and to use it for *Agrobacterium*-mediated genetic transformation.

The mandarin cultivars Thomas (*Citrus reticulata*) and Nules (*Citrus clementina*) and the intraspecific hybrid Fremont (*Citrus clementina* x *Citrus reticulata*) have great potential to be used in mandarin commercial production (PIO et al., 2006). However, these cultivars are affected by Huanglongbing (HLB). Currently, HLB is considered as the main citrus disease, associated with the bacteria *Candidatus* Liberibacter spp., which colonize phloem tissues (BOVÉ, 2006). Genetic engineering may be an alternative to HLB management. The expression of genes coding for antimicrobial peptides at the pathogen colonization site may be a good strategy to restrict the bacterial multiplication in infected plants. The expression of the *att*A gene, which codes for the antimicrobial peptide attacin A, showed to be effective in reducing *C. sinensis* susceptibility to *Xanthomonas citri* subsp. *citri* (BOSCARIOL et al., 2006; CARDOSO et al., 2010). The production of transgenic mandarin plants expressing the *att*A gene in the phloem tissue seems to be an adequate approach to manage HLB disease.

This study investigated the *in vitro* organogenesis of epicotyl and internodal-derived segments from Fremont, Thomas, and Nules mandarins. The protocol was then used to produce transgenic plants expressing the *att*A gene under the control of *Arabidopsis thaliana* sucrose transporter 2 promoter (AtSuc2) or *A. thaliana* phloem protein 2 (AtPP2) promoter, in an attempt to express the *att*A gene preferentially at the phloem tissue, aiming to reduce the susceptibility to *Ca.* Liberibacter spp.

Materials and Methods

Plant Material

Seeds were extracted from mature fruits of the mandarin cultivars Fremont (*Citrus clementina* x *Citrus reticulata*), Thomas (*Citrus reticulata*), and Nules (*Citrus clementina*). The seed coat was removed, and seeds were treated with sodium hypochlorite solution (0.5% active chloride; 20 min) for asepsis, followed by three rinses with sterile water. For *in vitro* germination, seeds were transferred to test tubes containing MS salts and vitamins solid culture medium (MURASHIGE; SKOOG, 1962), incubated at 27 ºC, in the dark (3 - 4 weeks), and transferred to a 16-h photoperiod (10 d). Epicotyl segments (0.8 - 1.0 cm) were extracted from *in vitro* germinated seedlings. Internodal segments (1 cm) were obtained from new elongated shoots from juvenile mandarin plants cultivated in a greenhouse. Stem pieces were collected, leaves were removed, and explants were disinfected with sodium hypochlorite solution (2.5% active chloride; 20 min), followed by three rinses with sterile distilled water.

In vitro organogenesis

Epicotyl and internodal-derived segments were horizontally cultivated in Petri dish (100 x 15 mm) containing MT salts and vitamins solid culture media (MURASHIGE; TUCKER, 1969), supplemented with BAP (0, 0.5, 1.0, 1.5, 2.0 mg L^{-1}). The explants were incubated in the dark, at 27 ºC, for 30 d, and then transferred to a 16-h photoperiod (65 µmol m^{-2} s^{-1}). The evaluation was

performed at 15 d after incubation, in a 16-h photoperiod, by determining the percentage of responsive explants and the number of shoots per explant for each treatment. The experimental design was completely randomized, with six replications per treatment. Each replication consisted of six explants, totaling 36 explants per treatment. Three independent experiments were performed for each cultivar. Data were analyzed by ANOVA, and the means were compared by the Tukey's test ($P < 0.05$). The influence of BAP concentrations on *in vitro* organogenesis was analyzed by regression analysis.

Genetic transformation

The experiments were performed with pCAtSuc2/*att*A and pCAtPP2/*att*A gene constructs, which contain the *att*A gene under the control of *Arabidopsis thaliana* sucrose transporter 2 (AtSuc2) or *A. thaliana* phloem protein 2 (AtPP2) promoters, respectively. Gene constructs were assembled into a pCAMBIA2201 vector and transferred to *Agrobacterium tumefaciens*, as reported by Tavano et al. (2015). The *npt*II gene, which confers resistance to the kanamycin antibiotic, was used as a selection gene.

Genetic transformation experiments were performed with epicotyl-derived explants from the mandarin cultivars Fremont, Thomas, and Nules, via *A. tumefaciens*. The co-culture medium consisted of MT salts and vitamins medium supplemented with BAP (1 mg L^{-1}), acetosyringone (100 mM), sucrose (25 g L^{-1}), ascorbic acid (50 mg L^{-1}), and agar (8 g L^{-1}), and the pH was adjusted to 5.5. During the co-culture period, the material was incubated in the dark (2 d; 24 °C). This protocol was used based on the results of the protocol adjustment in the first part of this study, where the best explant type and optimum BAP concentration were determined. After this period, the material was transferred to selection and regeneration medium, consisting of MT salts and vitamins medium supplemented with BAP (1 mg L^{-1}), sucrose (25 g L^{-1}), ascorbic acid (50 mg L^{-1}), agar (8 g L^{-1}), cefotaxime (100 mg L^{-1}), and kanamycin (100 mg L^{-1}), and the pH was adjusted to 5.8. The culture was incubated at 27 ºC, in the dark, for 30 d, and then transferred to a 16-h photoperiod. Shoots were *in vitro*-grafted onto *in vitro*-germinated Carrizo citrange (*C. sinensis* x *Poncirus trifoliata*) seedlings. This procedure has shown to improve the transgenic shoot development since the initial *in vitro* rooting of transgenic Citrus material is poor, and its further development may be hindered, even after plant acclimation. The putative transgenic plants were identified by PCR analyses. The DNA was extracted from leaves of acclimatized plants by the CTAB method (DOYLE; DOYLE, 1990). PCR analyses were performed to amplify the *att*A gene, using the primers Att-Psc2-F 5'ACATGTCCCGTTATTTGGTCTTTGAA3' and Att-Bst-R 5'GGTCACCTACCACTTATTACCAAAAGAC3', following the program: 94 ºC for 3 min; 35 times at 94 ºC for 30 s; 52 ºC for 30 s; 72 ºC for 1 min; and a final extension at 72 ºC for 4 min. The transformation efficiency was expressed as the percentage of PCR-positive plants in relation to the total explants inoculated with *A. tumefaciens*.

Southern blot analysis

DNA was extracted from leaves of PCR-positive acclimatized plants, using the CTAB method (DOYLE; DOYLE, 1990). DNA (20 - 60 µg) was digested with *Bam*HI (pCAtSuc2/*att*A) or *Hind*III (pCAtPP2/*att*A) restrictions enzymes (5 U μg^{-1}; 16 h). *Bam*HI- or *Hind*III-digested DNA-fragment were separated by electrophoresis on agarose gel (1%), transferred to nylon membrane (Hybond N^+ - GE Healthcare, Little Chalfont, UK), and fixed (80 ºC; 2 h). A PCR amplified fragment of the *att*A gene (150 ng) was labeled with alkaline phosphatase, using AlkaPhos direct labeling reagents (GE Healthcare, Little Chalfont, UK), and used as a probe. Hybridization, washing, and detection were performed by the Gene images CDP star detection kit (GE Healthcare, Little Chalfont, UK), following the manufacturer's instructions.

***att*A gene transcription analyses**

RNA was extracted from midrib leaves collected from plants identified as Southern blot-positive, using the TRIZOL reagent (Invitrogen, Carlsbad, USA). Isolated RNA was purified (RNAeasy plant mini kit - Qiagen, Hilden, Germany) and treated with DNAse (RNase-free DNase set - Qiagen). The first strand of cDNA was synthesized from DNAse-treated RNA (1 µg), using1 µl oligo (dT) (10 µM) and 1 µl M-MLV reverse-transcriptase enzyme (Invitrogen, Carlsbad, USA), as described by the manufacturer. RT-qPCR analyses were performed with the 7500 FAST™ Real-time PCR System (Applied Biosystems, Foster City, USA). The *att*A gene was amplified with the primers 5'TCGTCACCAAGAACATGCCTGACT3' and 5'AAGAATGGAGTGTTTGCCATGCCG3'. The ubiquitin (UBQ) gene was used to normalize the *att*A gene transcription. Primers 5'TTCGTCAGTTGACTAATCCT3' and 5'GTTGCTGTGTTGACTGTG3' were used to amplify the UBQ gene (BOAVA et al., 2011). Reactions contained 10 µl cDNA (25 ng), 0.6 µl primer (5 µM), 7.5 µl Fast Sybr green master mix (Applied Biosystems, Foster City, USA), and 1.9 µl RNase-free water. The following program was used: 95 ºC for 20 s; 40 cycles at 95 ºC for 3 s; and 60 ºC for 30 s; followed by 95 ºC for 15 s. A constant increase in the temperature between 60 ºC and 95 ºC was applied during the melting analyses. Two technical replications were used for each cultivar and gene construct. A non-transgenic plant and non-template sample (water) were used as negative controls. Data were analyzed by the LinRegPCR software (RAMAKERS et al., 2003), for primer efficiency calculation, and the REST 2009 software (PFAFFL et al., 2002), for relative quantification of the *att*A gene transcript, using the comparative

Ct method (ΔΔCt). Relative transcription of the *att*A gene was calculated in relation to the transgenic plant presenting the lowest *att*A transcript level.

Results and Discussion

In vitro organogenesis

In vitro organogenesis was observed in both explant types studied, *i.e*., the epicotyl and internodal-derived segments from the three mandarin cultivars. Shoot development was registered as early as six weeks after culture, regardless of the culture medium supplementation with BAP (Table 1; Figure 1). In all cultivars, epicotyl segments led to a higher % of responsive explants and number of shoots per explant (Table 1).

The results for the culture medium supplementation with cytokinin showed that, although BAP is not essential for *in vitro* organogenesis, the efficiency of shoot development increased up to the concentration of 1 mg L^{-1}, regardless of the cultivar (Table 1; Figure 1). Considering epicotyl-derived explants, the percentages of responsive explants varied from 73.15 to 82.41 in the mandarin cultivars studied, at 1 mg L^{-1} BAP concentration. When internodal-derived explants were used, this variable ranged from 40.74 to 54.63%, at the same cytokinin concentration. Similar results were obtained for the variable number of shoots developed per explant, whose maximum values for both explants and the three cultivars studied were obtained with BAP at 1 mg L^{-1}.

The success of genetic transformation programs depends on an efficient *in vitro* plant regeneration protocol. Considering citrus, epicotyl-derived explants are the most frequently used for genetic engineering in different cultivars (ALMEIDA et al., 2002; SILVA et al., 2005; GARCÍA-LUIS et al., 2006; NWE et al., 2014). Other explants, such as leaf discs- (KHAN et al., 2009), hypocotyl- (MAGGON; SINGH, 1995), cotyledon- (TAVANO et al., 2009), and internodal- (MARQUES et al., 2011) derived segments have also been tested; however, they presented lower efficiency. In this study, the adventitious shoot development occurred more frequently from epicotyl-derived explants, with more than 50% of responsive explants and more than four shoots developed per responsive explant (Table 1), regardless of the cultivar. Similar results have been reported for Volkamer lemon, sour orange, and Rangpur lime species, showing a higher number of responsive explants when epicotyl-derived explants were used, comparing with internodal or hypocotyl-derived explants (TAVANO et al., 2009; SORIANO et al., 2012).

Considering the regeneration efficiency from epicotyl-derived explants in other citrus species, some of them stand out with high regeneration efficiency, such as Troyer citrange, with 82 to 94% of responsive explants, and sweet oranges (Natal, Valencia, and Hamlin cultivars), with 71.42 to 85% of responsive explants (BORDÓN et al., 2000; MOREIRA-DIAS et al., 2001; ALMEIDA et al., 2002). The species considered as less responsive to *in vitro* organogenesis are Honey (*C. tangerine*) and Cleopatra (*C. reshni*) mandarins, with regeneration efficiency varying from 50 to 62.2%, respectively (SILVA et al., 2005; NWE et al., 2014). Some species, such as sour orange and Rangpur lime, are considered as recalcitrant to *in vitro* organogenesis, producing less than 50% of responsive explants (BORDÓN et al., 2000; ALMEIDA et al., 2002). In the present work, the cultivation of epicotyl-derived explants of Fremont, Thomas, and Nules cultivars in culture medium supplemented with 1 mg L^{-1} of BAP led to regeneration frequencies higher than 70% (Table 1; Figure 1). This result is similar to the regeneration efficiency reported in other studies involving three sweet orange cultivars (*C. sinensis*) (ALMEIDA et al., 2002) and seems to be adequate for genetic transformation experiments.

Genetic transformation

The genetic transformation was performed successfully resulting in transgenic plants of Fremont, Thomas, and Nules cultivars harboring the T-DNA that contains the *att*A gene under the control of AtSuc2 or AtPP2 promoters.

For Fremont, approximately 3200 explants were inoculated with *A. tumefaciens* carrying pCAtPP2/*att*A or pCAtSuc2/*att*A gene constructs, resulting in the production of three and eight transgenic plants, which was confirmed by the PCR analysis, for each construct, respectively (Table 2). For Thomas, about 3000 explants were inoculated with the *A. tumefaciens*, leading to the regeneration and confirmation of ten and 12 plants of pCAtPP2/*att*A and pCAtSuc2/*att*A gene constructs, respectively. Finally, for Nules, about 1600 explants were inoculated with *A. tumefaciens* carrying pCAtPP2/*att*A or pCAtSuc2/*att*A gene constructs, resulting in the production of three and two PCR-positive for each construct, respectively (Table 2). The genetic transformation efficiency ranged from 0.22% to 0.93%. The highest value was obtained for the experiments performed with Thomas cultivar and pCAtSuc2/*att*A gene construct (Table 2).

Genetic transformation is influenced by transgenic shoot regeneration efficiency after infection with *A. tumefaciens*. Some species are highly responsive to the *in vitro* organogenesis and genetic transformation, while others are less responsive or even recalcitrant to these processes. The genetic transformation efficiency reported for responsive genotypes, such as the sweet orange cv. Hamlin and Carrizo citrange, ranged from 14 to 47%, respectively (BOSCARIOL et al., 2006; DUTT; GROSSER, 2009). For recalcitrant species, such as Rangpur lime and sour orange, genetic transformation

efficiency was lower than 2.5% (GUTIÉRREZ et al., 1997; AZEVEDO et al., 2006).

In the present experiments, although the *in vitro* organogenesis frequency was higher than 70%, the genetic transformation efficiency obtained for Fremont, Thomas, and Nules mandarins was lower than 1%. This value is lower than the efficiency reported for other mandarin cultivars (KHAWALE et al., 2006; BACHCHU et al., 2011). Besides genotype dependence and regeneration frequency, the genetic transformation efficiency is influenced by other factors, including the *Agrobacterium* strain and density and the co-cultivation procedure (DUTT; GROSSER, 2009; YANG et al., 2016). The genetic transformation efficiency may also be influenced by gene constructs. A low genetic transformation efficiency was also observed for sweet orange cultivars and gene constructs containing the *uid*A gene under the control of AtSuc2, AtPP2, or CsPP2 phloem-specific promoters (MIYATA et al., 2012), reinforcing the idea that the gene construct may play an important role in the efficiency of genetic transformation. Future experiments, aiming to increase transformation efficiency, could include other transformation methods such as those evaluated by Dutt et al. (2018) in 'W Murcott' tangor.

The integration and transcription of the *att*A gene were confirmed by the Southern blot and RT-qPCR analyses, respectively. A total of 32 Southern blot positive plants were confirmed for the three mandarin cultivars and the two gene constructs studied. The T-DNA was inserted in different positions into the plant's genome. Most of the plants had one insertion event; however, two, three, or more than three integration events were also observed (Figure 2). Non-hybridization signal was detected in the non-transgenic plant (control). The transcription of the *att*A gene was confirmed in representative events from each gene construct for Fremont, Thomas, and Nules cultivars (Figure 3). Most of the transgenic plants showed a high level of the *att*A gene transcription, ranging from 10^1 to 10^4, in relation to the transgenic plants used as calibrator (Figure 3). The mandarin transgenic plants expressing the *att*A gene will be challenged with *Candidatus* Liberibacter spp. to investigate whether the expression of this antimicrobial gene will contribute to enhancing the resistance to HLB disease.

Table 1. *In vitro* organogenesis from epicotyl and internodal-derived segments from Fremont, Thomas, and Nules mandarins (mean of three experiments, totaling 108 explants per treatment).

cultivars	BAP (mg L^{-1})	responsive explants (%)		number of shoots/explant	
		epicotyl	internodal	epicotyl	internodal
Fremont	0.0	36.11	9.26	3.06	1.11
	0.5	56.48	23.15	4.61	2.17
	1.0	78.70	50.93	6.11	4.00
	1.5	57.41	15.74	4.56	1.78
	2.0	32.41	7.41	2.89	0.83
mean		52.22A	21.30a	4.24A	1.98b
SD		18.73	17.68	1.32	1.25
Thomas	0.0	34.26	7.41	3.28	1.39
	0.5	65.74	13.89	5.67	1.56
	1.0	73.15	40.74	5.89	3.89
	1.5	55.56	12.04	4.67	1.44
	2.0	36.11	5.56	3.39	0.72
mean		52.96A	15.93b	4.58A	1.80b
SD		17.40	14.27	1.23	1.21
Nules	0.0	30.56	16.67	3.22	2.06
	0.5	54.63	24.07	5.00	2.50
	1.0	82.41	54.63	6.56	5.17
	1.5	54.63	18.52	4.67	2.11
	2.0	31.48	12.96	3.17	1.83
mean		50.74A	25.37a	4.52A	2.73a
SD		21.28	16.84	1.41	1.38

Means followed by the same letter in the column are not significantly different by the Tukey's test ($P \leq 0.05$).

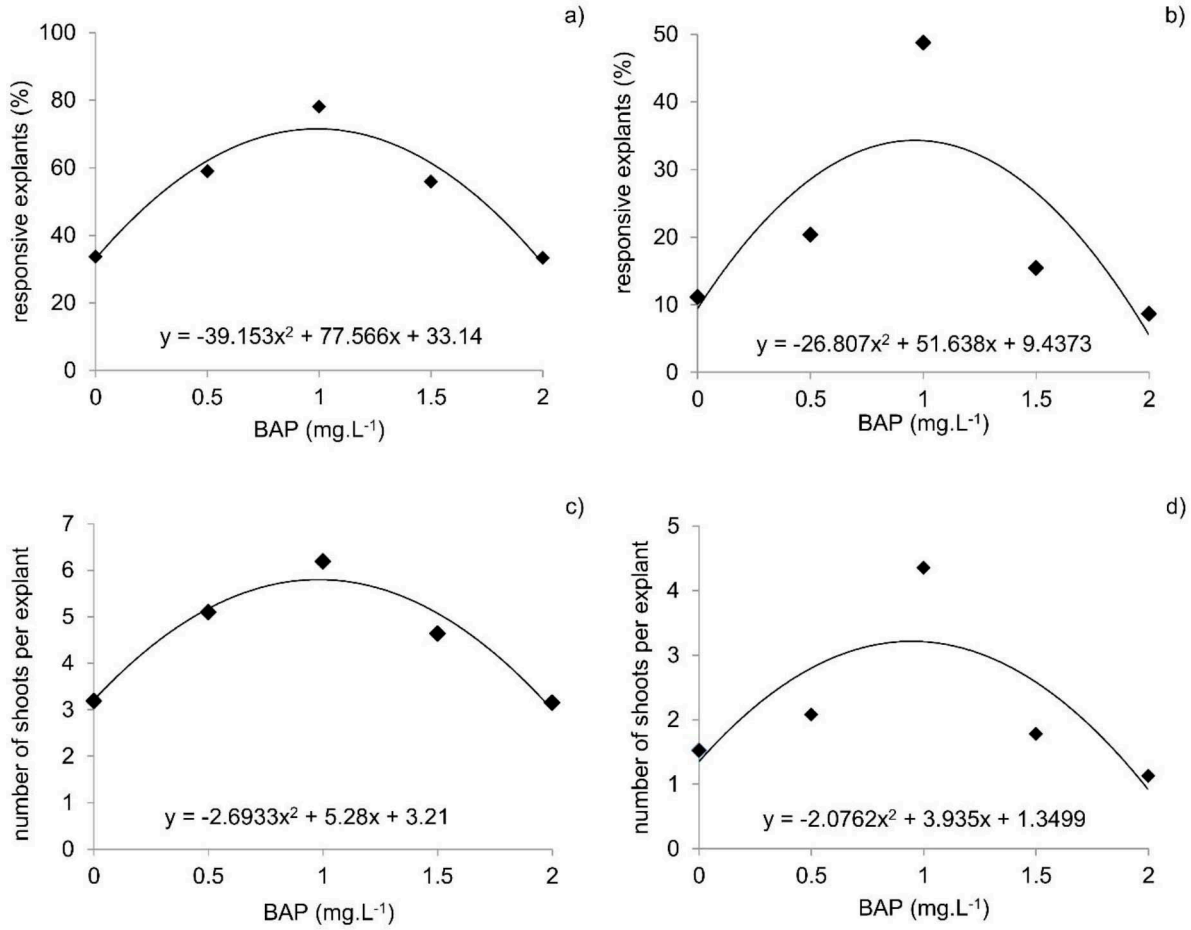

Figure 1. *In vitro* organogenesis of epicotyl and internodal-derived explants from Fremont, Thomas and Nules mandarin cultivars under the influence of the different concentration of BAP a-b) Mean of percentage of responsive explants derived from epicotyl (a) and internodal (b) segments for Fremont, Thomas, and Nules. c-d) Mean of number of shoots per explants derived from epicotyl (c) and internodal (d) segments for Fremont, Thomas, and Nules.

Table 2. Fremont, Thomas, and Nules mandarin genetic transformation experiments with gene constructs harboring the *attacin* A gene associated with phloem-specific promoters.

genotype	gene construct	no. PCR-positive plants/ total explants	genetic transformation efficiency (%)[a]	no. *Southern blot* + plants
Fremont	pCAtPP2/*att*A	3/1378	0.22	3
	pCAtSuc2/*att*A	8/1874	0.43	8
Thomas	pCAtPP2/*att*A	10/1743	0.57	7
	pCAtSuc2/*att*A	12/1297	0.93	12
Nules	pCAtPP2/*att*A	3/963	0.31	1
	pCAtSuc2/*att*A	2/709	0.28	1

[a] PCR-positive plants (x100) / total explants

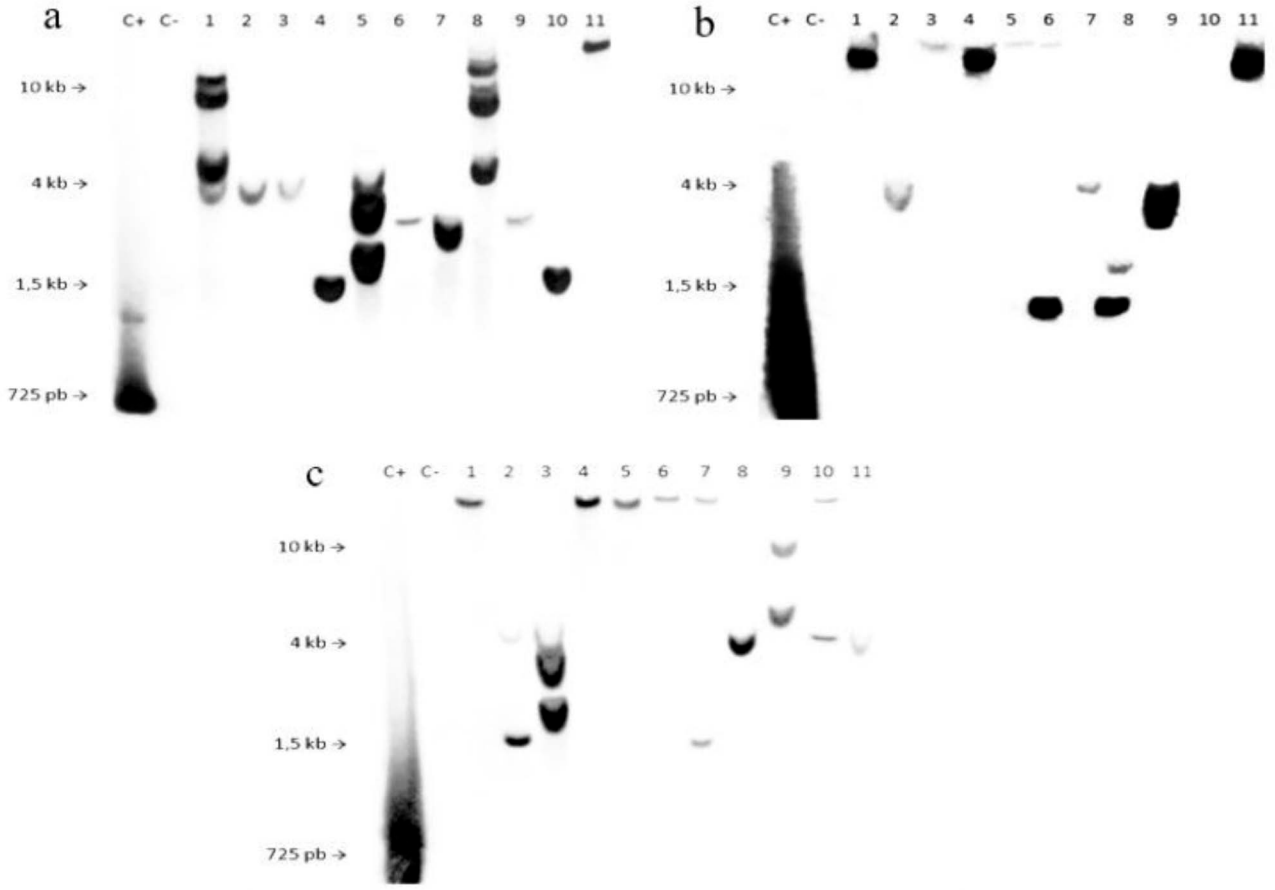

Figure 2. Southern blot analyses of transgenic plants of mandarin cultivars Thomas, Fremont and Nules transformed with pCAtPP2/*att*A (a) or pCAtSuc2/*att*A (b-c) gene constructs. a) Plants of cv. Fremont (events 1-3), Thomas (events 4-10) and Nules (event 11). Events 2, 3, 4, 6, 7, 9, 10 and 11 have one insertion. Events 1, 5 and 8 have more than three insertions. b) Plants of cv. Fremont (events 1-8), Thomas (event 9) and Nules (events 10-11). Events 1, 2, 3, 4, 5, 7, 9 and 11 have one insertion. Events 6 and 8 have two insertions. Event 10 not transformed. c) Plants of cv. Thomas (events 1-11). Events 1, 4, 5, 6, 8 and 11 have one insertion. Events 2, 7, 9 and 10 have two insertions. Event 3 has three insertions. C+: positive control (725 bp fragment of *att*A gene). C-: negative control (DNA from non-transgenic plant). Total DNA from transgenic and non-transgenic plants was digested with *Bam*HI (pCAtSuc2/*att*A) or *Himd*III (pCAtPP2/*att*A) and hybridized with a probe of the *att*A gene (150 ng).

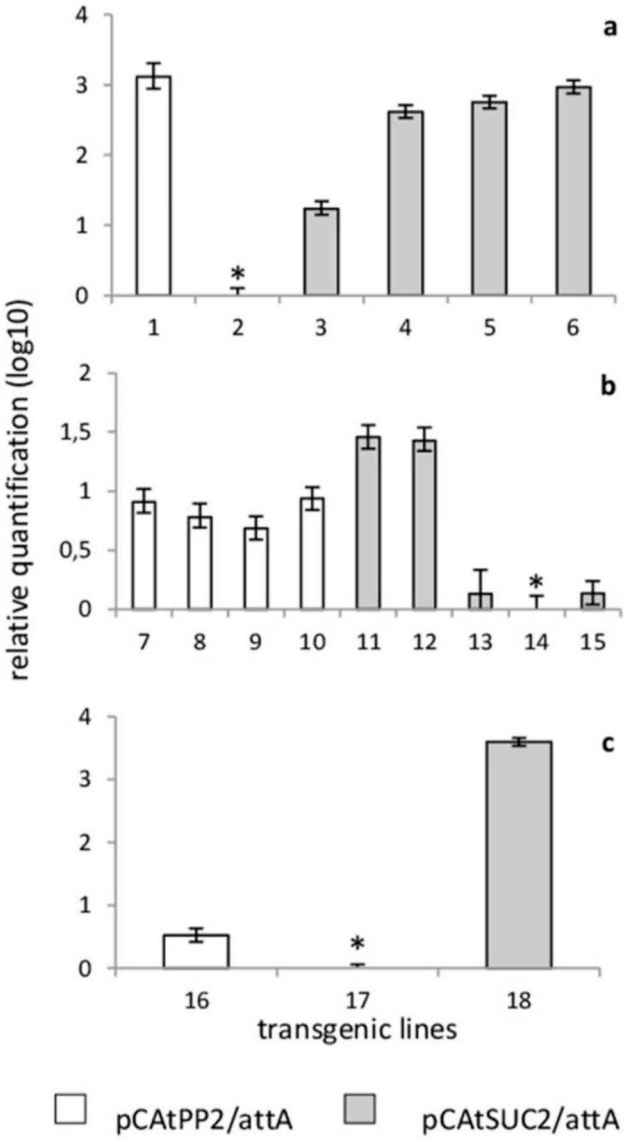

Figure 3. Relative quantification of the *att*A gene expression in transgenic plants of Thomas, Fremont, and Nules mandarin cultivars. a) Transgenic lines of Fremont cv. containing pCAtPP2/*att*A (bars 1 - 2) or pCAtSuc2/*att*A (bars 3 - 6) gene constructs. b) Transgenic lines of Thomas cv. containing pCAtPP2/*att*A (bars 7 - 10) or pCAtSuc2/*att*A (bars 11 - 15) gene constructs. c) Transgenic lines of Nules cv. containing pCAtPP2/*att*A (bar 16) or pCAtSuc2/*att*A (bars 17-18) gene constructs. Relative expression of the target gene was normalized with the ubiquitin (UBQ) gene and calculated in relation to the transgenic line that presented the lowest ΔCt value (*).

Conclusion

This study evaluated and confirmed an efficient *in vitro* organogenesis and plant regeneration protocol for three different mandarin cultivars. Epicotyl-derived explants in culture medium supplemented with BAP (1 mg L^{-1}) led to high regeneration frequency. The *in vitro* organogenesis protocol of mandarin cultivars allowed the production of transgenic plants from Fremont, Thomas, and Nules cultivars expressing the *att*A gene under the control of phloem-specific promoters.

Acknowledgments

The authors acknowledge Bebedouro Citrus Experiment Station (Estação Experimental de Citricultura de Bebedouro) for providing the plant material; São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP) for granting fellowship to LS and ECRT and for granting financial support to this project; National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq) for granting research fellowship to BMJM and FAAMF. The authors thank Maurel Behling and Sônia Maria De Stefano Piedade for support and advice on statistical analysis; Liliane Cristina Liborio Stipp and Renata Beatriz Cruz for technical support.

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