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Enhanced resistance to citrus canker in transgenic sweet orange expressing the sarcotoxin IA gene

Adilson K. Kobayashi · Luiz Gonzaga E. Vieira · João Carlos Bespalhok Filho · Rui Pereira Leite Jr · Luiz Filipe P. Pereira · Hugo Bruno C. Molinari · Viviani V. Marques

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Abstract Citrus canker, caused by the bacterial pathogen *Xanthomonas citri* subp. *Citri (*Xcc*),* is a serious disease reported in most citrus-producing areas around the world. Although different levels of field resistance to citrus canker have been reported in sweet oranges, they are usually not sufficient to provide adequate control of the disease. Ectopic over-expression of antibacterial genes is one of the potential strategies to increase plant resistance to bacterial diseases. Previous in vitro results showed that sarcotoxin IA, an antimicrobial peptide isolated from the flesh fly (*Sarcophaga peregrina*), can be efficient to control different plant pathogenic bacteria, including *Xcc*. Transgenic "Pera" sweet orange (*Citrus sinensis* [L.] Osbeck) plants constitutively expressing the sarcotoxin IA peptide fused to the PR1a signal peptide from *Nicotiana tabacum* for secretion in the intercellular space were obtained by *Agrobacterium*-mediated transformation using thin sections of mature explants. Citrus canker resistance evaluation in leaves of transgenic and non-transgenic plants was performed through inoculations with *Xcc* by infiltration and spraying. The *Xcc* population was up to 2 log unit lower in leaves of the transgenic plants compared to those of non-transgenic controls. Incidence of canker lesions was

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A. K. Kobayashi · L. G. E. Vieira · J. C. Bespalhok Filho · R. P. Leite Jr, · H. B. C. Molinari
Instituto Agronômico do Paraná - IAPAR, Rodovia Celso Garcia Cid, km 375, Londrina, PR 86001-970, Brazil

L. F. P. Pereira
Embrapa Café, Biothecnology Laboratory – IAPAR, Rod. Celso Garcia Cid Km 375, Londrina, PR 86001-970, Brazil

V. V. Marques
Fundecitrus – Fundo de Defesa da Citricultura, Av. Adhemar Pereira de Barros, 201, Araraquara, SP 14807-040, Brazil

Present Address:
A. K. Kobayashi · H. B. C. Molinari
Genetics and Biotechnology Laboratory, Embrapa Agroenergy, Brasília, DF 70770-901, Brazil

Present Address:
L. G. E. Vieira
Biotechnology Laboratory, UNOESTE, Rodovia Raposo Tavares, Km 572 - Limoeiro, Presidente Prudente, SP 19.067-175, Brazil

Present Address:
J. C. Bespalhok Filho
Universidade Federal do Paraná, R. dos Funcionários, 1540, Curitiba, PR 80035-050, Brazil

Present Address:
V. V. Marques (✉)
Biothecnology Laboratory, Fundo de Defesa da Citricultura – FUNDECITRUS, Av. Dr. Adhemar Pereira de Barros, 201 –, Araraquara, SP 14.807-040, Brazil
e-mail: vivianimarques@yahoo.com.br

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significantly higher in non-transformed controls (>10 lesions/cm^2) than in the transgenic plants (<5 lesions/cm^2) after injection infiltration or spraying with *Xcc* inoculum. Accumulation of sarcotoxin IA peptide in sweet orange tissue did not cause any deleterious effects on the growth and development of the transgenic plants, indicating this approach is suitable to provide resistance to citrus canker.

Keywords Citrus canker · Antimicrobial peptides · *Agrobacterium tumefaciens* · Mature tissue transformation; bacterial disease resistance

Introduction

Asiatic citrus canker, caused by the bacterial pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*), is a significant disease for citrus production in several countries with wet subtropical and tropical conditions (Gottwald et al. 2002). The disease is characterized by the presence of raised and corky lesions on leaves, branches and fruits of susceptible trees. These symptoms are due to parenchyma cell hypertrophy followed by extensive bacterial multiplication and exudation (Gottwald et al. 2002).

Economic losses may result from lower yield due to fruit and leaf abscission in addition to reduced value of diseased fruits for the fresh market. Quarantine restrictions may also constrain the commercialization of citrus fruits from canker-affected to disease-free areas (Gottwald et al. 2009). For many years, the basic measures for controlling citrus canker in the main sweet oranges-producing areas of São Paulo State in Brazil, and Florida in the USA, were the eradication of affected citrus trees. Millions of US dollars are spent on inspection and eradication programs in both citrus producing areas (Graham et al. 2004; Bronson and Gaskalla 2007). Currently, citrus canker control is based on exclusion measures in areas where the pathogen is not present, and implementation of an integrated management program that includes foliar spray of copper compounds and planting of less susceptible cultivars in areas to reduce canker incidence and severity (Graham et al. 2004; Behlau et al. 2008).

Although field resistance to citrus canker has been reported among citrus genotypes, hybrids and cultivars (Leite, 1990; Carvalho et al. 2015), the resistance present in most commercial citrus cultivars is not usually sufficient to provide acceptable canker control under favorable environmental conditions for disease development (Stall et al. 1982). Besides the lack of good natural resistance sources, conventional citrus breeding is limited by several factors including long juvenile period, high heterozygosis and crossing incompatibilities (Gmitter et al. 1992; Viloria et al. 2004). In contrast to the difficulties for conventional citrus breeding, genetic transformation is a useful technique to incorporate traits of interest into high value commercial varieties.

In order to improve resistance to bacterial diseases in plants by genetic engineering, different strategies have been proposed, including the over-expression of genes that code for antibacterial peptides, disease resistance proteins and transcription factors from non-plant origin that enhance natural plant defenses or are involved in the induction of programmed cell death at the infection site (Mourgues et al. 1998; Zhang et al. 2010; Wally and Punja 2010; Dutt et al. 2015). The constitutive expression of antimicrobial peptides (AMP) is one of those promising strategies to control plant pathogenic bacteria (Jaymes et al. 1993; Sharma et al. 2000; Cardoso et al. 2010; He et al. 2011; Furman et al. 2013).

Sarcotoxins are cecropin-like antibacterial peptides isolated from larvae of the common flesh eating fly, *Sarcophaga peregrina* (Kanai and Natori 1989). They belong to a group of antibacterial peptides in which two amphiphilic α-helices interact with bacterial cell membranes, causing the loss of their electrochemical potential (Nakajima et al. 1987). Among this group, sarcotoxin IA (STX IA), a 39-amino acid residue peptide, has been well-characterized (Yamada et al. 1990). In vitro experiments showed that synthetic sarcotoxin IA inhibited the growth of different plant pathogenic bacteria at low concentration, including *Xcc* (Ohshima et al. 1999).

The constitutive expression of the STX IA peptide in tobacco plants improved their resistance against two plant pathogenic bacteria, *Pseudomonas syringae* pv. *tabaci* and *Erwinia carotovora* (Ohshima et al. 1999). Furthermore, tobacco plants transformed with sarcotoxin IA under control of a modified promoter of the PR1a gene (Pathogenesis-Related 1a) showed improved resistance to both above-mentioned bacteria, as well as to *Rhizoctonia solani* and *Pythium aphanidermatum* (Mitsuhara et al. 2000). In addition, Mitsuhara et al. (2001) reported that STX IA possesses selective antibacterial activity, suppressing the growth of bacteria harmful to humans, such as *Clostridium ramosum, C. paraputrificum* and *Escherichia coli*

O157, with no detrimental effect on healthy intestinal microbiota, including the species *Bifidobacterium adolescentis*, *B. longum* and *Lactobacillus acidophilus.*

In this work, we describe *Agrobacterium*-mediated transformation of mature sweet orange (*Citrus sinensis* [L.] Osbeck) plants with the STX IA gene. The plants showed high level of sarcotoxin IA in leaves and exhibited resistance to *Xcc* infection compared to wild-type counterparts after inoculation with the *Xcc* bacterium.

Materials and methods

Bacterial strain and vector

The disarmed *Agrobacterium tumefaciens* strain EHA 105 carrying the plasmid pST10 (Okamoto et al. 1998) was used for transformation. This plasmid contained the STX IA coding region fused to the signal peptide from *Nicotiana tabacum* PR1a gene under the control of the double-enhanced strong constitutive 35S promoter from *Cauliflower mosaic virus* (CaMV) and the Omega region from *Tobacco mosaic virus* (TMV) (Fig. 1). The plasmid also contained the selectable marker gene *npt*II under the control of the *nopaline synthase* (*nos*) promoter and terminator sequences. For co-incubation experiments, EHA 105 cultures were grown in YMB medium (0.5 g l^{-1} K_2HPO_4, 0.1 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} NaCl, 5 g l^{-1} glucose, 10 g l^{-1} mannitol and 0.4 g l^{-1} yeast extract) containing 25 mg l^{-1} kanamycin, and incubated on a shaker at 28 °C for 24 h to produce cells in late log phase. The bacterial suspension was pelleted and adjusted to OD_{600} = 0.4 in fresh YMB medium plus 200 μM acetosyringone.

Plant transformation and regeneration

Pera sweet orange nursery trees maintained under greenhouse conditions were used as source of mature explants for transformation. Plants were pruned to induce new growth and elongated shoots (20 cm long) were used as source of mature tissue. Plant tissue culture conditions for genetic transformation followed the protocol as previously described (Kobayashi et al. 2003). Briefly, after removing the leaves, the shoots were surface sterilized for 15 min in 1% sodium hypochlorite solution followed by five rinses in sterile distilled water. Internodal stem explants were cut transversally into thin segments (1–2 mm) and incubated for 15 min in bacterial suspension with gentle shaking. The infected explants were blotted dry on sterile filter paper and placed horizontally on co-cultivation (CM) medium for 3 days. CM medium consisted of MS salts (Murashige and Skoog, 1962) supplemented with 1.8 μM 6-benzylaminopurine (6-BA), 0.7 μM gibberellic acid (GA_3), 3% *w/v* sucrose and 0.6% *w/v* Bacto agar, pH 5.7. After co-cultivation, the explants were transferred to shoot induction medium consisting of the same medium used for co-cultivation, plus 25 mg l^{-1} kanamycin for selection of transgenic shoots, and 200 mg l^{-1} cefotaxime and 200 mg $^{-1}$ timetin to control bacterial growth. Cultures were maintained in the dark at 26 °C. After 3 weeks, explants were transferred to elongation medium consisting of WPM salts (Lloyd and McCown, 1980) supplemented with 1.8 μM 6-BA, 0.7 μM GA_3, 3% *w/v* sucrose and 0.6% *w/v* Bacto agar, pH 5.7, plus 50 mg l^{-1} kanamycin, 200 mg l^{-1} cefotaxime and 200 mg l^{-1} timetin. Cultures were maintained at 26 °C, 45 μE m^{-2} s^{-1} illumination, 16 h/8 h photoperiod for 3–4 weeks.

Putative transgenic adventitious buds were regenerated into whole plants by in vitro shoot-tip grafting onto Carrizo citrange (*C. sinensis* [L.] Osbeck x *Poncirus trifoliata* L. Raf.) seedlings (Navarro 1992). A second step for grafting of the in vitro-growing plants on vigorous rootstocks of Rangpur lime (*Citrus limonia* Osbeck) allowed the rapid acclimatization and development of the plants under greenhouse conditions. Transgenic plants were propagated by grafting on Rangpur lime rootstocks.

Molecular analyses of the transgenic plants

Standard PCR techniques were used to detect the presence of the STX IA transgene in leaf samples from the regenerated putative transgenic plantlets. Primers 5′-GCCTCGGATCCATTCAAGATACAACATTTCT-3′ and 5′- AACTCGAGCTCATTTATCTGGCTGTA GCAGCAACAT-3′ were used for amplification of a 268 bp fragment. Reactions were performed in a thermal cycler under the following conditions: 29 cycles of 1 min at 94 °C, 45 s at 60 °C, 45 s at 72 °C, and a final extension period of 72 °C for 5 min.

Southern blot analysis was performed to confirm the stable integration of the STX IA gene in the transgenic plants. DNA was isolated from leaves according to Dellaporta et al. (1983). DNA samples (24 μg) were digested overnight with *Eco*RI, electrophoresed on 0.75% (*w/v*) agarose gel and transferred onto nylon membranes (Gibco BRL), according to Sambrock

et al. (1989). A 1289 bp fragment containing the sarcotoxin IA gene was used as template for labeling. The fragment was obtained from digestion of the pST10 plasmid with *Eco*RI and *Hind*III (Fig. 1). Labeling was performed using ^{32}P d-CTP and the Rediprime™ II labeling kit (Amersham Biosciences). Hybridization and washings were performed at 65 °C. Membranes were exposed to X-ray film for 72 h.

Leaf tissue was used to obtain crude protein extracts for Western analysis. Disks from fully expanded leaves were homogenized with 5 volumes of an extraction buffer (50 mM phosphate buffer pH 7.0, 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, 10 μg.l^{-1} leupeptin, 2 mM DTT). Protein content of the crude extracts was quantified according to Bradford (1976), using bovine serum albumin as standard. Protein extracts were fractionated by tricine SDS-PAGE (13.5% polyacrylamide) and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences), using a semi-dry transfer system (Bio-Rad Laboratories). Immunodetection was performed using a 1:1000 dilution of an anti-sarcotoxin IA antibody as a primary antibody with the alkaline phosphatase-conjugated anti-rabbit IgG as a secondary antibody. Synthesized sarcotoxin IA peptide was used as standard for the relative quantification of STX IA accumulation in the transgenic plants. For estimating peptide accumulation in the leaves, the size and intensity of each band (area = volume + intensity) were determined by the Kodak EDAS 120™ program after eliminating image background.

Xcc resistance assays

The *Xcc strain* 306 (Bacterial collection of the Paraná Agronomic Institute – IAPAR) isolated from citrus canker lesions on sweet orange leaves (Da Silva et al. 2002) was used as inoculum source. The bacterial inoculum was prepared by re-suspending bacteria harvested from 48 h old nutrient agar (NA) cultures (10 g l^{-1} peptone, 3 g l^{-1} meat extract, 20 g l^{-1} agar) in sterile distilled water.

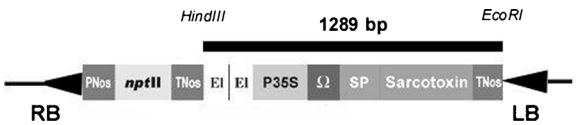

Fig. 1 Schematic diagram of the T-DNA of the pST10 plasmid (Okamoto et al. 1998) used for *Agrobacterium*-mediated transformation of Pera sweet orange. Bar represents the fragment used as probe for Southern blot analysis; RB, right border; LB, left border; Pnos, promoter of the Nopaline synthase gene; El, 5′-upstream sequence of CaMV 35S promoter; P35S, CaMV 35S promoter; Ω, 5′-untranslated sequence of TMV; SP, signal peptide coding sequence of PR1a gene from *Nicotiana tabacum*; TNos, terminator from Nopaline synthase gene; *npt*II, neomycin phosphotransferase II gene providing kanamycin resistance; Sarcotoxin IA, sarcotoxin coding gene

Five clonally propagated plants from 4 selected STX IA transgenic events (numbers 3, 5, 11 and 12) and a non-transformed control "Pera" sweet orange were assessed. Two techniques were used for challenge inoculation of the leaves: i) syringe-infiltration to estimate disease severity and *Xcc* titer, and ii) spray inoculation to evaluate disease incidence per leaf area. Five expanded young leaves (having surface area representing approximately 3/4 of fully expanded leaves) of similar age were used per plant for each of the challenge bioassays. Syringe infiltration was performed with a 10^4 cfu/ml bacterial suspension in water, while spray inoculation was applied by wetting all leaf surface with 10^6 cfu/ml of bacterial suspension. Both inoculation tests were performed in the morning, from 09.00 to 12.00, when stomata were fully open. After inoculation, plants were maintained in a wet chamber for 24 h to maintain high humidity favorable for bacterial growth in the leaves tissue.

Bacterial titer was determined in the infiltrated leaves by macerating leaf disks (0.64 cm^2) from the inoculated areas at 0, 1, 2, 4, 8 and 32 days after inoculation (DAI), and plating them onto Nutrient agar. The Petri plates were evaluated for bacterial colony development at 72 h after incubation at 28 °C. The bacterial population was expressed in log cfu/cm^2 of leaf tissue.

Disease severity and incidence were determined based on the severity of canker symptoms in leaves, by counting the number of canker lesions per infiltration-inoculated leaf area and estimating the number of lesions in spray-inoculated leaves, respectively, both at 32 days after inoculation. In each leaf, four areas with 1 cm^2 were used for calculate the mean and the percentage of lesions.

Statistical analysis

The experiment was a completely randomized design. Bacterial population data were log transformed prior to analysis and the mean number of lesions per cm^2 was determined. The means separation for the main effects were performed according to the Tukey's HSD test ($P < 0.05$).

Results

Out of 37 regenerated plantlets, 13 (35%) were confirmed to contain the STX IA gene by a primary PCR analysis. Fast acclimatization of in vitro transgenic plants was achieved by grafting on vigorous Rangpur lime rootstocks under greenhouse conditions. Transgenic sweet orange plants containing the STX IA gene started blooming 11 months after being transferred to greenhouse, confirming their mature stage.

Molecular analyses of the transgenic sweet orange plants

PCR analysis of transformed plants showed amplification of the expected 268 bp DNA fragment (Fig. 2), as well as the amplification of the *npt*II gene (data not shown). There was no PCR product in reactions with DNA of non-transformed plants or on non-template DNA reactions. Southern blot analysis of seven representative transgenic plants demonstrated different integration patterns, with only one insertion of the gene in all events, except for event number 3, which had two insertions. A weak hybridization signal observed for event number 1 indicated that this plant may be a chimera (Fig. 3).

Crude extracts from leaf disks of the transgenic events number 1, 3, 5, 11 and 12 were subjected to protein gel blotting and the transgene product was detected with the anti-sarcotoxin IA rabbit polyclonal antibody. An 8.2 kDa doublet protein band was recognized in transgenic plants numbers 3, 5, 11 and 12, but not in non-transgenic control plants (Fig. 4 and results not shown). When the synthetic STX IA peptide was used for protein gel analysis, a major signal was found at 4.2 kDa with a minor signal at 8.2 kDa (Fig. 4). As the monomer size of

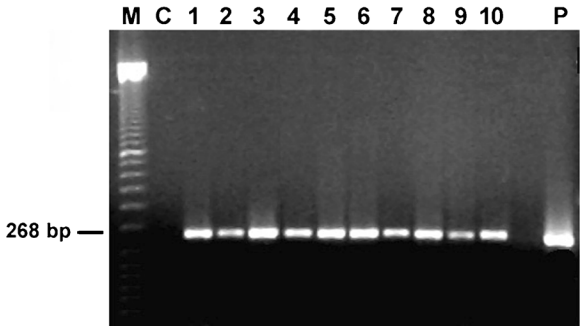

Fig. 2 PCR amplification of the sarcotoxin IA gene. Lane M, molecular marker; lane C, non-transformed control Pera sweet orange plant; lane 1–10, independent transgenic sweet orange events; lane P, positive control using pST10 plasmid as template

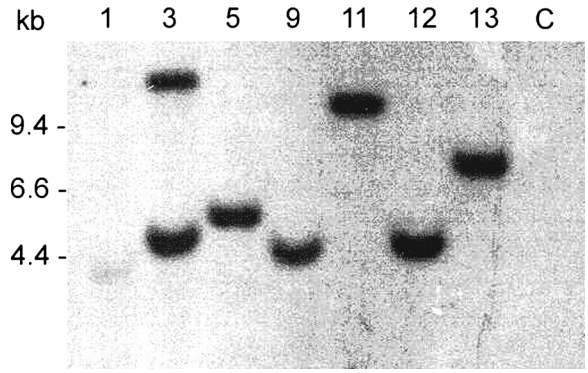

Fig. 3 Southern blot analysis of DNA from transgenic Pera sweet orange plants digested with *Eco*RI and hybridized to a specific probe of sarcotoxin IA gene to reveal T-DNA copy number. Lane C, non-transformed control; Lanes 1, 3, 5, 9, 11, 12, 13, independent transgenic events

STX IA is expected to be 4.2 kDa, the band at 8.2 kDa could likely be a dimer of the STX IA peptide, which has also been observed for STX IA in transgenic tobacco plants (Ohshima et al. 1999; Mitsuhara et al. 2000). The levels of sarcotoxin IA accumulation in transgenic events with higher concentrations ranged from approximately 13 to 20 μg/g of fresh leaf.

No phenotypic changes were observed in the transgenic plants accumulating the sarcotoxin IA peptide, indicating that the toxin was not deleterious to the sweet orange cells and tissues.

Growth and development of Xcc resistance in the transgenic sweet orange plants

Plants from the four independent events (3, 5, 11 and 12) displaying high levels of sarcotoxin IA peptide

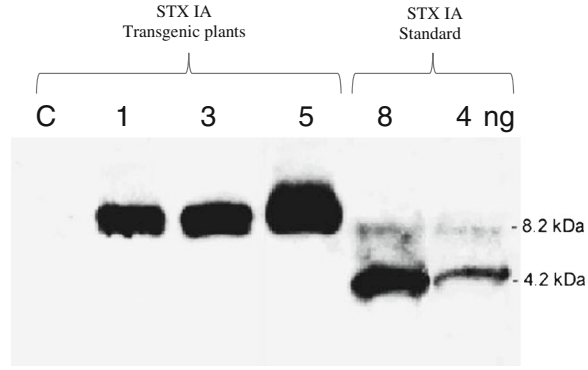

Fig. 4 Western blot analysis of sarcotoxin IA protein accumulation in transgenic Pera sweet orange plants. Lane C, non -transformed control; Lanes 1, 3, 5, independent transgenic events. Synthesized sarcotoxin IA, 8 and 4 ng, were used as standards for relative quantification

accumulation (Fig. 4 and results not shown) were inoculated with *Xcc* to evaluate the resistance against citrus canker. Care was taken to inoculate leaves that were at the same maturation stage, as susceptibility of citrus to *Xcc* infection depends on leaf age (Stall et al. 1982).

Our results showed that transgenic Pera sweet orange plants expressing the STX IA had lower *Xcc* titer and lesion number when compared to wild-type control plants. The population curve showed the same rate of *Xcc* multiplication at 4 DAI in the transgenic events compared to non-transgenic controls (Fig. 5). However, a 1 log difference was observed 8 DAI for STX IA transgenic events 3 and 11. At the end of the experiment (32 DAI), all transgenic events showed reduced bacterial populations when compared with those of untransformed controls (Fig. 5).

The most evident differences were observed in incidence of citrus canker lesions when comparing the STX IA plants versus the non-transformed controls. While the wild-type plants showed more than 11 lesions/cm^2 in their leaves, all the transgenic events had less than 5 lesions/cm^2 (Fig. 6). The events 3 and 5 had the lowest levels of disease incidence, with less than 3 lesions/cm^2 (Fig. 6). A large reduction in disease incidence was also observed in STX IA transgenic events 3 and 5 after spray inoculation (32 days), compared to that of non-transgenic controls (Fig. 7). Moreover, the infected transgenic plants had the disease symptoms expression and leaf abscission delayed, compared to controls (data not shown). Therefore, our results indicated that transgenic Pera sweet orange plants expressing the STX IA gene and accumulating its product at high levels significantly reduced bacterial population and incidence of symptoms when compared to non-transgenic control plants challenge-inoculated in parallel.

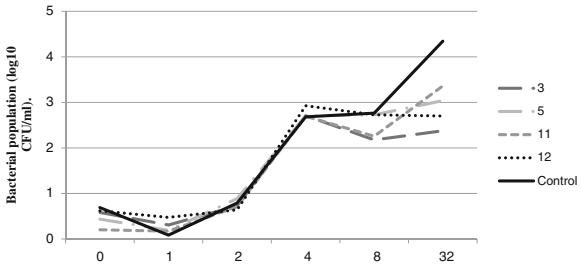

Fig. 5 Growth curve of *Xanthomonas citri* subsp. *citri* in leaves of STX IA transgenic plants and non-transformed control plants of sweet orange cv. Pera. Plants were inoculated with 10^4 CFU/mL of *Xcc* by syringe infiltration of. Each line represents value of the mean of three replicates

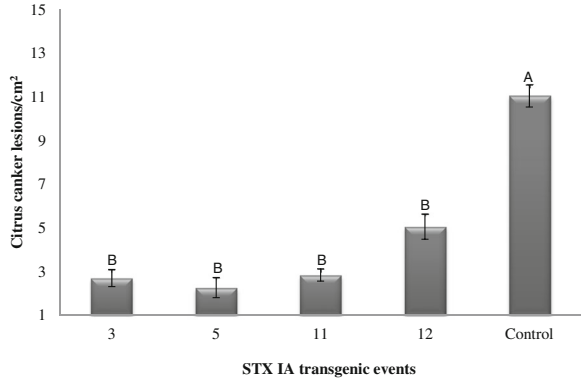

Fig. 6 Severity of citrus canker on leaves of STX IA transgenic events and non- transformed control "Pera" sweet orange, 32 days after inoculation by syringe infiltration. Bars labeled with different letters indicate significant differences among treatments at $p < 0.5$ level according to Tukey's HSD test

Discussion

In this work were used thin transversal segments of mature tissues to obtain sweet orange transgenic plants (Kobayashi et al., 2003). One of the advantages of using this type of explant is to reduce the number of regenerated non-transgenic plants or "escapes". Although the number of escapes was still high using thin segments of mature tissue (65%), it was comparable to protocols that employ 1-cm long stem segments as explants (Cervera et al., 1998). Buds cultured in vitro failed to elongate using this protocol. However, putative transgenic buds were micrografted in vitro as previously reported (Cervera et al., 1998; He et al., 2011) in order to regenerate whole transgenic plants. The use of mature explants is advantageous because less time is needed for

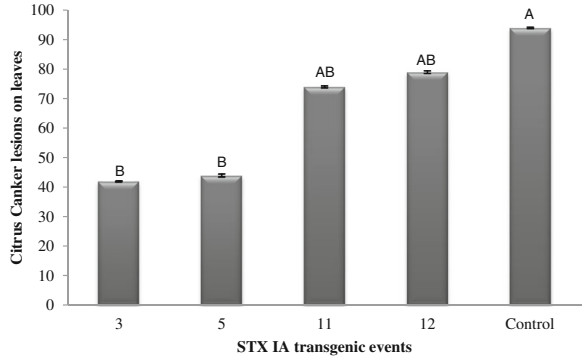

Fig. 7 Severity of citrus canker lesions on leaves of STX IA transgenic plants and non-transformed controls of "Pera" sweet orange, 32 days after spray inoculation. Bars labeled with different letters indicate significant differences among treatments at $p < 0.5$ level according to Tukey's HSD test

the evaluation of the inserted trait and other important agronomic characteristics in the transgenic citrus varieties (Rodríguez et al. 2008). In our case, flowering of Pera sweet orange transgenic plants were observed 11 months after transferring the plantlets to the greenhouse. The different DNA digestion patterns observed for the Southern blot analysis among the regenerated transgenic indicated that each plant was resulted from an independent transformation event. Under greenhouse conditions, the growth and development of the transgenic plants did not differ visually from those of non-transgenic control plants, indicating that sarcotoxin IA accumulation at high levels was not toxic to sweet orange tissues. The same was observed for the fruit produced in the transgenic trees. The first fruit set was irregular as expected for a greenhouse cultivated Pera sweet orange. However, 36 months after grafting on Rangpur lime, the trees produced fruits without any morphological or coloration alterations, as well as fruit quality parameters (Supplementary Fig. 1 and Table 1).

The sweet orange cultivar "Pera" was chosen for the transformation experiments because Pera is the most important citrus variety cultivar in Brazil and it is susceptible to *Xcc*. Transformation of Pera sweet orange with the *attacin A* gene for citrus canker resistance has been reported (Cardoso et al. 2010), but immature tissues were used as the source of explants in that case. Transgenic sweet orange plants accumulating high levels of the sarcotoxin IA peptide, as revealed by the Western blot analysis, showed enhanced resistance to citrus canker infection. Multiplication rate of the bacterium was reduced up to 100-fold in transgenic event number 3 compared to the non-transgenic control, resulting in a visible reduction in the disease symptoms. These results confirmed data showing that the STX IA peptide was efficient to inhibit in vitro growth of *Xcc* (Ohshima et al. 1999).

Accumulation levels of sarcotoxin IA observed in this work for certain transgenic events (13–20 μg/g fresh leaf) were much higher than those reported for transgenic tobacco plants transformed with the same vector (2 μg/g fresh weight, Ohshima et al. 1999). This difference may be related to the higher stability of STX IA in the intercellular space of citrus compared to tobacco tissues. In fact, large differences in degradation rates of this peptide in leaf intercellular fluids (ICFs) of several crop species have been observed. Among seven crops tested, the degradation rate of STX IA was higher in ICFs from tobacco leaves than those from rice and soybean leaves (Mitsuhara et al., unpublished results). However, further work with transgenic tobacco plants expressing high levels of STX IA (20 μg/g fresh weight), through an improved construct, also showed enhanced resistance to fungal infections (Mitsuhara et al. 2000). In this work, STX IA was expressed as a fusion peptide with the PR1a signal peptide for intercellular space secretion. Transport to the intercellular space is believed to be an essential feature of the strategy to prevent successful colonization of plant tissue by plant pathogenic bacteria using antibacterial peptides from insects (Düring et al. 1993), especially in the case of *Xcc*, a bacterium that colonizes the intercellular spaces of plant tissues. Moreover, the secretion into the intercellular space could be important to protect antibacterial peptides from cellular degradation (Sharma et al. 2000).

For other citrus pathogens that invade plant tissues through intercellular spaces, constitutive accumulation of the antimicrobial peptide in transgenic plants could effectively enhance host resistance considering that sarcotoxin IA has been demonstrated to be functional against a wide range of plant pathogens. Even though these citrus plants have not been assessed against fungal pathogens, the previous results reported for tobacco plants (Mitsuhara et al., 2000) suggested the potential for the management of different citrus diseases, reducing costs, time and cultural practices in the field. Additional experiments with a broad spectrum of intercellular pathogens will be performed to assess whether over-expression of STX IA gene in transgenic orange plants would increase resistance against them.

Field tolerance to *Xcc* differs among citrus genotypes, and it is related to the ontological stage of the leaves and fruits. For example, citrus cultivars and species with higher frequency, size, and duration of leaf flushes are more field-susceptible to *Xcc* than less vigorous cultivars or those whose foliage matures more rapidly (Gottwald et al. 1993). However, host range studies involving inoculation directly into mesophyll tissues often result in susceptibility of cultivars that show general field tolerance (Gottwald et al. 1993). Wounding for example, through the action of the citrus leaf miner *Phyllocnistis citrella*, also facilitates *Xcc* entrance to leaf mesophyll under field conditions enhancing host susceptibility (Graham et al., 2004). We showed here that the over-expression of the sarcotoxin IA peptide in "Pera" sweet orange plants significantly improved their mesophyll resistance to *Xcc* under greenhouse conditions.

Despite the fact that field experiments are required to fully address the effect of sarcotoxin IA against citrus canker in plants cultivated in areas where the disease is

endemic, the results presented here demonstrated that the strategy worked successfully in the greenhouse and at levels that make it highly promising for future field trials.

Although previous works showed that there are other peptides acting with antimicrobial activity against citrus canker disease (Cardoso et al. 2010; Furman et al. 2013), it is envisaged potential restrictions for large scale commercial use of the sarcotoxin IA transgenic citrus plants as well as those based on the use of AMPs of animal origin. In any case, it will be necessary to set up a risk assessment process to evaluate and monitor any genotypic and phenotypic characteristics associated with the insertion of the STX IA gene that may have adverse effects on the environment or tree phenology. For this, Brazil has adopted a regulatory system that establishes conditions for the release of genetically modified sweet orange in the environment (RN10, 2013; http://ctnbio.mcti.gov.br/resolucoes-normativas). In these normative, precautionary guidelines to avoid the dispersal of transgenic pollen in the field experiments are clearly described, making possible the assessment of genetically modified (GM) sweet orange plants in any development stage without genetic flow between GM and non-GM trees. Due to the general concerns of consumers and regulators about the use of AMPs of animal origin, future work should be addressed to the use of such type of peptides, or genes coding for them, of plant origin (Hao et al. 2016), ideally of citrus origin.

Finally, in this work, we showed that plants with high level of the antimicrobial peptide STX IA in leaves exhibited improved resistance to *Xcc* infection compared to non-transformed control plants.

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