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Transgenic sweet orange plants expressing a dermaseptin coding sequence show reduced symptoms of citrus canker disease

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ABSTRACT

Citrus canker provoked by *Xanthomonas axonopodis* pv. *citri* is a bacterial disease causing severe losses in all citrus-producing areas around the world. *Xanthomonas* infection is considered as an endemic disease in Northeast and Northwest Argentina, affecting as much as 10% of commercial citrus plantations. There is not known natural resistance neither in orange varieties nor in rootstocks used for grafting of commercial cultivars. To introduce resistance to this disease, plants of Pineapple sweet orange were transformed with a genetic construct allowing constitutive accumulation of dermaseptin. In comparison with non-transformed plants, transgenic plants showed symptom reduction levels of up to 50% in *in planta* assays performed under controlled conditions.

1. Introduction

Citrus canker is a major bacterial disease caused by *Xanthomonas axonopodis* pv. *citri* affecting most important citrus cultivars. Originally detected in Asia, the disease spread to the main citrus-producing areas around the world (Brunings and Gabriel, 2003; Gottwald et al., 2002). Due to its rapid propagation and its economic impact on exports and local markets, considerable resources were invested for its eradication. In Florida, where the disease was firstly detected in the United States, several large-scale eradication programs were conducted and more than 16 million trees had been destroyed by 2007 (Bronson and Gaskalla, 2007). Similar programs were implemented in Argentina and Brazil with variable results (Bassanezi et al., 2009; Canteros, 2004). Despite these efforts, the disease remains endemic and represents a potential threat to local economies. In particular, citrus canker is a serious economic problem for growers of Northwest and Northeast Argentina, two regions accounting for more than 98% of national citrus production (FederCitrus, 2011). Since a substantial percentage of lemon, grapefruit and orange is intended for export, outbreaks of this disease provoke immediate quarantines for the fruits harvested in the affected areas, with the subsequent losses of economic income. In the past, the damage caused by citrus canker provoked annual losses close to 36 million dollars (Huerga and San Juan, 2005).

Xanthomonas is extensively dispersed by the action of rain, wind, and insects, enters plant tissues through stomata and wounds, and multiplies into the apoplastic space (Gottwald et al., 2002; Graham et al., 2004). Visible symptoms of bacterial infection include tissue maceration, pustules and necrosis, and are apparent after a period of 5–7 days (Gottwald et al., 2002; Graham et al., 2004). Leaf, shoot and fruit tissues are more susceptible during early developmental stages. Severe infections can induce defoliation, twig dieback, fruit blemishes and early fruit-drop, leading to major economic losses (Brunings and Gabriel, 2003; Gottwald et al., 2002). Bacterial count in each individual lesion is variable and depends upon host susceptibility. As a rule, lesions do not alter the nutritional quality of the fruits, but negatively affect their aspect and commercial value (Gottwald et al., 2002).

There is no natural resistance against citrus canker in commercial orange varieties or other citrus species used as rootstocks. Current disease control consists of preventive sprayings with copper-based bactericides to reduce the amount of *Xanthomonas*

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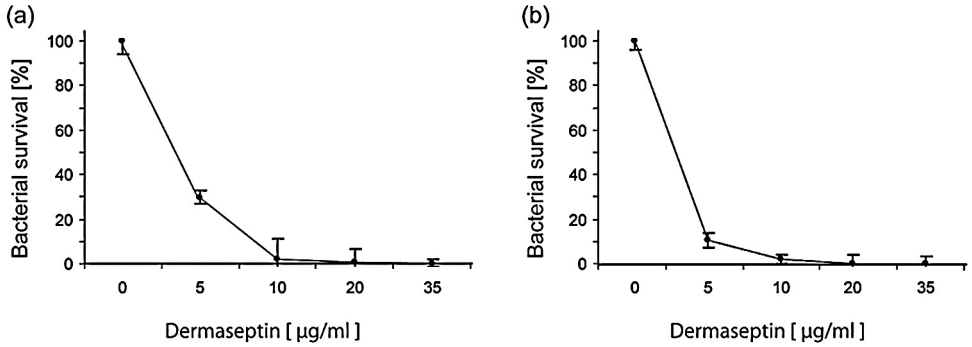


Fig. 1. Bacterial growth inhibition assay. *Xanthomonas axonopodis* pv. *citri* (a) and *Xanthomonas campestri* pv. *campestri* strain 8004 (b) were incubated with the indicated concentrations of dermaseptin as described in Section 2. Bacterial survival was scored as the percentage of bacterial growth in dermaseptin-free medium. Results shown represent the mean ± SD values from three independent assays.

inoculum (Gottwald et al., 2002). However, this approach only confers temporary protection and contributes to environmental contamination due to the spreading effect of rain and wind. On the other hand, once presence of disease is detected, the only effective control to prevent its dissemination relies on the destruction of the affected trees. Due to these considerations and to the long periods required for the conventional breeding of citrus species, the transgenic approaches offer considerable advantages to introduce resistance to this disease.

A wealth of research to develop resistance to bacterial infections in plants has been produced in the last decade and several biotechnological strategies are currently available (Collinge et al., 2010; McDowell and Woffenden, 2003). One of these approaches, based on the transgenic expression of antimicrobial peptides has been successfully implemented in different crops, including potato, rice and apple (Ko et al., 2002; Osusky et al., 2005; Rivero et al., 2012; Sharma et al., 2000). In the case of citrus species, a significant attenuation of canker symptoms have been previously demonstrated by expression of an attacin A coding sequence isolated from *Tricoplusia ni* (Boscariol et al., 2006). Alternative strategies, based on the expression of a spermidine synthase sequence isolated from apple and of the C-terminal region of *X. axonopodis* pv. *citri* PthA protein, also conferred considerable degrees of resistance in orange plants (Fu et al., 2011; Yang et al., 2010).

Dermaseptins are cationic antimicrobial peptides present in *Phyllomedusa* spp. comprising 27–34 amino acid residues that, like other antimicrobial peptides, bind to the phospholipid head groups of bacterial membranes, leading to leakage of cellular components and cell death (Amiche and Galanth, 2011; Mor and Nicolas, 1994; Mor et al., 1994). Dermaseptins exhibit a broad-spectrum activity against Gram-positive and Gram-negative bacteria (Navon-Venezia et al., 2002; Osusky et al., 2005; Rivero et al., 2012; Yaron et al., 2003), fungi (De Lucca et al., 1998), yeasts (Coote et al., 1998), and protozoa (Hernandez et al., 1992). In this work, we show that constitutive expression of a dermaseptin coding sequence in sweet orange plants strongly reduces the frequency and intensity of citrus canker symptoms.

2. Materials and methods

2.1. Bacterial strains and culture conditions

X. axonopodis pv. *citri* and *X. campestris* pv. *campestris* strain 8004 were kindly provided by Dr. A. Castagnaro (EEAOC, Argentina) and Dr. A. Vojnov (Centro César Milstein, Argentina), respectively. Bacteria were grown at 28 °C in Peptone–Yeast extract–Malt extract (PYM) nutrient medium (5 g/L Bacto-peptone, 3 g/L Bacto-yeast extract and 3 g/L Bacto-Malt extract), supplemented with D-glucose to a final concentration of 1% (w/v). For growth on plates, PYM was solidified with 1.5% (w/v) agar to make PYM-A. For *in planta* assays, one single colony was chosen, transferred to liquid PYM medium and grown overnight at 200 rpm on a rotary shaker at 28 °C. Bacterial cells were harvested by centrifugation and resuspended in 10 mM $MgCl_2$ to a final concentration of 1×10^5 c.f.u./ml.

2.2. Growth inhibition assays

Growth inhibition of *X. axonopodis* and *X. campestris* by synthetic dermaseptin (MALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ) was assayed according to Osusky et al. (2005). Briefly, 90 µl of bacterial culture in PYM liquid medium (1×10^7 cells/ml) were incubated in Eppendorf tubes containing 10 µl of dermaseptin dilutions. Control incubation containing only water was included. Incubations were performed at 25 °C for 4 h under continuous shaking, diluted in PYM medium and spread on PYM-A plates. Colony counting for each treatment was performed after incubation for 48 h at 28 °C.

2.3. Genetic constructions

A DNA cassette containing the dermaseptin coding sequence from *Phyllomedusa sauvagii*, the *Cauliflower mosaic virus* 35S promoter, the *Tobacco mosaic virus* Ω enhancer, the esporamine pre-peptide signal peptide (apoplastic export signal) and the nopaline synthase transcription termination sequence (Rivero et al., 2012) was sub-cloned at the *Hind* III site of the pBIN19*sgfp* binary vector (Chiu et al., 1996). The resulting construction was named pBIN19*sgfp*-Der (Fig. 2a). Transformation with the pBIN19*sgfp* vector allows the selection of transgenic callus in kanamycin-containing medium or by direct visualization of GFP expression.

2.4. Plant transformation

Internodal explants were obtained from of 6 to 12-old month sweet orange (*Citrus sinensis* L. Osbeck cv. Pineapple) seedlings grown in greenhouse conditions. The stem segments employed for transformation were approximately 1-cm long. For transformation, explants were co-cultivated with *Agrobacterium tumefaciens* (EHA105 strain) carrying the pBIN19*sgfp*-Der construction. Transformants were selected in Murashige and Skoog medium containing 100 mg/L kanamycin and the regenerating buds were subsequently screened under a stereomicroscope for GFP fluorescence. Positive shoot tips were *in vitro* grafted on decapitated seedlings of Troyer citrange (*Poncirus trifoliata* (L.) Raf. × *C. sinensis* (L.) Osbeck) as previously described (Peña et al., 1995). After 3–4 weeks, scions were preliminarily screened by PCR for presence of the dermaseptin and GFP sequences using the specific primer pairs Der-Fw (5′-tggctctttggaagactatgc-3′)

Fig. 2. Genetic construction used to transform sweet orange plants. (a) Expression *cassette* of the pBIN19*sgfp*-Der construction. 35S, CaMV 35S promoter; nosP, nopaline promoter; nosT, nopaline synthase transcription termination sequence; Ω, *Tobacco mosaic virus* translational enhancer; *Es*, esporamine apoplastic signal peptide; *der*, *P. sauvagii* dermaseptin; *nptII*, neomycin phosphotransferase; *sgfp*, green fluorescence protein; RB and LB, right and left borders. Arrows indicate the annealing sites of forward (Fw) and reverse (Rv) primers used to amplify the dermaseptin sequence. The probe sequence (the upper bar) and *Bgl* II site used in Southern blot analyses are indicated. Scheme is not in scale. (b) Transgenic sweet orange plants grafted on rough lemon (*Citrus jambhiri* Lush) rootstocks grown in growth chamber. Upper picture shows the seven transgenic and NT plants; middle panel, Der-1 and NT plants; and lower panel, Der-6 and NT plants.

and Der-Rv (5′-gctgatactatttctcaaggaactcaa-3′), and GFP-Fw (5′-atggtgagcaagggcgagga-3′) and GFP-Rv (5′-ggaccatgtgatcgcgcttc-3′), respectively. Then, positive plantlets were side-grafted on vigorous 6-month-old rough lemon (*Citrus jambhiri* Lush) rootstocks and grown in a growth chamber at 26 °C under a 16 h-light photoperiod.

2.5. Leaf infiltration assays

Bacterial suspensions of *A. tumefaciens* (5×10^6 c.f.u./ml) and *X. axonopodis* pv. *citri* (1×10^5 c.f.u./ml) were infiltrated at the abaxial surface of fully expanded *Nicotiana benthamiana* leaves according to the procedure reported by Metz et al. (2005). The experiment was repeated 3 times and each assay included 5 plants. Infiltrations were carried out on 3 leaves per plant.

2.6. RNA extraction and RT-PCR analysis

For RNA extraction, 200 mg of transgenic and control sweet orange leaves were ground under liquid nitrogen in TRiZOL Reagent (Invitrogen). RNA samples were resuspended in 50 μl of RNase-free water and incubated with RQ1 RNase-Free DNase (Promega) to remove DNA traces prior to RT-PCR. cDNA synthesis was performed using the iScript cDNA Synthesis kit (Bio Rad Laboratories). A PCR assay lacking reverse transcriptase was performed as a control for DNA contamination. Expression of the dermaseptin sequence in transgenic plants was assessed using the Der-Fw and Der-Rv primers. The actin B coding sequence, used as internal control, was detected using the Act-Fw (5′-tggtcgtaccaccggtattgtgtt-3′) and Act-Rv (5′-tcacttgcccatcaggaagctcat-3′) primers. Twenty-four cycles were performed for both transcripts and analyzed by electrophoresis in agarose gel 2%.

2.7. Southern blot analysis

Total DNA was extracted from leaves as described by Dellaporta et al. (1984). The DNA (10 μg) was digested with *Bgl* II (Fermentas), electrophoresed in a 0.8% agarose gel and blotted onto Hybond N^+ Nylon membranes (Amersham). Specific DNA sequences were detected by hybridization with a ^{32}P-labeled DNA probe comprising the complete dermaseptin sequence. Labeling of the probe was performed by random priming using a Prime-a-Gene kit (Promega). Pre-hybridization and hybridization were carried out at 63 °C in Church's hybridization solution (Church and Gilbert, 1984) for 2 and 16 h, respectively. After hybridization, membranes were washed in 2× SSC, 0.1% SDS for 15 min at room temperature, then in the same solution for 15 min at 63 °C, and finally wash in 0.2× SSC, 0.1% SDS at 63 °C for 15 min. The blots were then exposed to X-ray film or analyzed using the Storm 840 PhosphorImager system (Amersham).

2.8. In planta infection assays and statistical analysis

Transgenic and non-transgenic sweet orange plants were propagated by grafting on Rough lemon rootstocks. Non-transgenic plants were used as controls. All leaves used in infection assays were of the same age and physiological condition. Infection assays with sweet orange plants were performed in a growth chamber at 26 °C and a 16 h-light photoperiod. Inoculation with *X. axonopodis* pv. *citri* (1×10^5 c.f.u./ml) was performed using the method reported by Yang et al. (2010) with minor modifications. To this end, leaf abaxial surfaces were previously punctured with sterile needles at two adjacent areas located at both sides of the leaf midvein. Each area comprised 8 punctures separated from each other by 2-mm distance. Bacterial suspension was sprayed on the abaxial surface of leaves from a distance of 3 cm. Each punctured area was sprayed three times at short intervals. Each assay included 9–12 fully expanded leaves per each transgenic plant and assays were repeated 3 times. Observations of canker disease symptoms were made using a hand-held magnifier and were photographed using a 2000-C stereomicroscope (Zeiss). Frequency of canker formation is expressed as (total canker number/total punctures) × 100. In addition, according to the number of canker developed, each individual leaf was classified as low (1–4 cankers), moderate (5–8 cankers) or high (9–16 cankers) infection categories. Results were shown as the percentage of leaves belonging to each infection category for corresponding transgenic plant.

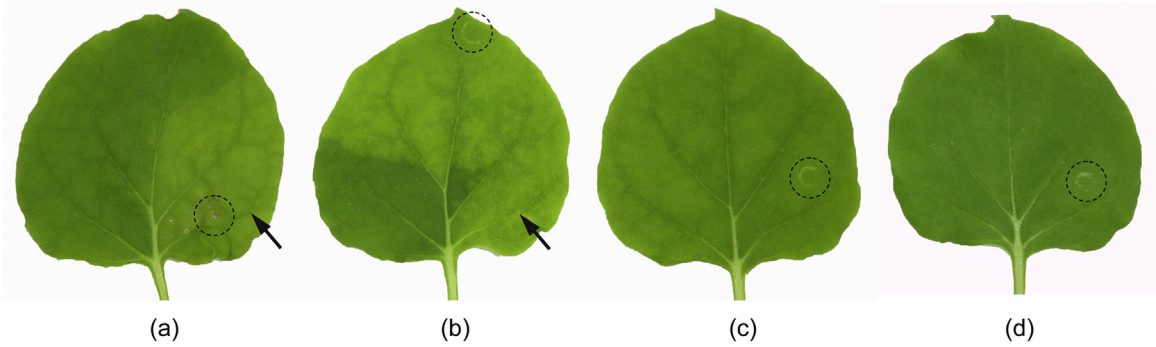


Fig. 3. Infiltration assays on *N. benthamiana* leaves. (a) Leaf infiltrated with *X. axonopodis*, showing typical chlorosis symptoms (arrow). (b) Leaf co-infiltrated with *X. axonopodis* plus *A. tumefaciens* harboring the pBIN19*sgfp* binary vector, showing similar chlorosis symptoms. (c) Leaf co-infiltrated with *X. axonopodis* plus *A. tumefaciens* harboring the pBIN19*sgfp*-Der construction, showing reduction of infection symptoms. (d) Leaf infiltrated with dilution buffer, showing no infection symptoms. Dashed circles indicate the infiltration entry sites. Pictures are representative of three independent assays.

A statistical analysis was performed using the Kruskal–Wallis test to compare the susceptibility of the 7 transgenic plants and the non-transformed citrus control. Multiple comparisons between transgenic and non-transformed plants were performed using the Dunn's contrasts method.

3. Results

3.1. Antimicrobial activity of dermaseptin against Xanthomonas spp.

Though the inhibitory effects of dermaseptin had been previously established for a wide spectrum of microorganisms, there was no information regarding its activity on *Xanthomonas* growth. To address this point, two different *Xanthomonas* strains (*X. axonopodis* pv. *citri* and *X. campestris* pv. *campestris* strain 8004) were tested in *in vitro* assays using a synthetic dermaseptin peptide (see Section 2). As shown in Fig. 1, growth of both strains was significantly inhibited by dermaseptin concentrations higher than 5 μg/ml.

3.2. Dermaseptin transient expression in N. benthamiana leaves reduces Xanthomonas induced chlorosis

A genetic construction allowing dermaseptin constitutive expression was generated on the basis of the pBIN19*sgfp* binary vector (pBIN19*sgfp*-Der; Fig. 2a). To test its functionality, *N. benthamiana* leaves were co-infiltrated with *X. axonopodis* pv. *citri* plus *A. tumefaciens* harboring the pBIN19*sgfp*-Der construction. Parallel co-infiltration assays were carried out with *X. axonopodis* pv. *citri* alone and with *X. axonopodis* pv. *citri* plus *A. tumefaciens* harboring the pBIN19*sgfp* vector as positive and negative controls, respectively. At 4 days post-infiltration, the control leaves exhibited typical chlorotic symptoms (Fig. 3a and b), while the leaves co-infiltrated with pBIN19*sgfp*-Der showed signs of attenuated chlorosis (Fig. 3c). Although accumulation of the transgenic peptide was below the detection level of our immunological assays, presence of dermaseptin transcripts in leaves co-infiltrated with pBIN19*sgfp*-Der was confirmed by RT-PCR (data not shown).

3.3. Sweet orange transformation and analysis of transgenic plants

Seven orange plants named as Der-1 to -7 were selected. These plants were indistinguishable from the non-transgenic plant as shown in Fig. 2b. Southern blots assay were performed to confirm transgene integration and to estimate the transgene copy number in the plant genomes (Fig. 4a). Accordingly to the band patterns, Der-1 to –7 have 4, 3, 5, 4, 5, 1 and 4 transgene copies, respectively. Dermaseptin mRNA expression of the transgenic plants was analyzed by semi-quantitative RT-PCR determining the relative transcript level in each plant (Fig. 4b). All lines showed mRNA expression although Der-3 and Der-7 express the lowest level of mRNA. We do not found a correlation between copy number and mRNA expression.

3.4. In planta infection assays

To evaluate their susceptibility to *X. axonopodis* pv. *citri*, three independent infection assays including the seven transgenic plants and a non-transformed citrus control were conducted according to the procedure described in Section 2. The leaf punctures sprayed with the bacterial inoculum were examined up to 24 days post infection (d.p.i.) under a dissecting microscope and scored for the presence of typical disease symptoms (tissue maceration, chlorosis and canker). In total, about 144–192 punctures per plant were analyzed for canker development in each assay. Results from a representative assay are shown in Table 1. Lesion development was attenuated or absent and individual canker exhibited smaller sizes in all transgenic leaves (up to 40% in size reduction) as compared to non-transformed controls. Similarly, chlorosis intensity and maceration symptoms showed lower levels compared to controls (Fig. 5). In particular, Der-5 and Der-6 plants showed visible canker development in only 33% and 43% of the inoculated punctures, respectively, indicating a reduction of near 50% in canker frequency with regards to controls (Table 1). For each experimental data set, a statistical analysis was performed using the Kruskal–Wallis test to compare the susceptibility of the 7 transgenic plants and the non-transformed citrus control. A representative analysis, showing a statistical significance (P value = 0.034) is presented in Supplemental Table 1. Multiple comparison analyses between transgenic and control plants were also performed using the Dunn's contrasts method (dms, Supplemental Table 2). With the exception of Der-4, the calculated values were statistically significant for all other transgenic plants, indicating that they were less susceptible to canker disease.

For a better comparison of individual phenotypes to infection, the inoculated leaves were classed as belonging to "low", "moderate" or "high" canker frequency categories, and the percentage of leaves for each category was calculated for the seven transgenic plants (Fig. 6a). Remarkably, canker frequency was reduced in six of the seven transgenic plants. In addition, the time course of canker development was determined in two transgenic plants (Der-5 and Der-6; Fig. 6b). Canker development was clearly delayed in these two plants.

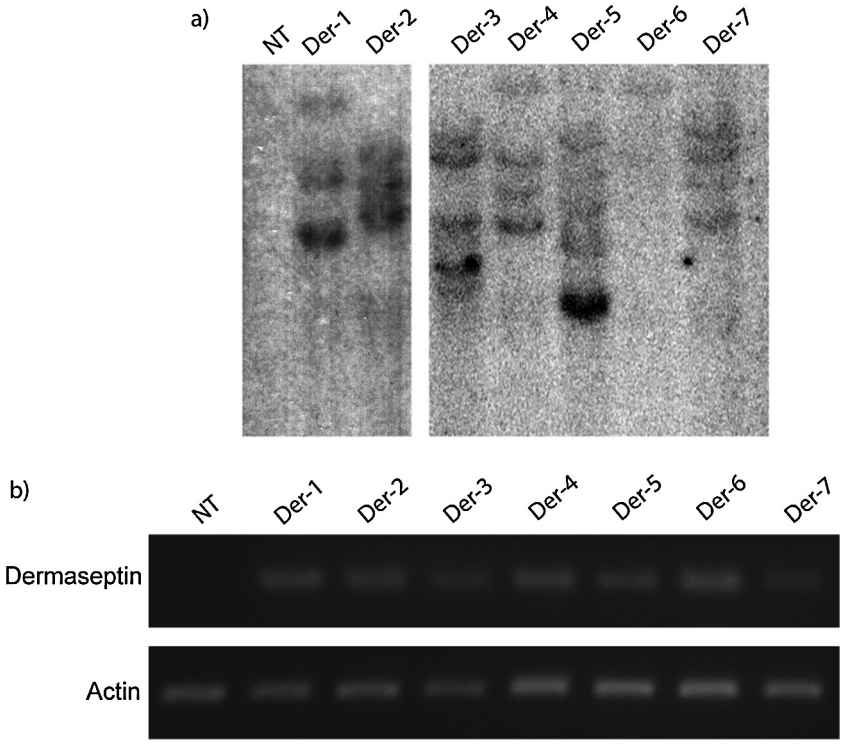


Fig. 4. Southern blot and RT-PCR analyses of transgenic plants. (a) Southern blot analysis of Der-1 to -7 transgenic and non-transformed (NT) sweet orange plants. Genomic DNA was digested with *Bgl II* and hybridized with a specific dermaseptin probe as described in Section 2. (b) RT-PCR analysis of Der-1 to -7 plants. PCR amplifications of dermaseptin and actin sequences were performed with Der-Fw and Der-Rv and Act-Fw and Act-Rv primers, respectively.

Table 1
Canker formation frequencies from *in planta* infection assay. Transgenic and non-transformed plants were assayed following the protocol described in Section 2. Each assay included 9–12 leaves inoculated with a bacterial suspension containing 1×10^5 c.f.u./ml. Canker numbers were scored at 24 d.p.i. Frequency of canker formation is expressed as (total canker number/total punctures) × 100. "–" indicates not sampled. *In planta* infection assays were repeated 3 times with similar results.

Line/*Leaf*	# Canker per leaves												Total punture	Total canker	(Total canker/total punture) × 100
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*			
NT	11	14	7	14	15	15	14	9	15	14	13	15	192	156	81.25
Der-1	3	7	3	3	16	16	4	8	8	6	12	9	192	95	49.48
Der-2	9	11	12	8	3	6	9	4	12	3	13	–	176	90	51.14
Der-3	12	5	7	8	5	3	9	13	8	–	–	–	144	70	48.61
Der-4	13	12	12	13	6	13	11	14	9	11	5	–	176	119	67.61
Der-5	8	6	9	14	8	3	1	2	2	–	–	–	144	53	36.81
Der-6	13	12	8	9	10	3	6	6	5	3	–	–	160	75	46.88
Der-7	2	5	5	6	2	7	9	14	5	15	16	–	176	86	48.86

Scoring of canker development was performed at 24 d.p.i.

4. Discussion

Different strategies based on the induction of specific defense responses were previously described to introduce canker disease resistance into citrus plants. Thus, constitutive expression of a PthA truncated protein from *X. axonopodis* pv. *citri* in orange plants resulted in the interference of normal *Xanthomonas* infection (Yang et al., 2010). More recently, canker disease attenuation in sweet orange plants has been reported by transformation with a spermidine synthase sequence to induce increased accumulation of intracellular H_2O_2 (Fu et al., 2011). Although these strategies confer partial resistance to canker disease, they might also represent potential limitations. In the first case, resistance depends mainly on altering a specific host–pathogen interaction that could be overcome by appearance of resistant bacterial strains. On the second, it involves a mechanism that triggers multiple plant responses, with the potential risk of negative effects on the physiology and agronomic performance. In contrast, a more promising approach combining broad-spectrum activity and efficient antibacterial mechanisms makes use of the genetic transformation with antimicrobial peptide sequences. Such approach has been successfully implemented in different plant species (Osusky et al., 2005; Rivero et al., 2012). Previous work in this direction has been already reported in citrus species by constitutive expression of an attacin A peptide from *T. ni* (Boscariol et al., 2006) in *C. sinensis* plants. In this work, a symptom reduction of up to 40% was determined in infection assays performed with *X. axonopodis* pv. *citri*, as deduced from lesion area measurements of infected leaves.

Based on this background, and on our own results with dermaseptin-expressing potato plants (Rivero et al., 2012), we decided to evaluate the effect of dermaseptin against *Xanthomonas* spp. As compared with other lytic peptides, dermaseptin exhibits high antibacterial and antifungal activity at micromolar levels and is not significantly toxic to humans (Amiche and Galanth, 2011; Kastin, 2006; Mor et al., 1994). Since its activity to *Xanthomonas* had not been previously demonstrated, we carried out a series of growth inhibition assays with *X. axonopodis* pv. *citri* and *X.*

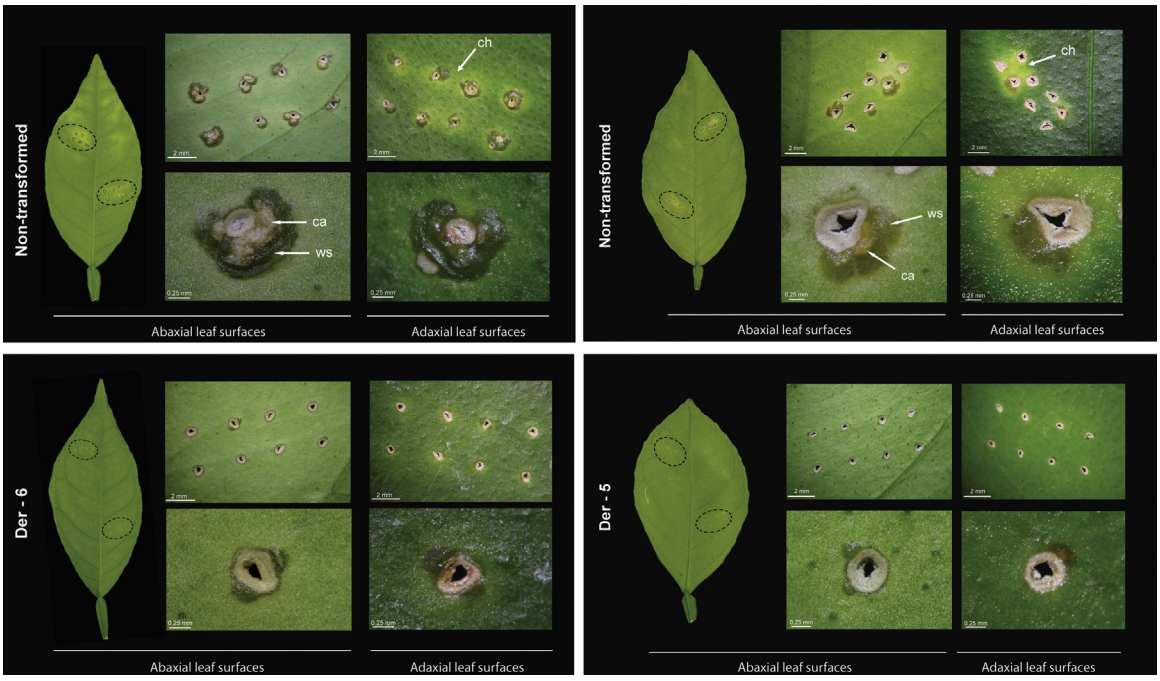
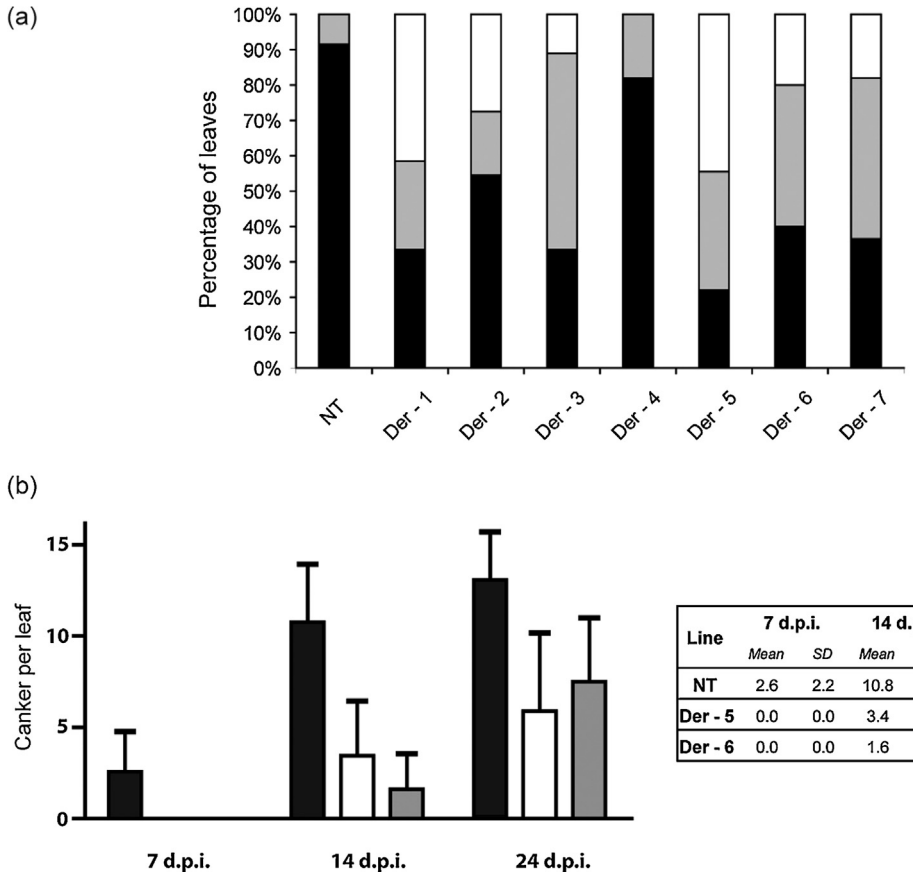


Fig. 5. Resistant phenotypes of transgenic sweet orange plants. Non-transformed and Der-5 and Der-6 plants were infected with *X. axonopodis* pv. *citri* and the results recorded at 24 d.p.i. Areas inoculated on leaves (dashed circles) are shown at left. Note the contrasting aspect of cankers (ca), water soaking (ws) and chlorotic tissue (ch) on the abaxial and adaxial surfaces of control and Der-5 and Der-6 leaves.



Line	7 d.p.i.		14 d.p.i.		24 d.p.i.	
	Mean	*SD*	*Mean*	*SD*	*Mean*	*SD*
NT	2.6	2.2	10.8	3.2	13.0	2.6
Der - 5	0.0	0.0	3.4	3.0	5.9	4.3
Der - 6	0.0	0.0	1.6	2.0	7.5	3.5

Fig. 6. Canker development in transgenic and non-transformed sweet orange plants. According to the number of cankers, infected leaves were classed into low (white bars: 1–4 cankers), moderate (gray bars: 5–8 cankers) or high (black bars: 9–16 cankers) symptom frequency categories. Each infection assay included 9–12 leaves from each plant. (a) Percentage of leaves ranked into each frequency category in non-transformed (NT) and transgenic plants (Der-1 to -7) measured at 24 d.p.i. Results are representative of 3 independent assays. (b) Time-course of canker development. Symptom development was monitored at 7, 14 and 24 d.p.i. in transgenic plants Der-5 (white bars) and Der-6 (gray bars) and non-transformed (NT; black bars). Canker numbers corresponding to each plant are indicated on the graphic. In each case, results represent the mean ± SD values from 144 to 192 punctures.

campestris pv. *campestris* and showed that these species are susceptible at relatively low concentrations of the lytic peptide.

On this basis, the pBIN19*sgfp*-Der construction, allowing constitutive expression of dermaseptin, was generated. To assess the functionality of pBIN19*sgfp*-Der, a series of co-infiltration assays with *X. axonopodis* and *A. tumefaciens* carrying the genetic construction were performed in *N. benthamiana* leaves. Results from these assays showed considerable reduction of chlorotic symptoms and suggested that dermaseptin was produced at effective levels.

After sweet orange transformation, transgene integration was evaluated by Southern blot analyses and mRNA expression was confirmed by RT-PCR. No reliable protein measurements could be performed by immunological tests because, despite repeated attempts to obtain high-affinity antibodies, the lower dermaseptin level detected by our antisera was around 1 ng.

Transgenic sweet orange plants challenged with *X. axonopodis* pv. *citri* in controlled infection assays showed symptom reductions of up to 50%, as measured by the frequency of canker-developing wounds on total inoculated wounds. In addition, the smaller size of cankers present in transgenic plants and the delay observed in symptom development suggest a considerable decrease of disease severity. Due to the lack of effective antibodies, no statistical correlations could be obtained between dermaseptin accumulation and resistance levels in the transgenic plants. Because growth inhibition and leaf infection assays are essentially not comparable, the minimum dermaseptin accumulation needed to confer *in planta* resistance could not be inferred.

Taking into account the wide antimicrobial activity showed by dermaseptin (Rivero et al., 2012), this peptide could be employed to introduce resistance to other bacterial and fungal pathogens affecting citrus. In this regard, it would be of interest to test the susceptibility of dermaseptin-expressing plants to *Candidatus* Liberibacter, the causal agent of Huanglongbing (HLB) disease (Bové, 2006) and to *Xylella fastidiosa*, the causal agent of citrus variegated chlorosis (CVC) disease (Hartung et al., 1994).

Since controlled infection assays cannot be directly extrapolated to real agricultural conditions, the results obtained in this work must be confirmed in greenhouse and field trial assays. If the reduction in canker frequency can be reproduced in these conditions, the strategy described in this work could advantageously be employed to restrict *Xanthomonas* spreading and to improve the efficiency of disease management.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.07.019.

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