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Highlights

- Canker-resistant transgenic Citrange troyer rootstocks were generated.
- Overexpression of Snakin-1 strongly reduces the development of canker symptoms.
- Transgenic lines show reductions of around 75% in the number and size of cankers.

Abstract

Citrus canker is a major disease caused by *Xanthomonas citri* pv. citri. Snakin-1 is an antimicrobial peptide, which was previously shown to be effective against different bacterial and fungal diseases in potato, wheat and lettuce when expressed in transgenic plants. We generated transgenic Citrange Troyer citrus rootstocks constitutively expressing this peptide and 5 different transgenic lines were challenged against virulent *X. citri* isolates. Challenge assays conducted *in*

vitro using detached leaves and *in planta* by infiltration revealed a significant reduction of the number and size of canker lesions in some of the transgenic lines.

KEYWORDS: Citrus canker; antimicrobial peptides; Citrange troyer; Snakin.

Citrus are among the most widely grown and economically important fruit tree crops (Gmitter et al., 2012). Commercial varieties are produced by grafting over compatible hybrid rootstocks such as Citranges. Citrus canker (Brunings and Gabriel, 2003) and Huanglongbing (HLB) (Bové et al., 2006; Coletta-Filho et al., 2004) are among the most important citrus diseases. Citrus canker is caused by *Xanthomonas citri* 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). It was reported in Argentina since 1928 (Canteros, 2001a, 2001b; Canteros de Echenique et al., 1985; Fawcett and Bitancourt, 1949). Despite the efforts for eradication (Bassanezi et al., 2009; Canteros, 2004), the disease remains endemic and represents a potential threat to local economies.

Several biotechnological strategies are currently available to get resistance against plant bacterial infections (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, lettuce and apple (Sharma et al., 2000; Ko et al., 2002; Osusky et al., 2005; Rivero et al., 2012; Darqui et al., 2018;). Antimicrobial peptides (AMPs) are part of innate immunity, establishing a first line of defense against pathogens. Most of them are cysteine (Cys)-rich peptides expressed in nearly all organs constitutively or in response to infections. They are classified in different families such as cyclotides, defensins, thionins, LTP (lipid transfer protein), hevein-like, vicilin-like and knottin-like peptides and snakins (Stotz et al., 2013; Tang et al, 2018). In the case of citrus species, a significant attenuation of canker symptoms and other bacterial diseases has been demonstrated by overexpression of antimicrobial peptide coding sequences such as *attacin A* from *Trichoplusia ni* (Boscariol et al., 2006; Cardoso et al, 2010), dermaseptin from *Phyllomedusa spp* (Furman et al., 2013), a modified plant thionin

(Stover et al., 2013; Hao et al., 2016) and the human lysozyme and β-defensin 2 (Guerra-Lupián et al., 2018). Other strategies, based on the expression of an apple spermidine synthase sequence (Fu et al., 2011), a synthetic peptide called D2A21 (Hao et al., 2017) and a C-terminal region from *Xanthomonas citri* PthA protein (Yang et al., 2010) also conferred considerable degrees of resistance against different bacterial pathogens in orange plants.

Snakin-1 (SN1) is an AMP isolated from *Solanum tuberosum* which exhibits remarkable antimicrobial activity *in vitro* (Berrocal-Lobo et al., 2002; Segura et al., 1999) and *in vivo* in potato plants (Almasia et al., 2008). Besides, heterologous expression of SN1 in wheat and lettuce has demonstrated antimicrobial activity against *Blumeria gramimis* and *Gaeumannomyces graminis* (Faccio et al., 2011; Rong et al., 2013) and *Rhizoctonia solani* and *Sclerotinia sclerotiorum* (Darqui et al., 2018) respectively.

Citrus rootstocks exert a high degree of influence to scions in fruit production and plant susceptibility to citrus canker or other important diseases such as Huanglongbing (HLB) (Nariani, 1981; Albrecht et al., 2012). As it was already shown, there is an extensive and non-specific movement of proteins between companion cells and sieve elements, as well as long-distance transport of macromolecules between scions and rootstocks (Paultre et al., 2016). This protein movement would allow pathogen tolerance triggered in a rootstock to be establish also in the scion. Therefore, rootstock transformation can be a potential strategy to avoid public perception issues since the non-transgenic nature of the harvested fruits is maintained. In this work, we show that constitutive expression of the *S. tuberosum* SN1 coding sequence strongly reduces the number and intensity of citrus canker symptoms in the transgenic rootstock Citrange Troyer, validating our strategy of expressing AMPs against citrus associated bacterial infection.

Epicotyls of Citrange Troyer (*Poncirus trifoliate* (L.) Raf. x *C. sinensis* (L.) Osbeck) seedlings were transformed using *Agrobacterium tumefaciens* carrying the binary vector pPZPK-SN1 (Almasia et al., 2008) as previously described (Cervera et al., 1998). Regenerated shoots from stems explants were grafted *in vitro* on decapitated and etiolated seedlings and after 3–4 weeks,

scions were screened for the presence of the transgenes by PCR using SNK-F/SNK-R primers (SNK-F: 5'-ATGAAGTTATTTCTATTAACT3´; SNK-R: 5'-TCAAGGGCATTTAGACTTGCC-3'). Ten stable transgenic lines (SN1-4, SN1-5, SN1-7, SN1-19, SN1-20, SN1-32, SN1-33, SN1-36, SN1-40 and SN1-43) were generated. Expression of the transgene was confirmed and quantified by RT-qPCR (Fig. S1 of Supplementary data) using *Solanum tuberosum* SN1 specific primers (StSN1-Fw/StSN1-Rv) and *CsGAPC2 (Glyceraldehyde-3-phosphatedehydrogenase C2)* as citrus internal reference gene (CsGAPC2-Fw/CsGAPC2-Rv primers) (Mafra et al., 2012). Lines SN1-19, SN1-32 and SN1-43 showed the highest levels of SN1 mRNA. Lines were re-grafted in rough lemon rootstocks (*Citrus jambhiri* Lush.) for growing and acclimation in the greenhouse (18–24◦C) (Fig. S2 of Supplementary data).

Detached leaves from 5 transgenic lines (4, 5, 36, 40 y 43) and non-transgenic Citrange Troyer plants (NT) of the same age and physiological condition were used for infection assays performed in a growth chamber (26◦C/16 h-light photoperiod). Inoculation with *X. citri* pv. citri was performed using the method reported by Yang et al. (2010) with minor modifications. Leaf abaxial surfaces were previously punctured with sterile needles in adjacent areas at both sides of the leaf midvein. Each area comprised 6 punctures in the main leave, 2 punctures in the small leave (right) form the trifolia and 2 other punctures in the remaining small leave (left) (for mock-inoculation). Bacterial suspension (1×10^5 c.f.u./ml) was inoculated by pipetting on the abaxial surface. Each assay was repeated 3 times including 4-10 fully expanded leaves per plant. Canker disease symptoms were observed at different times post-inoculation using a hand-held magnifier (Fig 1 a). Frequency of canker formation was calculated as (total canker number/total punctures) ×100 (Table 1). All evaluated lines showed a reduction in canker frequency compared to non-transgenic controls (NT). Lines SN1-5 and SN1-36 showed the lowest frequency of cankers (25%) at 21 dpi followed by SN1-40 (37.5%), SN1-43 (68.7%) and SN1-4 (72.2%). The time course of canker development was also determined from 5 to 21 dpi (Fig. 1 b). The development of cankers was dramatically delayed in all transgenic lines. For example, at 7 dpi all transgenic

lines exhibited less than 20% of canker frequency while non-transformed already exhibited a value of 100%. At the end of the assay, leaves from lines SN1-4 and SN1-43 showed a moderate frequency of cankers (~70%) at later times (17-21 dpi), compared to lines SN1-5, SN1-36 and SN1-40 that reached a lower maximum frequency (< 40%) at earlier times (10-12 dpi) without further development of symptoms. For a better comparison of individual infection phenotypes, the inoculated leaves were classified as low (0-2 cankers), moderate (3–4 cankers) or high (5–8 cankers) canker frequency categories, and the percentage of leaves for each category was counted at 21 dpi (Fig. 1 c). All transgenic lines showed fewer leaves corresponding to the high category compared to NT leaves. Most of the leaves from lines SN1-5, SN1-36 and SN1-40 were classified to the low category while leaves from lines SN1-4 and SN1-43 were classified to high category. Canker areas were also measured at 21 dpi. Results showed a significant reduction of canker size in all transgenic lines compared to NT control (Fig. 2). Lines SN1-5, SN1-36, and SN1-40 showed the striking behavior coincident with the results for canker frequency. Acquired resistance was also analyzed by an alternative *in planta* infiltration method (Stall et al., 1982). By assaying a parallel experiment in quadruplicate, 25-days old leaves of transgenic lines SN1-4, SN1-5, SN1- 36 and control plants (Duncan grapefruit) were infiltrated either with 5 $x10^3$ cells of *X. citri* or mock inoculated under controlled conditions. Canker disease symptoms were observed at 50 dpi and lesions per cm^3 were counted. Leaves from control plants showed significantly more lesions than SN1-5 and SN1-36 transgenic lines, thus confirming their tolerance to infection. Line SN1-4, showed not statistically significant differences compared to control (Fig. 3).

In conclusion, transgenic SN1 Citrange Troyer plants challenged with *X. citri* under controlled infection assays showed symptom reductions of up to 75%, as measured by the frequency of canker development on total inoculated wounds and *in vivo* infiltration assays. In addition, the smaller size of cankers and the delay observed in symptoms development suggest a considerable decrease of disease severity.

Author contribution statement

GC, VG, MAV, BIC and CAR designed the experiments. GC, VG, MAV, CG, BIC, GJ, CH and CAR performed experiments. LB, NIA, VN, CVR, AMG, NF, CCL, KK, MLG, BIC, EH and CAR analyzed the data. CAR and EH wrote the manuscript. All authors read and approved the final manuscript.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

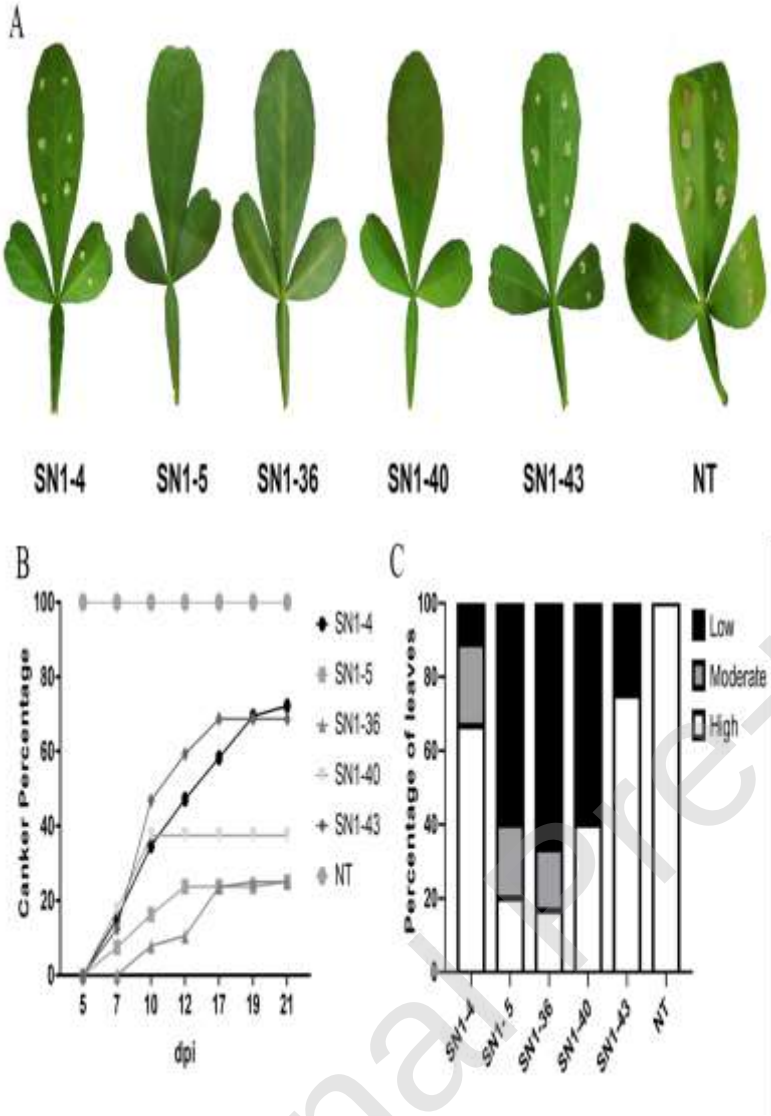

Figure 1. Enhance resistance of transgenic lines in detached leaves assays. Non-transgenic (NT) and SN1-4, SN1-5, SN1-36, SN1-40, SN1-43 plants were inoculated.with *X. citri* pv. citri. **(a)** Canker development at 21 dpi. One representative leave is shown for each line. **(b)** Time-course for canker development monitored at 5, 7, 10, 12, 17, 19 and 21 dpi. **(c)** Percentage of leaves ranked into different canker frequency categories in NT and transgenic plants measured at 21 d.p.i.

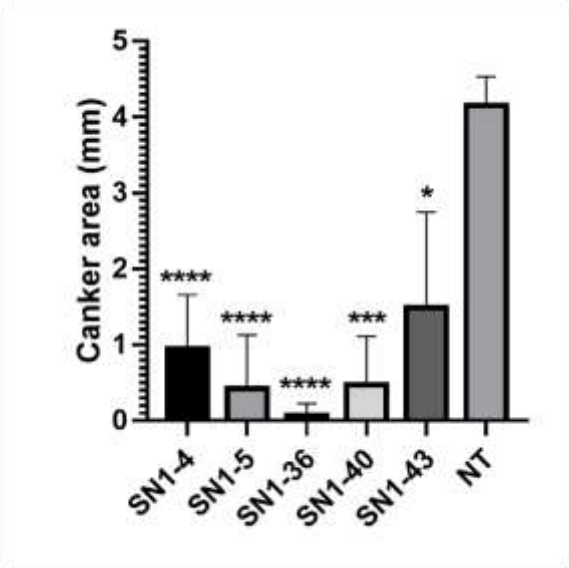

Figure 2. Canker size in transgenic lines and non-transgenic (NT) controls. Mean values and standard errors for total canker area at 21 dpi are shown. *, *** and **** indicate P <0.05, P < 0.001 and P < 0.0001 values respectively and indicate significant differences from NT using a two-tailed unpaired t-test.

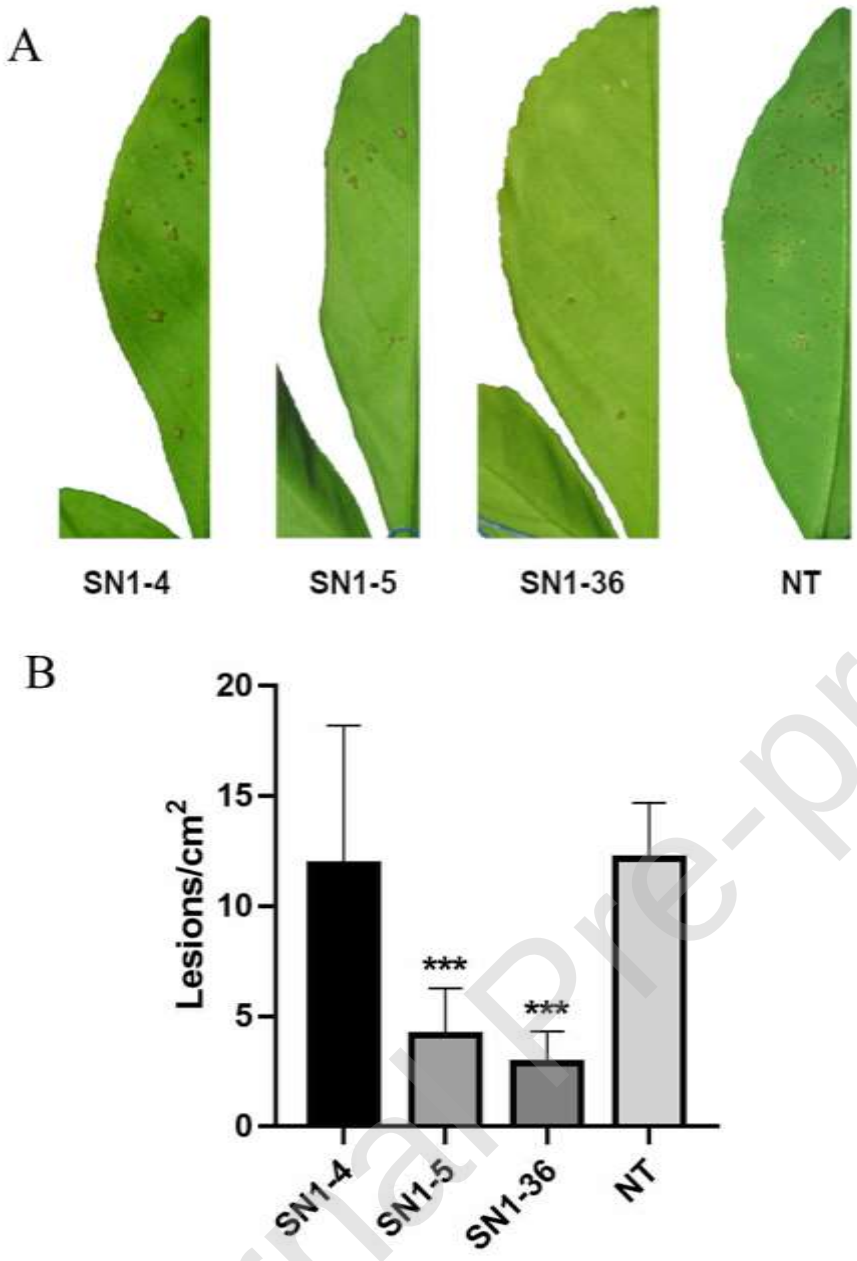

Figure 3. Enhance resistance of transgenic lines in *in planta* infiltration assays. Non-transgenic Duncan (NT) and SN1-4, SN1-5 and SN1-36 plants were infiltrated with *X. citri* pv. citri. **(a)** Canker development at 50 dpi. One representative leave is shown for each line. (b) Number of lesions per cm^2. Differences from the NT were tested using a two-tailed unpaired t-test. *** indicate $P < 0.001$. Assays were repeated 2 times with similar results.

Line/leaf	Number of cankers per leaves										Total puncture	Total canker	(Total canker/Total puncture) x 100
	1	2	3	4	5	6	7	8	9	10			
NT	8	8	8	8	8	-	-	-	-	-	40	40	100.00
SN1-4	4	8	6	7	4	8	2	8	5	-	72	52	72.22
SN1-5	0	8	0	0	0	5	3	4	0	0	80	20	25.00
SN1-36	0	0	0	4	8	0	-	-	-	-	48	12	25.00
SN1-40	0	8	0	7	0	-	-	-	-	-	40	15	37.50
SN1-43	8	6	8	0	-	-	-	-	-	-	32	22	68.75

Table 1. Canker formation frequencies in transgenic lines and non-transgenic (NT) controls. Each assay included a pool of 4–10 leaves inoculated with a bacterial suspension (8 punctures per leave). Number of cankers was scored at 21 dpi and canker frequency is expressed as (total canker number/total punctures) × 100. Assays were repeated 3 times with similar results.