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Commentary

Genetic Transformation of Rangpur Lime (*Citrus limonia* Osbeck) With the *bO* (bacterio-opsin) Gene and its Initial Evaluation for *Phytophthora nicotianae* Resistance

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Abstract. Transgenic plants expressing the bacterio-opsin (*bO*) gene can spontaneously activate programmed cell death (pcd) and may enhance broad-spectrum pathogen resistance by activating an intrinsic defense pathway in plant species such as tobacco and potato. In this work, we produced transgenic Rangpur lime plants with the *bO* gene, via *Agrobacterium tumefaciens*-mediated transformation, and evaluated these plants for *Phytophthora nicotianae* resistance. Two transgenic lines were successfully regenerated and transformation was confirmed by GUS activity assay, PCR analysis, Southern, Northern and Western blot analyses, in addition to detecting the expressed bO protein by an immunological approach. Evaluation for *Phytophthora nicotianae* resistance was carried out by plant inoculations with the pathogen and quantification of the affected area. One of the two transgenic lines showed greater tolerance to the fungal pathogen as compared to the control, with significantly smaller stem lesions after pathogen challenge. This increase in pathogen tolerance is correlated with a significantly higher level of transgene expression in this line when compared with the other transgenic line. This is the first report of the introduction of a potentially important gene into Rangpur lime to provide novel pathogen tolerance.

Key words: *Agrobacterium tumefaciens*, disease, gummosis, root rot, rootstock, transgenic

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Abbreviations: bO, bacterio-opsin; CTV, citrus tristeza virus; HR, hypersensitive response; SAR, systemic acquired resistance.

Introduction

Brazil is the world leader in citrus production, with approximately 20.1 million tons per year (FAO, 2006). The majority of the citrus is based on sweet orange cultivars budded on Rangpur lime (*Citrus limonia* Osbeck). This rootstock may represent over 70% of the total cultivated area in the country. The intensive use of this rootstock is largely due to its vigor in the nursery, early production after planting, good resistance to water stress, and tolerance to citrus tristeza virus (CTV). On the other hand, Rangpur lime is less resistant to *Phytophthora* spp., leading to considerable increase on the incidence and severity of diseases caused by these pathogens (Feichtenberger, 2001).

Phytophthora species are the most important soilborne problem that affect citrus trees; they may damage any part of the plant at any age. Several diseases are caused by *Phytophthora* species, such as dumping-off in seedlings, crown rot, fibrous root rot and brown rot of fruit. However, foot rot and gummosis may be the most relevant pathologies, leading to serious damage. Foot rot is characterized by an injury in the trunk bark or the roots near ground level, whereas gummosis is a rotting of the bark anywhere in the tree, leading to necrosis and abundant gum exudation. Nursery trees and young orchard trees may be girdled and rapidly killed by this disease. Older trees are normally partially girdled, causing canopy decline, defoliation, dieback, and short grown flushes (Timmer et al., 1993).

Although it is difficult to estimate, the worldwide losses due to these diseases are very high in all economically important citrus-growing regions (Erwin et al., 1996; Graham and Menge, 2000). *Phytophthora nicotianae* Breda de Haan (*P. parasitica* Dastur) and *P. citrophthora* (Smith & Smith) Leonian are the most frequent fungal species infecting citrus in Brazil. However, *P. nicotianae* has been the predominant species associated with disease, found in more than 95% of groves and nurseries in the São Paulo State (Medina Filho et al., 2004).

The search for resistant or tolerant cultivars is important to prevent and control diseases (Widmer et al., 1998), and in the case of *Phytophthora*-induced diseases, the use of resistant or tolerant rootstocks with adequate horticultural characteristics is certainly a key strategy for control (Broadbent, 1997). However, perennial breeding programs, such as for citrus, may take several years to produce a new cultivar. The recent development of citrus genetic transformation may provide an additional biotechnological tool for citrus cultivar improvement that could help plant breeders obtain disease resistant material in a shorter time. This advantage can be achieved due to the fact that genetic transformation can modify one specific trait, while keeping the other desirable characteristics in the genome (Brasileiro and Dusi, 1999).

Citrus genetic transformation has been successfully applied in sweet orange, sour orange, lemon, lime, grapefruit, *Poncirus trifoliata* and some of its hybrids (Peña et al., 2003). Specific research on genetic transformation for disease resistance has focused on methods associated with specific pathogen resistance, such as with the introduction of the CTV coat-protein gene in sour orange, Mexican lime and grapefruit (Gutiérrez-E et al., 1997; Domínguez et al., 2000; Febres et

al., 2003). In some cases, the expression of the CTV coat-protein gene led to an increase in CTV resistance (Olivares-Fuster et al., 2003). However, the constitutive expression of the p23 coat-protein gene led to CTV-like lesions in Mexican lime (Ghorbel et al., 2001). The use of the PR-5 tomato protein to confer *Phytophthora citrophthora* resistance was also studied for 'Pineapple' sweet orange (Fagoaga et al., 2001).

The hypersensitive response (HR) is a common manifestation of plant disease resistance that is characterized by rapid cell death around the point of infection, in some cases restricting the systemic spread of pathogens (Yalpani et al., 1991). In plants, programmed cell death is thought to be activated during the hypersensitive response to certain non virulent pathogens and in the course of several differentiation processes (Mittler et al., 1995; Lam, 2004). The bacterio-opsin gene (*bO*) encodes a protein that functions as a light driven proton pump in *Halobacteria halobium*. However, its expression can induce an HR-lesion phenotype in transgenic plants, and in the absence of the pathogen, *bO*-expressing plants have elevated expression of defense-related genes associated with the HR, including chitinase, glucanase, and salicylic acid (Mittler et al., 1995). Using a site-specific mutagenesis approach, it was demonstrated that the *in planta* activities of *bO* requires its ability to conduct protons through membranes but not its association with retinal (Pontier et al., 2002).

Potato plants expressing the *bO* gene were found to have heightened fungal resistance, which was correlated with an increase in the levels of salicylic acid and transcripts for several pathogenesis-related proteins that are hallmarks of systemic acquired resistance (SAR) (Abad et al., 1997). There is also considerable evidence of disease protection induced by *bO* in tobacco (Pontier et al., 2002; Lam, 2004). Therefore, this work had the objective to produce transgenic Rangpur lime plants expressing the *bO* gene, and then to evaluate these plants for an increase in *Phytophthora nicotianae* tolerance due to enhanced defense induction.

Material and Methods

Genetic transformation

Plant material: Seeds were extracted from ripe fruits of Rangpur lime (*Citrus limonia* Osbeck) and dried at room temperature for 24 hours. The seed integument was removed, and surface sterilization was carried out by treatment with a 67% commercial sodium hypochlorite solution (2.5% active chlorine) for 15 min followed by three rinses in sterile distilled water. The seeds were germinated in test tubes (150 × 25 mm) containing 15 mL MT medium (Murashige and Tucker, 1969) with 25 g L^{-1} sucrose and incubated at 27 ± 2°C in the dark for 20 d followed by 10-15 d in a 16-h photoperiod (40 µmol m^{-2} s^{-1}). Epicotyl segments approximately 1.0-cm-long were collected for the transformation experiments.

Agrobacterium *strain*

Agrobacterium tumefaciens GV3101, carrying the plasmid EL154 for *bO* gene expression, was used. The vector was constructed with pBIN19 as backbone, and contains the sequence for a *bO* gene that is tagged with a hexahistidine tag at the

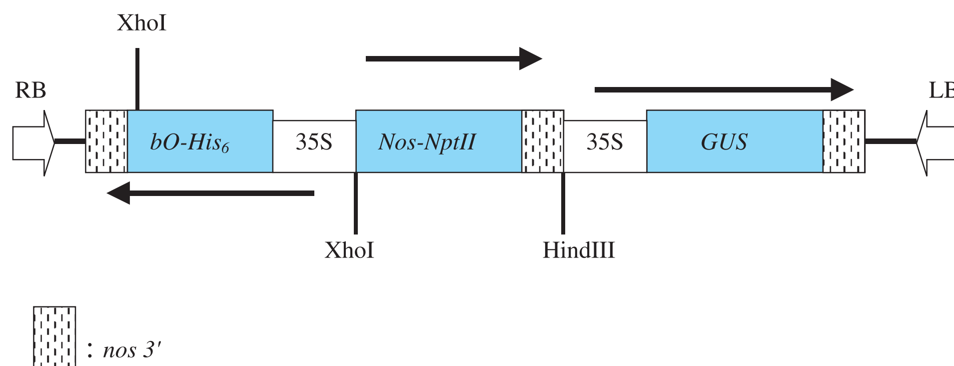

Figure 1. Binary vector EL154. The vector was constructed with pBIN19 as backbone with only the relevant components between the left (LB) and right borders (RB) shown here schematically. *bO-His$_6$*: *bO* gene tagged with a hexahistidine tag at the C-terminus for immunodetection; 35S: cauliflower mosaic virus 35S promoter; *Nos-NptII*: neomycin phosphotransferase gene driven by the nopaline synthase promoter for kanamycin resistance selection; *GUS*: β-glucuronidase gene from bacteria. The 3′ terminator sequence from the nopaline synthase gene is indicated by hatched boxes as indicated.

3′-end and is driven by the 35S promoter from Cauliflower Mosaic virus. The plasmid also contains the *nptII* selection gene for kanamycin resistance (Figure 1). A 35S-*GUS*-nos expression cassette is also present in the vector to facilitate identification of putative transformed plants. The bacteria were cultured in solid YEP medium (10 g L^{-1} peptone, 10 g L^{-1} yeast extract, 5 g L^{-1} sodium chloride, 15 g L^{-1} agar) containing kanamycin (100 mg L^{-1}) and rifampicin (50 mg L^{-1}), for 48 h. A single colony was then transferred to a 250 mL Erlenmeyer flask with 50 mL liquid YEP medium, supplemented with antibiotics and cultured at 180 rpm at 28°C for 16 h. The bacterial suspension was centrifuged at approximately 4147*g* (5°C/20 min) and resuspended in liquid MT medium.

Transformation, selection and regeneration

Epicotyl segments were incubated with the bacteria suspension for 20 minutes. Following incubation, explants were blotted dry and plated on regeneration EME medium (Grosser and Gmitter, 1990) supplemented with sucrose (25 g L^{-1}) and benzylaminopurine (BAP) (2.0 mg L^{-1}) in the dark, at 27°C, for a 3-day period. After co-culture, segments were transferred to regeneration medium, supplemented with kanamycin (100 mg L^{-1}) and cefotaxime (500 mg L^{-1}). Explants were sub-cultured every 2 weeks. Developed shoots were transferred to EME medium supplemented with gibberellic acid (1.0 mg L^{-1}), kanamycin (100 mg L^{-1}) and cefotaxime (500 mg L^{-1}) for elongation. Shoot basal ends and leaf segments were assayed for β-glucuronidase (GUS) activity. Leaves or stem segments were excised from 1-2-cm plants and then incubated in the dark at 37°C for 24 h in an X-Gluc solution (Jefferson, 1987). GUS+ shoots were micrografted onto Carrizo citrange (*C. sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) seedlings. After 45 days, well-developed *in vitro* grafted plantlets were transferred to a commercial potting mix composed primarily of pinus bark, and kept under high relative humidity (plastic cover) for 60 days in a growth chamber for acclimatization, at

27 ± 2ºC, with 16-h photoperiod (40 μmol m^{-2} s^{-1}). After this period, plants were transferred to a greenhouse.

Analysis of putatively transformed tissue

For the detection of specific T-DNA sequences, DNA was extracted from leaves of *in vitro* plantlets (Dellaporta et al., 1983). PCR amplification was performed using 1 μL DNA (50-100 ng), 0.2 μL each dNTP (10 mM), 1 μL $MgCl_2$ (25 mM), 2 μL buffer (10×), 0.3 μL Taq polymerase (5 U/μL) and 0.3 μL each of *bO* primers (10 μM). The primers: 5′-CAA GCT CAA ATT ACT GGA CGT CC-3′ and 5′-CCG GAG CCT CAG CCT CAC CGA A-3′ were used to amplify a 750 bp specific fragment from the *bO* gene sequence. Samples were subjected to 34 cycles of 1 min at 95°C, 45 s at 52°C and 1 min at 72°C.

Southern blot analysis was performed to confirm stable integration of the *bO* gene into the transgenic plants. Total plant DNA was extracted according to the DNAzol method (Plant DNAzol™), supplemented with two rinses with phenol, for additional purification. A total of 20 μg DNA was digested with *Xho*I or *Hin*d III, separated in a 1% agarose gel, and then transferred to a nylon membrane (Hybond-N^+, Amersham Biosciences). The *bO* gene probe was prepared by PCR, and the respective fragment was labeled using the Gene Images™ Random Primer Labelling Module kit (Amersham Biosciences). Hybridizations were performed at 60°C, and were detected with the CDP-Star™ kit (Amersham Biosciences).

For Northern blot analysis, total RNA isolation was performed from leaf tissues using Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's instructions. Aliquots of the RNA preparations (20 μg) were fractionated by electrophoresis on 0.8% denaturing agarose gel containing formaldehyde, and then blotted onto nylon membrane (Hybond-N^+). The *bO* gene probe was prepared by PCR, and the same kits for Southern blot were used for Northern blot analysis according the manufacturer's instructions.

The expression of bO protein was detected by preparing total protein extracts from leaves by directly grinding the tissues in liquid nitrogen followed by the addition of Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5% β-mercaptoethanol). Extracts were then incubated on ice for 5 min, after heating at 100ºC for 5 min. The samples were centrifuged at 13,000*g* for 5 min at room temperature, and the pellets discarded. Similar amounts of proteins as determined by Coomassie Blue staining were separated on 12% SDS-PAGE gels and blotted onto a nitrocellulose membrane. The blot was then probed with the Tetra-His antibody (Qiagen Co.) at a 1:3,500 dilution. The detection was performed using a chemiluminescence detection system (SuperSignal™ West Pico Chemiluminescent Substrate, Pierce).

Evaluation of tolerance to Phytophthora nicotianae in Rangpur lime transgenic lines

Plants of Rangpur lime genetically transformed with the EL154 vector were propagated through grafting, resulting in ten plants from each of two lines. Similarly, non-transformed Rangpur lime plants were also propagated to serve as experimental controls.

Initial inocula of *Phytophthora nicotianae* were multiplied on carrot-agar (CA) culture medium (Kaosiri et al., 1978). *Phytophthora* mycelia at four days after transfer were used for inoculating plants by the pathogen infected needle method (Siviero et al., 2002), which involves punching the stems with the infected needle, at 10 cm above the grafting region. Afterwards, the inoculation point was protected with sterile wet cotton. Twenty-five days after inoculation, the length and width of the lesions were determined by an area reader (Licor™ LI-3000). The initial presence of gum in the lesions was also recorded.

Results and Discussion

Genetic transformation

There is high variation in the transformation efficiency among different citrus species, the best results being achieved in sweet oranges (*Citrus sinensis* L. Osbeck), especially the Hamlin cultivar (Mendes et al., 2002). Rangpur lime (*Citrus limonia* Osbeck), in contrast, is a relatively difficult citrus species to transform (Almeida et al., 2003). We generated two GUS+ sprouts of Rangpur lime from approximately 300 explants co-cultivated with *Agrobacterium tumefaciens*. Insertion of the *bO* gene in the genome of these recovered plantlets was confirmed by PCR analysis, showing amplification of the correct sized fragment in both transgenic plant lines (Figure 2a). DNA from PCR+ plants was hybridized to a specific probe to observe *bO* gene integration in the transgenic Rangpur lime plants. The two plants were analyzed, and their transformation was confirmed, with three likely insertion events of the transgene in their genomes (Figure 2b). The transcript levels for *bO* in these transgenic Rangpur lime plants were examined by Northern blot, and high levels of hybridization signal were seen in transgenic line 1, while relatively low levels were detected for transgenic line 2 (Figure 2c).

We have also confirmed by immunoblot analysis the stable expression of bO protein, as well as the different levels of expression in the two transgenic lines that we recovered. Figure 3 shows the detection of the bO protein via antibodies specific for the polyhistidine tag fused to the bO coding sequence in our construct (Pontier et al., 2002). Transgenic tobacco plants expressing the same *bO* transgene that was previously characterized were used as positive controls (Figure 3, lane 2). Similar to the Northern blot analysis shown in Figure 2, transgenic line 1 showed much higher levels of bO protein than that of line 2, although it is clearly detectable in the latter. The different expression levels of the transgene observed for the two independent transgenic lines of Rangpur lime likely result from variations in the genome location of the inserted genes as well as their mode of integration.

Lesions likely related to *bO* transgene expression (HR-like leaf lesions) were observed in both lines of transgenic Rangpur lime obtained. As characterized in line 2, plants had lesions, which gradually increased in density as leaves aged, varied in shape and size, and usually turned yellow in color (Figure 4). Spontaneous formation of HR-like lesions in the absence of pathogen has been reported in a number of transgenic plants that express the *bO* gene (Mittler et al., 1995; Abad et al., 1997; Pontier et al., 2002). In some cases, the activation of programmed cell death was accompanied by the induction of multiple defense mechanisms and

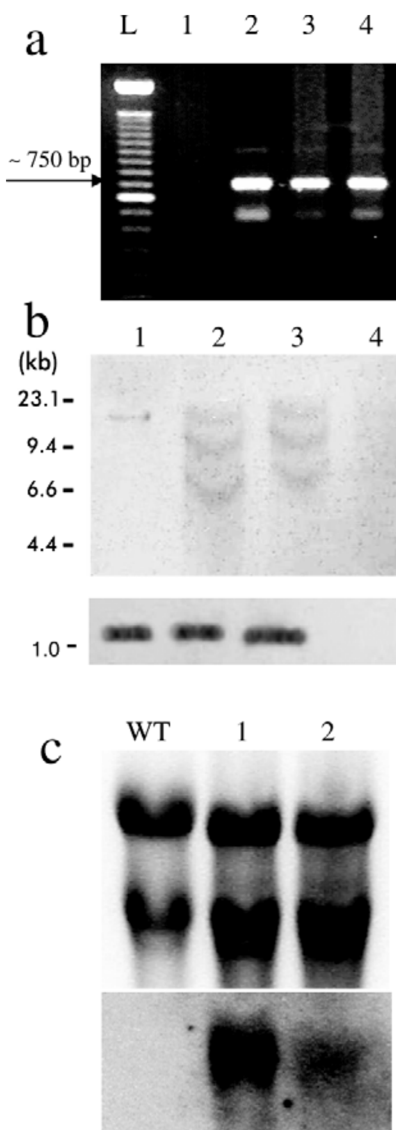

Figure 2. Molecular characterization of transgenic Rangpur lime tissues. a. PCR analysis: lane L, 100 bp ladder; lanes 1 and 2, no template and EL154 plasmid controls; lanes 3 and 4, GUS+ lines 1 and 2. b. Detection of *bO* gene via Southern blot. Lane 1, digested EL154 plasmid as positive control; lanes 2 and 3, DNA from transformed lines 1 and 2, respectively; lane 4, non-transformed plant control. In the top panel, DNA samples were digested with *Hin*dIII; in the lower panel, DNA samples were digested with *Xho*I. Both blots were probed with labeled *bO* coding sequence. c. RNA quantification by ethidium bromide staining (above) and detection of *bO* transcripts in transgenic plants by Northern blot. WT, untransformed Rangpur lime plants; 1, line 1; 2, line 2.

the induction of enhanced resistance, similar to systemic acquired resistance (SAR) (Mittler and Lam, 1996). However, previous reported studies with *bO* have been with tobacco and tomato only.

The present work is the first report of citrus transformation with the *bO* gene and also the first introduction of a gene of potential agronomic application in

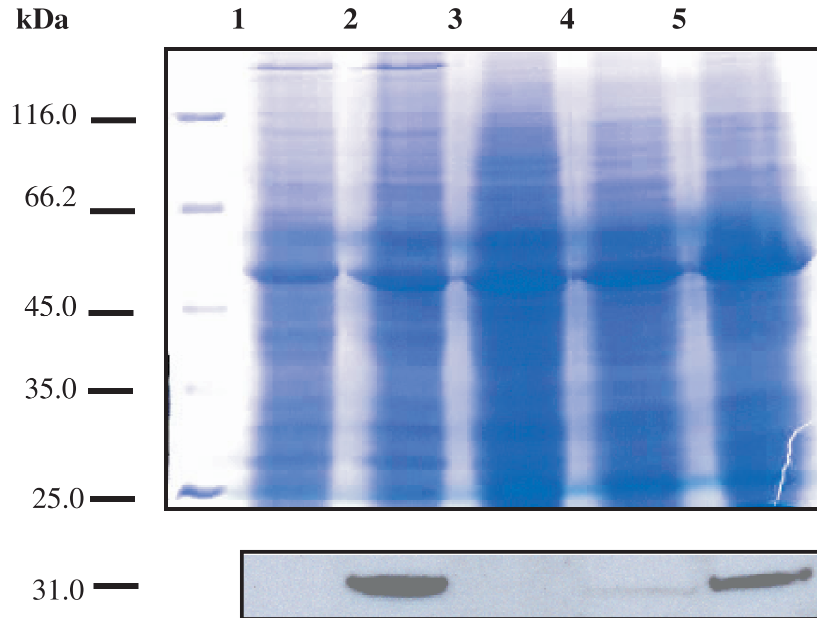

Figure 3. Protein immunoblot analysis of bO-His-expressing plants using Tetra-His tag antibody. Total protein (top panel) or anti-tetra-His antibody reactivity (bottom panel). (1) non-transformed tobacco plant (negative control), (2) transgenic tobacco expressing His-tagged bO (positive control), (3) non-transformed Rangpur lime, (4) transgenic Rangpur line 2, (5) transgenic Rangpur line 1. Approximate sizes of the protein markers are shown at the left.

Figure 4. Transgenic Rangpur lime leaves showing lesions likely related to *bO* transgene expression (HR-like leaf lesions). Various leaves from transgenic Rangpur lime are shown to illustrate the spontaneous lesions that appear on the tissues.

Table 1. Presence of gum, length and area of lesions caused by *P. nicotianae* in the wood of Rangpur lime plants (Piracicaba, SP, 2004).

Treatments	Plants with gum (%)	Length (cm)	Area (cm^2)
Transgenic (Line 1)	30	0.90 b	0.32 b
Transgenic (Line 2)	40	1.04 ab	0.46 ab
Control	60	1.37 a	0.54 a
CV (%)		13.35	10.28

Means followed by the same letter do not differ (Tukey, $P<0.05$)

Rangpur lime, a widely used rootstock in the Brazilian citrus industry. Development of the transformed plants appeared normal, without any significant change in the cultivar phenotype aside from sporadic appearance of HR-like leaf lesions with mild chlorosis observed in the older leaves.

Evaluation of tolerance of bO expressing Rangpur lime to P. nicotianae

At 25 days after inoculation, we evaluated the bioassay based on the initial observation of gum presence in the lesions and on measuring the length and area of the lesions caused by *P. nicotianae* (Table 1 and Figure 5). Gum was observed in more than half of the non-transformed control Rangpur lime plants (60%), while the transgenic plants showed significantly lower exudation, reducing by more than 50% in the case of line 1 (30%). The presence of gum in lesions usually precedes bark rupture, which is a characteristic symptom of *P. nicotianae* in citrus. This includes trunk, fiber and feeder roots. Therefore, a reduction in exudations in the transgenic lines, especially line 1, should reflect less severe illness (Feichtenberger, 2000).

The two transgenic lines of Rangpur lime also had smaller lesions than the wild type upon challenge with *P. nicotianae* with the stem inoculation assay. Consistent with the higher expression level of the *bO* transgene, line 1 transgenic plants were more tolerant to the pathogen in terms of lesion size compared with line 2 or wild-type Rangpur lime plants (Figure 5).

Plants expressing the *bO* gene could spontaneously activate programmed cell death (PCD) and may show heightened fungal resistance. Tobacco plants expressing *bO* gene also exhibited increased resistance to pathogens such as *Pseudomonas syringae* pv. tabaci (Mittler et al., 1995) and Tobacco Mosaic Virus (Pontier et al., 2002). In potatoes, increased resistance to some fungal pathogen was also observed (Abad et al., 1997). Site-specific mutagenesis studies have shown that the PCD and defense-inducing activities of *bO* in plants require their ability to conduct protons. Thus, we have hypothesized that the leakage of protons through a plasma-localized bO protein could activate an HR-like response in plants (Pontier et al., 2002). Our present results indicate that *bO* expression may provide a simple approach to activate defense mechanisms and enhance pathogen resistance in transgenic citrus trees. Furthermore, they suggest that ionic homeostasis at the plasma membrane level may also be an important control for defense activation in plants other than the *Solanacea*. The positive correlation between the level of expression of the *bO* transgene (both RNA and protein levels) and the resultant phenotypes in the two transgenic lines provide additional evidence for

Figure 5. Variations in size of lesions by *P. nicotianae* in stems of Rangpur lime. A. Non-transformed; B. Transgenic (Line 2); C. Transgenic (Line 1).

the causal relationship between the presence of the transgene and the increased disease tolerance observed. Similar dependence of phenotypes on *bO* expression levels were noted in previous studies with tobacco and tomato (Mittler et al., 1995; Pontier et al., 2002; Abad et al., 1997).

Only one previous study related to transgenic citrus for tolerance to gummosis caused by *Phytophthora* sp. has been reported. The transformation of 'Pineapple' sweet orange with a gene expressing the PR-5 protein from tomato resulted in the regeneration of nine plants. In some transgenic lines, protection against *Phytophthora citrophthora* was reported, with high survival rates in the presence of the pathogen after a year (Fagoaga et al., 2001). On the other hand, there is no reported work with transgenic rootstocks concerning the protection against this disease. Our present work suggests that the *bO*-expression vector used in this study could be applied for enhanced disease tolerance in citrus plants. Furthermore, the transgenic Rangpur lines that we have produced could be used as rootstocks to ascertain whether this engineered disease tolerance phenotype can be propagated systemically in grafted trees.

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