

# Evaluation of tolerance to Pierce's disease and *Botrytis* in transgenic plants of *Vitis vinifera* L. expressing the pear PGIP gene

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## SUMMARY

Polygalacturonase-inhibiting proteins (PGIPs) are plant cell-wall proteins that specifically inhibit fungal endo-polygalacturonases (PGs) that contribute to the aggressive decomposition of susceptible plant tissues. The inhibition of fungal PGs by PGIPs suggests that PGIPs have a role in plant tolerance to fungal infections and this has been observed in transgenic plants expressing PGIPs. *Xylella fastidiosa*, the causal agent of Pierce's disease (PD) in grapevines, has genes that encode cell-wall-degrading enzymes, including a putative PG. Therefore, we hypothesized that PGIP expression could confer tolerance against this bacterium as well as against the fungal pathogen *Botrytis cinerea*. To test this hypothesis, *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' were transformed to express pear fruit PGIP-encoding gene (*pPGIP*) under the control of the CaMV 35S promoter. Substantial pear PGIP (pPGIP) activity was found in crude extracts from leaves and in xylem exudate of transgenic lines obtained from independent transformation events, but not in untransformed controls. pPGIP activity was detected in xylem exudate of untransformed scions grafted on to transgenic rootstocks expressing *pPGIP*. Leaves of transgenic plants infected with *B. cinerea* had reduced rates of lesion expansion. The development of PD was delayed in some transgenic lines with increased pPGIP activity. PD-tolerant transgenic lines had reduced leaf scorching, lower *Xylella* titres and better re-growth after pruning than the untransformed controls.

## INTRODUCTION

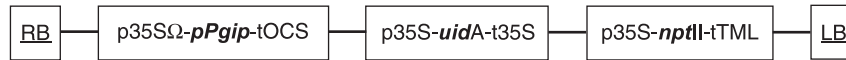
To infect plants successfully, some pathogens need to penetrate the polysaccharide cell-wall barrier to gain access to the contents of the cells. Pathogens accomplish this tissue maceration by

secreting a number of hydrolytic enzymes, among which are endo-polygalacturonases (PGs), capable of degrading cell-wall pectin polymers (De Lorenzo *et al.*, 2001). Polygalacturonase-inhibiting proteins (PGIPs) are leucine-rich repeat glycoproteins located in the plant cell wall. They specifically inhibit fungal PGs, especially those with mixed endo/exo functions (Cook *et al.*, 1999). By inhibiting pathogen PGs, PGIPs reduce plant host cell-wall degradation and may favour the release of oligogalacturonide plant cell-wall fragments, which then could serve as elicitors of a variety of defence responses that plants can activate against the microbe (Cervone *et al.*, 1989). Recently, D'hallewin *et al.* (2004) reported that grapefruit PGIP directly affected the development of *Botrytis cinerea* and *Penicillium italicum* hyphae *in vitro* when the fungi were grown on complex pectin carbon sources, suggesting that direct interference of PGIP with fungal development, perhaps due to the impact of the protein on pectin digestion per se, may be playing the role of supplying carbohydrates as nutrient reserves in the spores are depleted. PGIPs have been identified in nearly all plants in which they have been sought (Powell *et al.*, 2000). The characterization of several PGIPs within the same plant and PGIPs from different plant species indicates that the apparently broad antifungal effect of PGIP is the consequence of multiple isoforms, each of which has narrow specificity (Cook *et al.*, 1999; Desiderio *et al.*, 1997).

One of the first well-characterized PGIPs was identified and cloned from *Pyrus communis* L. cv 'Bartlett' (Stotz *et al.*, 1993). The pear fruit PGIP gene (*pPGIP*), encodes a 36.5-kDa polypeptide containing a putative signal sequence of 24 amino acids and seven potential N glycosylation sites. Heterologous expression of *pPGIP* in transgenic tomato plants improved their tolerance to *B. cinerea*, manifested by a decrease in symptom development, i.e. slowed expansion of lesions and associated tissue maceration (Powell *et al.*, 2000). These results reinforce the concept that improvement of tolerance could be obtained by transforming crop plants to express PGIP.

In grapes, two diseases, bunch rot and Pierce's disease (PD), have been identified as significant contributors to crop loss. *B. cinerea* is the most important fungal pathogen responsible for

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**Fig. 1** Schematic representation of binary plasmid pDU94.0928. Abbreviations: p35S, CaMV 35S promoter; t35S, CaMV 35S terminator;  $\Omega$ , TMV U1  $\Omega$  enhancer; tOCS, octopine synthase terminator; *pPgip*, pear PGIP gene; *uidA*,  $\beta$ -glucuronidase (GUS) gene; *nptII*, neomycin phosphotransferase gene; tTML, tumour morphology large 3' terminator; RB, right border; LB, left border.

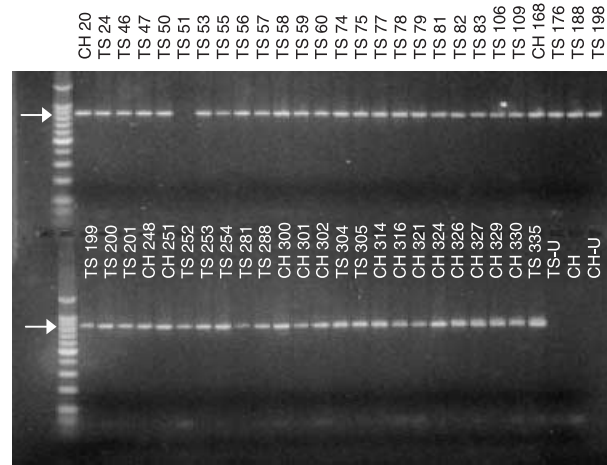
bunch rot of grape berries, a serious disease in most wine-producing countries. PGs and other hydrolases are among the enzymes produced by the fungus, and these enzymes probably participate in the rapid destruction of the berry integrity, resulting in extremely high losses in years with favourable conditions for fungal infection.

*Xylella fastidiosa* is a xylem-limited bacterium that causes PD in grapevines. The genome of the *X. fastidiosa* strain 9a5c that causes citrus variegated chlorosis (CVC), a very important disease in Brazil, has been sequenced, revealing a putative PG-encoding gene truncated due to a frame shift mutation (Simpson *et al.*, 2001); however, the putative PG gene is intact in the PD-causing Temecula strain of *X. fastidiosa* (Van Sluys *et al.*, 2003). PD symptoms include yellowing and gradual necrosis of grapevine leaves, uneven cork development and presence of petioles attached to the cane after leaf fall (Flaherty *et al.*, 1992). The disease progresses rapidly, resulting in occlusion of xylem vessels and consequent water stress. Vine death often occurs within 2 years (Goodwin *et al.*, 1988). The putative *Xylella* PG may contribute to bacterium virulence and systemic colonization of the host by degrading the pectin-containing pit membranes that separate adjacent vessels, releasing nutrients for the pathogen, triggering host vessel blockage and/or aiding in initial invasion (Harakava *et al.*, 2001).

The purpose of this study was to investigate the hypothesis that expression of *pPGIP* in grape could improve tolerance to diseases caused by *B. cinerea* and *X. fastidiosa*. In order to test this hypothesis, pre-embryogenic calli originating from anthers of *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' were transformed with *pPGIP* and the resulting transgenic plants were tested for PGIP activity and protein, and tolerance to *B. cinerea* and *X. fastidiosa* infections.

## RESULTS

A total of 36 lines of 'Thompson Seedless' (TS) and 15 of 'Chardonnay' (CH) were obtained from independent transformation events with *Agrobacterium tumefaciens* containing *pPGIP* under the control of the CaMV 35S promoter (Fig. 1). These 51 lines were successfully multiplied *in vitro* and transferred to the greenhouse for further analysis. No significant differences in morphology were observed between transgenic and untransformed plants. A fragment of the expected size of 850 bp was amplified from DNA of 50 of the 51 putative transgenic lines tested (Fig. 2).



**Fig. 2** DNA analysis. PGIP coding sequences were amplified from 50 out of 51 independent lines of Thompson Seedless (TS) and Chardonnay (CH). TS-U and CH-U are untransformed controls of TS and CH, respectively. The arrows on the left indicate the position of the expected PCR product for the amplification of *pPGIP* coding sequence. A 100-bp DNA ladder (Promega) was used as reference.

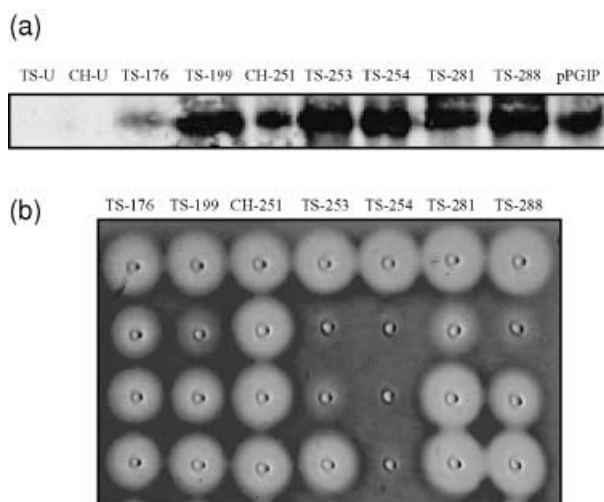
This fragment was not detected in DNA from untransformed controls and transformed line TS-51 (Fig. 2). All of the transgenic lines were analysed for PGIP activity by testing dilutions of leaf protein extracts to inhibit the mixture of PGs produced in *B. cinerea* cultures using a semiquantitative radial diffusion assay. Using this assay, 47 of the 51 lines of TS and CH transformants (92%) showed activity. The lines displaying PGIP activity were grouped in three categories of high, moderate and low expressors (Table 1, Fig. 3). A majority of the TS transgenic lines (20/36, 55.5%) had high activity, 11/36 (30%) showed moderate levels and five showed low or no detectable PGIP activity. Three of the lines containing *pPGIP* (based on genomic DNA PCR analysis, Fig. 2) apparently did not express the gene. These three lines had no detectable *B. cinerea* PG inhibition and also did not show GUS activity (data not shown). In the case of the CH lines, the differences in PGIP activity among lines were evenly spread among high, moderate and low categories (Table 1). Western blotting was performed with 33 of the 47 transgenic lines that displayed PGIP activity. In all cases a protein with a size similar to *pPGIP* was detected (Table 1, Fig. 3).

*pPGIP* protein and activity were found in leaves, roots and stems of transgenic plants both by antibody cross-reactivity and

**Table 1** Transgenic lines of cvs. Thompson Seedless and Chardonnay that displayed high, moderate or low activity in the agar diffusion assay. Lines that only showed activity with 200 ng total protein leaf extracts were included in the low activity group, and lines that showed activity with 100 ng and 20 ng total protein leaf extracts were included in the moderate and high activity groups, respectively. Lines underlined were also tested in Western blot analysis.

	Thompson Seedless	Chardonnay
High activity (100–200 U of PGIP/mL of leaf extract)	24, <u>50</u> , 53, 55, <u>58</u> , <u>60</u> , <u>74</u> , <u>77</u> , <u>78</u> , <u>79</u> , <u>106</u> , <u>109</u> , 198, 200, 201, <u>253</u> , <u>254</u> , <u>304</u> , <u>305</u> , 335	<u>314</u> , <u>316</u> , <u>321</u> , <u>329</u> , 330
Moderate activity (100–10 U of PGIP/mL of leaf extract)	<u>56</u> , <u>57</u> , <u>75</u> , 81, 82, <u>83</u> , <u>176</u> , 188, <u>199</u> , <u>281</u> , <u>288</u>	<u>168</u> , 302, 324, <u>326</u> , <u>327</u>
Low activity (< 10 U of PGIP/mL leaf extract)	<u>46</u>	<u>20</u> , 248, <u>251</u> , <u>300</u> , <u>301</u>
No activity	47, 51, 59, 252	

One unit (1 U) of PGIP activity was defined to be that amount of leaf extract that reduced the activity of 10  $\mu$ L PG enzyme extract (75 nmol/min/mL) by 50%.



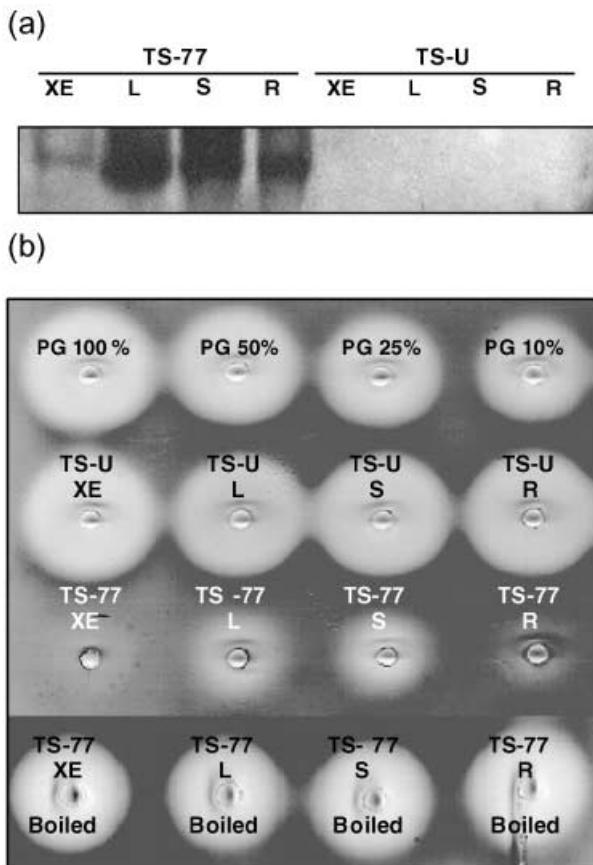
**Fig. 3** Protein analysis. (a) Western blotting performed on seven independent transgenic lines of cvs. Thompson Seedless (TS) and Chardonnay (CH). Total protein (50  $\mu$ g) from extractions from grape leaves was analysed using antibodies prepared with a deglycosylated pPGIP. TS-U and CH-U are untransformed controls of TS and CH, respectively. (b) Inhibition of the PG activity from culture filtrates of *B. cinerea* was determined by radial diffusion assay in agarose using the same transgenic lines analysed above by Western blotting. A clear zone is indicative of PGs degrading the glycosidic bonds present in the polygalacturonic acid substrate. First upper row represents boiled transgenic leaf samples (200 ng of each leaf extract) incubated with 15  $\mu$ L of *B. cinerea* PG extract (75 nmol/min/mL). The second, third and fourth rows correspond to 200, 100 and 20 ng total protein leaf extracts, respectively, incubated with 15  $\mu$ L of *B. cinerea* PG extract (75 nmol/min/mL).

by inhibition of *B. cinerea* PGs, as expected for a gene driven by the CaMV 35S promoter (Fig. 4). To determine if pPGIP could be translocated from transgenic rootstocks, untransformed scions of TS (Fig. 5) and CH were grafted on transgenic rootstocks expressing pPGIP. PG inhibition was close to 100% in 10 $\times$  diluted xylem exudates from the grafted untransformed scions. Xylem exudate from a transgenic scion grafted on to an untransformed rootstock had

less PGIP activity than the reverse combination, indicating a larger contribution of the root system to the exudate composition. No PGIP was identified in xylem exudate from untransformed rootstock/scion combination. No GUS activity was detected in any exudates.

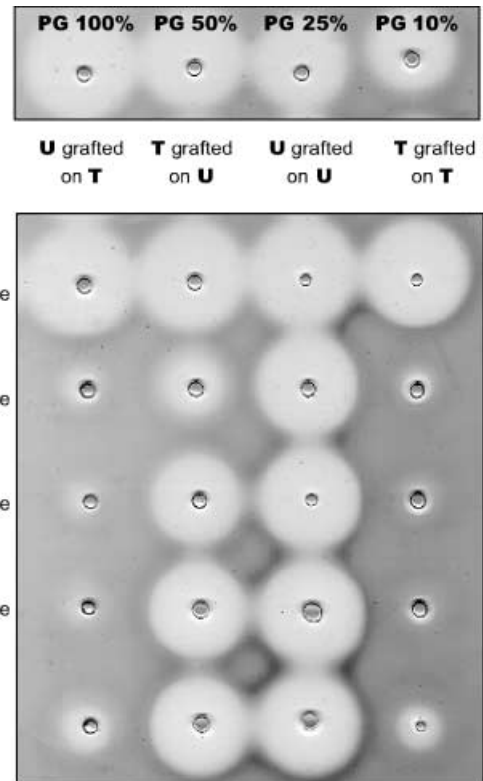
Eighteen lines, displaying high (11 lines), moderate (five lines) and low (two lines) pPGIP activity, were assayed for *Botrytis* tolerance. Lesion expansion in leaves was significantly retarded in transgenic grapevines inoculated with *B. cinerea* (Table 2). Lesions in control leaves appeared more transparent (Fig. 6). Plants transformed with a construct containing only the *uidA* and *nptII* genes behaved much like the untransformed controls (Table 2). At least some reduced lesion expansion was found in all 18 transgenic lines tested. Most of these were tested in two or three independent experiments. The lesion reduction was statistically significant at  $P \leq 0.05$  in 12 of the 18 lines. Lesion size reduction was less prominent and not always statistically significant at  $P \leq 0.05$ , in six lines, which had displayed moderate or low levels of PGIP activity (data not shown).

One TS pPGIP-expressing line and two CH pPGIP-expressing lines exhibited less severe PD symptoms (leaf marginal necrosis) than the untransformed controls (Fig. 7). The concentration of bacteria in stem segments immediately below the apical pruning point was measured by ELISA in the CH lines. The results showed that *X. fastidiosa* was present in both untransformed and transgenic stems, although the concentration was considerably lower in the transgenic lines (Fig. 8). Specific hydraulic conductivity, measured 40 cm above the point of inoculation (POI), 28 weeks after inoculation, was reduced in all infected plants. The infected transgenic line 327 had a higher conductivity but no differences were found between infected untransformed plants and infected-transgenic lines 329 (Fig. 8) and 50 (data not shown). Microscopic observations showed that most of the vessels of the infected plants had developed tyloses (structures that protrude into the xylem vessels). These results indicate that the vessel blockage that developed 40 cm above the POI 28 weeks after inoculation was high, both in untransformed and in transgenic



**Fig. 4** PGIP presence in xylem exudate and in extract from different organs of Thompson Seedless plants representing an untransformed control (TS-U) and transgenic line TS-77. (a) Western blot analysis of PGIP. Performed with 10  $\mu$ g of total protein from xylem exudates (XE), and extracts of leaf (L), stem (S) and root (R) obtained from the untransformed control (TS-U) and transgenic line 77 (TS-77). PGIP was detected using antibodies prepared with deglycosylated pPGIP. (b) Semi-quantitative determination of PGIP activity in different organs. The inhibition of the PG activity from culture filtrates of *B. cinerea* was determined by radial diffusion assay in agarose on the same samples. Top: the clear zones of PG activity standards (100, 50, 25 and 10%) resulted from adding 15, 7.5, 3.75 and 1.5  $\mu$ L PG (75 nmol/min/mL), respectively, to the gel containing the substrate. Two hundred nanograms of protein, obtained from each of the organ extracts from TS-U (row 2) or TS-77 (rows 3 and 4), were incubated with PG enzyme. In row 4 the organ extracts were boiled to inactivate PGIP.

plants, and that tyloses might be one of the determinant factors in this blockage. In addition, we noticed that more lateral shoots developed at the apices of the transgenic plants after pruning at 90 cm above the POI. In the few cases in which the control plants developed an apical lateral shoot, it was considerably shorter than lateral shoots that developed on the transgenic shoots. When the main shoot of infected plants was pruned at the base of the stem, transgenic plants developed longer shoots than did the infected control plants (Fig. 7).



**Fig. 5** Semi-quantitative determination of PGIP activity obtained from the xylem exudates of different graft combinations. First row contains PG activity standards corresponding to 100, 50, 25 and 10% PG activity obtained by adding 15, 7.5, 3.75 and 1.5  $\mu$ L PG (75 nmol/min/mL), respectively, to the substrate in the gel for radial diffusion. The second row shows boiled samples; rows 3–6 correspond to different dilutions of xylem sap. U and T are Thompson Seedless untransformed and transgenic line 77, respectively.

In order to establish the production of PG activity by *X. fastidiosa* in grapevines displaying PD symptoms, protein extracts from petioles of uninfected and infected CH and TS grapevines were tested using the radial diffusion assay. PG activity was detected in extracts from both infected and uninfected plants, although this activity was not found in all the samples tested. Western blots showed a 35-kDa, cross-reactive polypeptide, using polyclonal antibodies raised against a truncated C terminal peptide encoded by the putative PG sequence of the CVC strain of *X. fastidiosa*. This 35-kDa band was seen in extracts of all PG-positive samples, both from infected and from uninfected tissues (data not shown).

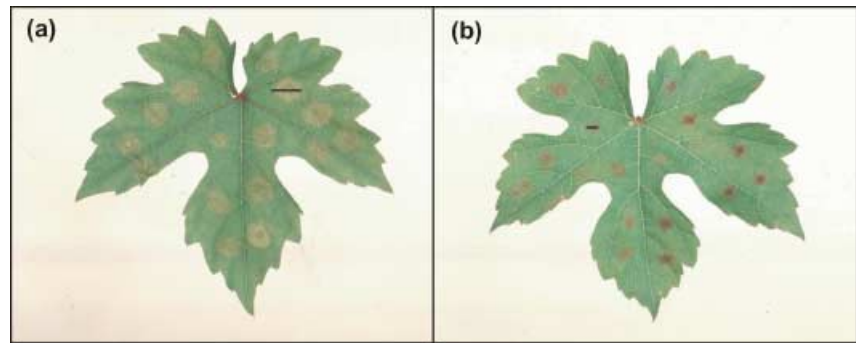
## DISCUSSION

Among the numerous pathogens of the grapevine, *Botrytis cinerea* is possibly the most significant agricultural problem. In this paper we show that the expression of pPGIP and concomitant increase in *Botrytis* PG-inhibiting activity in two important cultivars of *V. vinifera* increase their tolerance to disease caused by this

**Table 2** Lesion size (mm) on transgenic and control leaves inoculated with *B. cinerea* 8 or 9 days after inoculation. The 'gus/pgip' are the transgenic lines transformed with the binary plasmid pDU94.0928, carrying the neomycin phosphotransferase II gene (*nptII*), the  $\beta$ -glucuronidase gene (*gus*) and the *pPGIP*, whereas, the 'gus transgenic' are lines transformed using binary plasmid pGV3850p35SGUSINT carrying only *nptII* and *gus*. Data presented are means of four or five replicates  $\pm$  standard error. ANOVA and Tukey tests indicated that, within a set of data for an individual experiment, the lesion size of the *pPGIP* transgenic lines was significantly smaller ( $P \leq 0.05$ ) than the lesion size of the untransformed controls and the lesion size of the lines transformed with only *nptII* and *gus*.

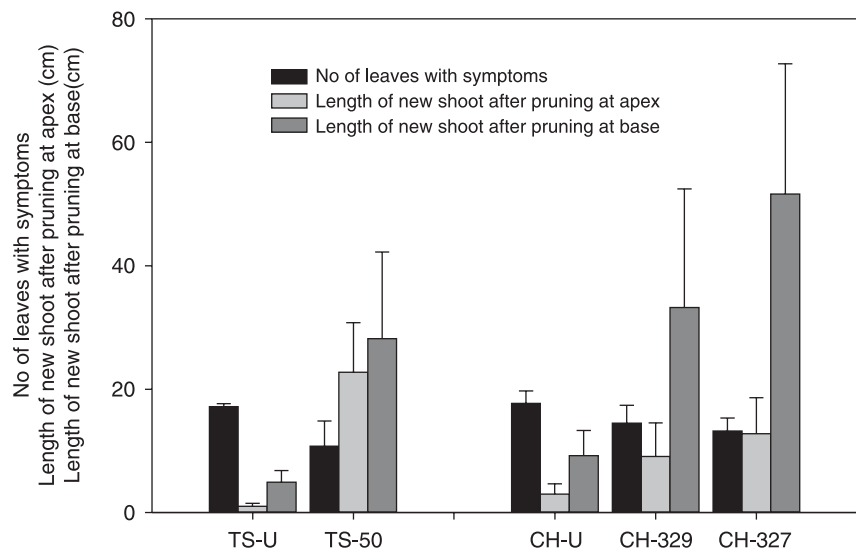
	Chardonnay			Thompson Seedless		
	Untransformed control	<i>gus</i> transgenic	<i>gus/pgip</i> transgenic 329	Untransformed control	<i>gus</i> transgenic	<i>gus/pgip</i> transgenic 60
Exp. 1 (Sep. 2002)	8.2 ( $\pm$ 0.32)	NT	6.9 ( $\pm$ 0.25)	10.2 ( $\pm$ 0.53)	NT	6.8 ( $\pm$ 0.21)
Exp. 2 (Nov. 2002)	9.4 ( $\pm$ 0.6)	NT	6.3 ( $\pm$ 0.55)	9.8 ( $\pm$ 0.59)	NT	7.9 ( $\pm$ 0.23)
Exp. 3 (Mar. 2003)	8.9 ( $\pm$ 0.21)	9.4 ( $\pm$ 0.37)	7.4 ( $\pm$ 0.33)	12.0 ( $\pm$ 0.51)	10.9 ( $\pm$ 0.8)	5.7 ( $\pm$ 0.89)

NT, not tested.



**Fig. 6** *B. cinerea* infection of control (a) and transgenic line CH-329 (b) leaves. Bars show the diameter of the lesions. The lesions of the transgenic leaf (right) are smaller and less transparent than those on the untransformed control (left).

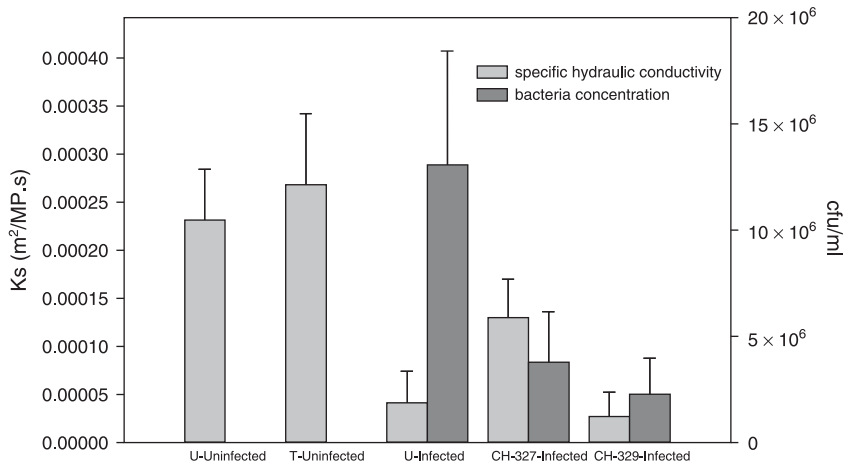
**Fig. 7** Number of leaves with PD symptoms and re-growth after pruning. Infection of untransformed and transgenic plants of lines TS-50, CH-329 and CH-327 was confirmed by ELISA. The total number of leaves evaluated was represented by all the leaves in the main shoot, which varied from 25 to 28 in CH and 20–22 in TS. At the moment we measured symptom development, upper leaves were symptomless. Main shoot was pruned 90 cm above the point of inoculation 10 weeks after inoculation and below the point of inoculation 28 weeks after inoculation. TS-U and CH-U are untransformed controls of cvs. 'Thompson Seedless' and 'Chardonnay', respectively. Standard errors of the means are represented by error bars.



pathogen as judged by disease symptoms on infected leaves. Furthermore, we provide the first demonstration that PGIP reaches the xylem and moves through the graft union. We found that the development of PD, caused by a xylem-limited bacterium, was delayed in three transgenic lines with increased PGIP activity.

Our results with *B. cinerea* confirm those obtained by Powell *et al.* (2000), who found that growth of the fungus on tomato

leaves was reduced between 15 and 25% in transgenic tomato plants expressing *pPGIP*. They also reported that the lesions had a more transparent, water-soaked appearance, when developing on untransformed controls. Likewise, over-expression of PGIPs in *Arabidopsis* significantly reduced *Botrytis* disease symptoms (Ferrari *et al.*, 2003). In grape, two PGIP isomers have been purified from berries of *V. vinifera* L. cv. Pinotage (De Ascensao, 2002).



**Fig. 8** Specific hydraulic conductivity and bacteria concentration. U and T are untransformed controls and transgenic lines CH-327 and CH-329 of cv. 'Chardonnay', respectively. Standard errors of the means are represented by error bars.

One of these, designated PGIP-B, showed a strong inhibitory activity against PGs from *B. cinerea*. Heterologous expression of the encoding gene, called *pgip1*, in tobacco plants slowed *B. cinerea* disease development *in planta* (De Ascensao, 2002). Bezier *et al.* (2002) recently isolated from *V. vinifera* L. cv. 'Chardonnay' a PGIP gene almost identical to *pgip1*. This gene was induced in response to *B. cinerea* infection. Taken together, these results suggest a potential strategy for the improvement of tolerance to bunch rot in grape berries by over-expression of PGIP-encoding genes. We have previously shown with transgenic tomato lines expressing *pPGIP* that plants with reduced disease symptoms on leaves also had reduced symptoms in fruit (Powell *et al.*, 2000). Because transgenic pPGIP was detected in roots and stems of our transgenic grapes and *pPGIP* expression in transgenics is controlled by the 35S promoter, it is expected that *pPGIP* will also be expressed in the fruit clusters. We plan to test for the development of bunch rot in berries.

A useful strategy to enhance further the resistance of grapevine against *B. cinerea* would be to add additional putative defence genes (pyramiding expression) such as *Vst1* to plants expressing *pPGIP*. *Vst1* encodes stilbene synthase, a key enzyme involved in the synthesis of resveratrol, an abundant phytoalexin in grapevine, whose over-expression in grape rootstock 41B was shown to reduce *Botrytis* growth *in vitro* (Coutos-Thevenot *et al.*, 2001). The expression of other PGIPs could also be considered; for example, the grapefruit PGIP that has been shown to interfere directly with *B. cinerea* and *Penicillium italicum* hyphae development *in vitro* when the fungi were grown on complex pectin carbon sources (D'hallewin *et al.*, 2004). Thus, expression of genes encoding other PGIPs or enzymes influencing production of particularly efficacious antifungal compounds may provide an increased tolerance by grapes to *B. cinerea*.

Some of the transgenic lines were also less susceptible to *X. fastidiosa*. Delayed bacterial movement and population increase along the stem might account for the improved growth of

infected transgenic grapevines. The inhibition by PGIP of a *Xylella* PG could readily influence these events, but further information is needed to explain these results. The *Xylella* PG antibody used in Western blots recognizes a protein isolated from *E. coli* transformed to express the PG gene of the PD strain of *X. fastidiosa* (C. Roper, personal communication). The proteins extracted from the transformed *E. coli* also show PG activity (C. Roper and L. C. Greve, personal communication). In addition, infection of grapevines with a PG knock out strain of *X. fastidiosa* has led to a substantial reduction in PD symptom development relative to inoculations using unmodified *X. fastidiosa* (Roper *et al.*, 2004). These observations lead us to conclude that *Xylella* produces a PG that is important in PD development. However, our analysis showed PG activity in proteins isolated from both infected and uninfected grape petioles, and Western analysis of these proteins revealed a cross-reactive band estimated to have a molecular weight of c. 35 kDa (data not shown). Because we detected no proteins cross-reactive with the anti-*Xylella* PG antibody in extracts that had no PG activity, we conclude that the cross-reactive protein represents the PG we measured. However, its molecular weight is considerably different from that predicted by the full-length sequence encoding the PG of the PD-causing Temecula strain of *X. fastidiosa* (Van Sluys *et al.*, 2003) and confirmed in Western analysis of the proteins isolated from the *X. fastidiosa* PG gene-expressing *E. coli* mentioned above. Continuing studies are aimed at determining whether and where the bacterial PG gene is expressed in *Xylella*-infected grape tissues and at determining if the PG activity is inhibited by pPGIP.

It will be important to validate the transgenic plants under field conditions to evaluate PD and bunch rot susceptibilities, a process that will take many years, especially to evaluate the disease susceptibility of fruit tissues. Nevertheless, the expression of pPGIP has provided useful information that suggests future strategies for the control of PD, bunch rot and perhaps other diseases. pPGIP in xylem vessels may represent protein synthesized in

procambial vessel initials that was not broken down during the programmed cell death process that leads to vessel differentiation or it may be secreted into the vessels through pit membranes that serve as transfer pathways from neighbouring parenchyma cells. We favour the latter, presuming that protein turnover during vessel differentiation would eliminate procambial cell proteins. Furthermore, because the pPGIP has a signal sequence that directs its secretion to the apoplast (Stotz *et al.*, 1993), we would anticipate that any pPGIP produced in procambial cells would not have remained in them. The presumed secretion is relevant to PD development because *X. fastidiosa* is xylem-limited and any anti-*Xylella* gene product must therefore be delivered to this tissue. In fact, the pPGIP signal sequence could be used to direct other anti-*Xylella* products to the xylem. Finally, we have demonstrated that the transgene PGIP product moves through the graft union. This movement to the scion implies that a few transgenic rootstocks could be used with any scion variety provided that the anti-*Xylella* compound is synthesized in effective concentrations in roots.

## EXPERIMENTAL PROCEDURES

### Grape transformation

Pre-embryogenic calli derived from *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' immature anthers were inoculated with *Agrobacterium tumefaciens* strain EHA101 harbouring the binary plasmid pDU94.0928, carrying the neomycin phosphotransferase II gene (*nptII*), the  $\beta$ -glucuronidase gene (*gus*) and the pear *PGIP*, each under the control of the CaMV 35S promoter (Fig. 1). In addition, calli were transformed using the binary plasmid pGV3850p35SGUSINT carrying only *nptII* and *gus*. Putative transformed calli were selected on PT medium (Hanson *et al.*, 1999) supplemented with 100  $\mu$ g/mL kanamycin and 300  $\mu$ g/mL cefotaxime and then transferred to a modified WP medium (York Moi, personal communication) for germination. Plantlets were multiplied on half-strength MS medium (Murashige and Skoog, 1962) plus 0.05  $\mu$ M NAA, acclimatized and transferred to the greenhouse. Plants were grown in pots with a modified UC mix in the greenhouse for 4–6 months. Various combinations of grafted plants were created by bench grafting 4–6-month-old grapevine plants. After grafting, plants were allowed to grow for 2 months in the greenhouse before they were used for xylem exudate analysis.

### Molecular and biochemical analysis

Genomic DNA was isolated from young leaves of plants (4–6-month-old grapevines) growing in the greenhouse with DNeasy Plant Mini Kit (Qiagen). A primer that binds the CaMV 35S promoter (GACGTAAGGGATGACGCACAAT) was used together with

one that binds the PGIP coding region (GGTGAAGTCCATCTGAG) for the PCR amplification of inserted segments. These two primers generate a DNA fragment of about 850 bp. This fragment was not present in genomic DNA from control, non-transformed grape plants.

PG preparations were obtained from *B. cinerea* strain Del 11 isolated from grapevine. The fungus was cultured on a modified Pratt's medium (Fergus, 1952) supplemented with 1 g/L Difco yeast extract and 3 g/L glucose on a rotary shaker in the dark. Three-day-old cultures were filtered through a 4.25- $\mu$ m glass fibre filter and a 0.22- $\mu$ m filter to remove mycelium and then concentrated by dialysis against polyethyleneglycol through a 6000–8000 molecular-weight cut-off membrane (Spectra/Por). Such culture filtrates contain a variety of PG isoforms (Sharrock and Labavitch, 1994).

Plant tissue extracts were assayed for *B. cinerea* PG inhibiting activity. Finely crushed tissue (0.15 g, from fully expanded young leaves of 4–6-month-old grapevine plants) was homogenized in 1 mL extraction buffer consisting of 1 M NaCl, 0.1 M sodium acetate buffer (pH 5). The homogenate was centrifuged at 16 000 g for 5 min. Total protein concentration of the supernatant was determined using the Bradford (1976) reagent. Inhibition of PG activity from culture filtrates of *B. cinerea* was determined by a radial diffusion assay in agarose (Taylor and Secor, 1988). The same test was used to determine PG activity in petiole extracts of plants infected with *X. fastidiosa*. In addition, more quantitative PGIP activity measurements of leaf extracts were based on the PG assay of Gross (1982), which measures the generation of reducing groups released from polygalacturonic acid (PGA). For this assay, 1 mL of 0.2% PGA in a buffer containing 50 mM sodium acetate, 10 mM EDTA (pH 5) was incubated with 10  $\mu$ L *B. cinerea* PG (75 nmol/min mL)  $\pm$  leaf extract. D-Galacturonic acid was used as standard. One unit (1 U) of PGIP activity was defined to be that amount of leaf extract that reduced PG activity by 50%. For Western analysis, extracted proteins were precipitated with two volumes of cold acetone, separated on a 10% polyacrylamide gel containing sodium dodecyl sulphate (SDS-PAGE), and electro-transferred to a membrane for the detection of PGIP with polyclonal antibodies developed using deglycosylated pear PGIP as an antigen (Stotz *et al.*, 2000). Secondary antibodies, conjugated with horseradish peroxidase, were used to visualize the reaction by chemiluminescence (Perkin Elmer). Detection of *X. fastidiosa*-PG was performed using polyclonal antibodies raised against a C-terminal fragment of CVC *X. fastidiosa* PG, expressed in *E. coli* and secondary antibodies conjugated with alkaline phosphatase (Harakava *et al.*, 2001).

Herbaceous stems of the scion were cut ~10 cm above the graft union or ~10 cm above the crown in 'own rooted' grapevines to collect xylem exudate. The cut surface was washed several times with deionized water and then allowed to bleed for 15 min. The exudate was collected in Eppendorf tubes on ice and

filter sterilized through a 0.22- $\mu$ m filter before further analysis. Xylem exudate was also extracted by pressure displacement using a pressure chamber. In addition, xylem exudate was collected from scions (~10 cm above the graft union) of four types of grafted plants: (1) untransformed scion on transgenic roots and (2) the reciprocal, (3) untransformed scion on untransformed roots and (4) transgenic scion on transgenic roots, and assayed for PGIP activity.

### Botrytis inoculation

Ten lines of 'Thompson Seedless' and eight lines of 'Chardonnay', both including lines with high (TS-50, TS-60, TS-74, TS-79, TS-106, TS-254, CH-314, CH-316, CH-321, CH-329 and CH-330), moderate (TS-56, TS-75, TS-82, CH-326 and CH-327) and low (TS-46 and CH-20) PGIP activity, were inoculated with conidia of *B. cinerea* strain Del 11 grown on PDA medium. Conidia were harvested from sporulating cultures by washing with sterile modified Pratt's medium and filtering through cheesecloth. Leaves corresponding to the 4th (Chardonnay) or 7th (Thompson Seedless) nodes from the apical meristems of untransformed and transgenic plants growing in the greenhouse were explanted, washed with 70% ethanol, rinsed with de-ionized water and dried on blotting paper. They were then placed in Petri dishes on plastic grids positioned over wet filter papers and inoculated with a suspension of spores by applying 8–10 droplets of conidial suspension ( $10^3$  conidia per drop) on the upper surface of each leaf. Petri dishes were incubated at 4 °C as described by Powell *et al.* (2000). Lesion growth was measured 8–9 days after inoculation in leaves obtained from 4–6 plants per line. Data were statistically analysed with a one-way analysis of variance (ANOVA) using SAS 6.1 (SAS Institute, Cary, NC) at a significance of  $P < 0.05$ . Mean values were compared using the Tukey test when statistically significant differences were detected.

### Xylella inoculation

Five lines of 'Thompson Seedless' and six lines of 'Chardonnay', both including lines with high (TS-50, TS-74, TS-201, CH-329 and CH-330), moderate (TS-56, TS-188, CH-168 and CH-327) and low (CH-248 and CH-251) PGIP activity, were inoculated with *X. fastidiosa* bacterial suspensions. Five to six plants of each line were mechanically inoculated 10–20 cm above the soil level with 20- $\mu$ L droplets ( $10^8$  cfu/mL) of the Temecula strain using a pin-prick technique (Hopkins, 1980). At least two additional plants were treated with *X. fastidiosa*-free PBS or left untreated. Untransformed plants were subjected to the same treatments. Plants were pruned twice. The first pruning was carried out 10 weeks after inoculation, cutting back the main shoot to 90 cm above the point of inoculation. The second pruning was per-

formed 28 weeks after inoculation, by cutting back the main shoot to the base. *X. fastidiosa* infection was confirmed by ELISA (Krivanek and Walker, 2004). Hydraulic conductivity was measured on a 20-cm-long stem segment 40 cm above the POI as described by Schultz and Matthews (1993).

### Author recommended internet resources

International grape genome programme: <http://www.vitaceae.org>

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