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ORIGINAL ARTICLE

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Expression of the grapevine stilbene synthase gene *VST1* in papaya provides increased resistance against diseases caused by *Phytophthora palmivora*

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Abstract The phytoalexin resveratrol (trans-3,5,4′-trihydroxy-stilbene), a natural component of resistance to fungal diseases in many plants, is synthesized by the enzyme trihydroxystilbene synthase (stilbene synthase, EC 2.3.1.95), which appears to be deficient or lacking in susceptible plants. Earlier workers isolated a stilbene synthase gene (*Vst*1) from grapevine (*Vitis vinifera* L.), which has subsequently been introduced as a transgene into a range of species to increase resistance of hosts to pathogens to which they were originally susceptible. Papaya (*Carica papaya* L.) is susceptible to a variety of fungal diseases, including root, stem, and fruit rot caused by the pathogen *Phytophthora palmivora*. Since resveratrol at 1.0 mM inhibited mycelium growth of *P. palmivora* in vitro, we hypothesized that papaya resistance to this pathogen might be increased by transformation with the grapevine stilbene synthase construct pVst1, containing the *Vst*1 gene and its pathogen-inducible promoter. Multiple transformed lines were produced, clonally propagated, and evaluated with a leaf disk bioassay and whole plant response to inoculation with *P. palmivora*. RNA transcripts of stilbene synthase and resveratrol glycoside were induced in plant lines transformed with the grapevine pVst1 construct shortly after pathogen inoculation, and the transformed papaya lines exhibited increased resistance to *P. palmivora*. The immature transformed plants appear normal and will be advanced to field trials to evaluate their utility.

Keywords *Carica papaya* · Genetic transformation · Phytoalexin · Resveratrol (trans-3,4′,5-trihydroxy-stilbene) · Root rot

Abbreviations BA: Benzyladenine · 2,4-D: 2, 4-Dichlorophenoxyacetic acid · IBA: Indolebutyric acid · MS: Murashige and Skoog plant culture medium · NAA: Naphthaleneacetic acid

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Introduction

Papaya (*Carica papaya* L.), one of the more important fruit crops of the tropics, has limited resistance to a range of fungal pathogens that may kill the plants or, at a minimum, reduce productivity and fruit quality (Nishijima 1994). Viral diseases, such as that caused by the papaya ringspot virus (PRV), are even more deadly. A severe epidemic of PRV disease in the early 1990s nearly destroyed Hawaii's papaya industry until a high level of resistance to the local strain of PRV was obtained through genetic transformation of the papaya cultivar 'Sunset' with a PRV coat protein gene to produce 'SunUp'. Transgenic SunUp was crossed with the more widely grown cv. Kapoho to produce the hybrid cv. Rainbow, which is now widely grown in Hawaii for production of papayas in the presence of PRV (Gonsalves 2002). However, because the high susceptibility of cv. Sunset to *Phytophthora palmivora* was transmitted to the transgenic hybrid Rainbow, serious threats of yield loss due to damping off, root rot, fruit rot, and stem rot diseases remain. Severe decline and death of papaya trees due to root rot in poorly drained areas has been attributed to *P. palmivora* (Nishijima 1994). Heavy yield losses are frequently caused by this pathogen under less severe conditions, especially during the cool, rainy, winter season. Simultaneous control of both PRV and fungal

pathogens is needed to decrease dependence on fungicides, increase productivity, and improve pre- and post-harvest fruit quality.

Phytoalexins have been shown to be important natural components in the defense of plants against fungal infection (Jeandet et al. 2002; Kuc 1995). Several crops, including grapevine and peanut, synthesize the stilbene-type phytoalexin resveratrol (trans-3,4′, 5-trihydroxystilbene) when attacked by pathogens (Langcake and Pryce 1977). Stilbene synthesis is induced in grapevine by inoculation with the pathogens *Botrytis cinerea* or *Plasmopara viticola* (Blaich and Bachmann 1980; Langcake 1981). The subsequent level of resistance to *P. viticola* was positively correlated with the capacity of grapevine to synthesize stilbene (Dercks and Creasy 1989). Resveratrol is synthesized by the enzyme trihydroxystilbene synthase (stilbene synthase or resveratrol synthase) (EC 2.3.1.95), using as substrates one molecule of 4-coumaroyl-CoA plus three molecules of malonyl-CoA (Rupprich and Kindl 1978). Since these two substrates are commonly present in plants, introduction of a single gene encoding stilbene synthase may be sufficient to synthesize resveratrol in heterologous plant species for increased resistance against fungal pathogens.

Stilbene synthase genes isolated from grapevine have been transformed, with variable results, into tobacco (Hain et al. 1993), oil-seed (Thomzik 1993), tomato (Thomzik et al. 1997), rice (Stark-Lorenzen et al. 1997), barley and wheat (Fettig and Hess 1999; Leckband and Lorz 1998), kiwi (Kobayashi et al. 2000), alfalfa (Hipskind and Paiva 2000), apple (Szankowski et al. 2003), and a grapevine rootstock (Coutos-Thevenot et al. 2001). A significant increase in resistance to the pathogen *B. cinerea* was reported for stilbene synthase-transformed tobacco plants (Hain et al. 1993). In transformed tomato plants, stilbene synthase produced a 65% reduction in disease incidence following inoculation with *Phytophthora infestans*, but susceptibility to the pathogens *Alternaria solani* and *B. cinerea* was not decreased (Hipskind and Paiva 2000; Thomzik et al. 1997). Similarly, the stilbene synthase gene did not enhance resistance against *B. cinerea* in transgenic kiwi (Kobayashi et al. 2000). However, resistance against *B. cinerea* was enhanced in transgenic barley and wheat plants (Leckband and Lorz 1998). Rice plants transformed with the stilbene synthase gene exhibited enhanced plant resistance against the rice blast fungal pathogen *Pyricularia oryzae* (Lorenzen et al. 1997). Transgenic alfalfa had significantly increased resistance to *Phoma medicaginis* (Hipskind and Paiva 2000). Thus, although pathogen control by transgenic stilbene synthase enhancement of resveratrol can be significant, it cannot be predicted, and so must be evaluated empirically.

Herein, we report results of experiments to evaluate the stilbene synthase gene *Vst*I from grapevine under the control of its own inducible promoter (Hain et al. 1993) in increasing resistance of transgenic papaya to disease caused by *P. palmivora*.

Materials and methods

Plant materials and transformation procedure

Previously published procedures (Fitch et al. 1990) were used for producing papaya somatic embryogenic cultures from seedling hypocotyls of papaya cv. Kapoho. The embryogenic tissues were co-transformed with a plasmid construct containing the stilbene synthase gene *Vst*1 from grapevine (Hain et al. 1993) under the control of its own pathogen-inducible promoter plus a selectable marker plasmid construct containing the hygromycin resistance gene (*hpt*) driven by the cauliflower mosaic virus (CaMV) 35S promoter. Particle bombardment was used for gene insertion with conditions optimized for papaya embryogenic callus (Fitch 1993). After a 10-day recovery period for the callus in induction medium without antibiotic selection, tissues were transferred to selection medium containing 50 mg l^{-1} hygromycin. Bombarded papaya tissue cultures were cultured for 3 months with subcultures transferred at 3- to 4-week intervals or until hygromycin-resistant callus was selected and regenerated into plants using published methods (Fitch 1993).

Transgenic plant multiplication and growth conditions

Putative transformed plants were multiplied using the micropropagation methods of Fitch (1993). The cloned transgenic lines were grown in the greenhouse at 25°C and relative humidity ranging from 30 to 60% under natural daylight for about 3 months, at which time plants were subjected to molecular and pathological evaluation.

Genomic DNA extraction, PCR, and Southern blot analysis

The youngest fully expanded leaves of approximately 3-month-old greenhouse-grown papaya plants were excised, immediately frozen in liquid nitrogen, and stored in a −80°C freezer until extraction of genomic DNA. Genomic DNA was isolated by a published CTAB method (Doyle and Doyle 1990). PCR amplification of the *Vst*1 gene was carried out using a set of primers (GAC AGT TCC ACC TGC ATA G upstream 5′ primer and GAG GAA ATT AGA AAC GCT CAA CGT GCC downstream 3′ primer) specific for the *Vst*1 gene (based on Primer designer software, version 4; Sci Ed Central, Durham, N.C.). The PCR reactions were carried out in 50 µl volume consisting of 1 µl template DNA, 1 µl each primer (50 µmol), 4 µl dNTPs (2.5 mM each), 5 µl 10× *Taq* buffer (Promega), 1 U *Taq* polymerase (Promega), and 38 µl H_2O. The PCR reaction conditions used were: 30 cycles of 30 s denaturation at 94°C, 30 s primer annealing at 58°C, and 1 min primer

extension at 72°C. A final 5 min incubation at 72°C was allowed for complementation of the amplified products. The amplified PCR products were separated by electrophoresis in 0.8% agarose gel (Sambrook and Russell 2001).

Plants that tested PCR-positive for the stilbene synthase gene were examined by Southern analysis (Southern 1975) to confirm integration of the transgene. For Southern blot analysis, aliquots (30 μg) of genomic DNA were restriction digested overnight with *Eco*RI to release a 1.8 kb fragment containing the *Vst*1 gene, which was flanked by two *Eco*RI sites. Digested DNA was fractionated by electrophoresis in a 0.8% agarose gel followed by alkali-blotting onto Hybond N+ membranes (Amersham) according to the manufacturer's instructions. Blots were hybridized with a 1.8 kb *Vst*1 chemifluorescent probe labeled with AlkPhos Direct (Amersham) according to the manufacturer's instructions. The number of stilbene synthase genes inserted into the papaya genome was estimated from the number of unique genomic DNA fragments in restriction digests of transgenic lines.

Pathogen growth inhibition by resveratrol in vitro

Spore germination and mycelium growth of the papaya pathogens *P. palmivora* and *Colletotricum gloeosporiodes* and the soil blight pathogen *Phytophthora capicisi* were evaluated in an 8% V8 juice agar medium containing various concentrations of resveratrol according to procedures reported by Sela-Buurlage et al. (1993) and Coutos-Thevenot et al. (2001). Resveratrol (Sigma) solutions were solubilized in 1% ethanol, filter-sterilized, and added to V8 juice agar medium at 50°C to give final concentrations ranging from 0 to 1 mM. The control with 0 mM resveratrol was supplemented with an equal amount of ethanol. After the medium solidified, a small plug of agar from a 7- to 8-day-old pathogen culture was placed in the center of each Petri plate containing resveratrol-V8 juice agar medium. The diameter of pathogen mycelium was measured 4 days after inoculation. Relative mycelium growth was calculated using the 0 mM resveratrol control as 100%. The mycelium growth diameter was recorded as the average of three directional diameter measurements. Each resveratrol concentration was evaluated in three plates. Data presented are the means of two independent experiments.

Induction of stilbene synthase mRNA and northern blot analysis

Individual greenhouse-grown, 3-month-old papaya plants were thoroughly sprayed with 10 ml of a 1×10^4 spores ml^{-1} zoospore suspension of *P. palmivora*. The youngest mature inoculated leaves were harvested at 0, 5, 24, 48 and 72 h after spraying and stored frozen at −80°C until extracted for RNA analysis. For the 0 h time point, leaves were harvested immediately after the spray. The remaining plants were enclosed in clear plastic bags to maintain saturated relative humidity until harvest. For each time point, the single youngest mature leaves from three separate plants were pooled for RNA extraction. Fifteen plants were used for the five times of harvest. Total RNA was extracted from the inoculated leaf samples according to the methods of Bugos et al. (1995).

Total RNA (10 μ) from each pooled sample was separated on a 1.5% agarose formaldehyde denaturing gel, then blotted onto Hybond N+ membranes (Amersham) by capillary transfer. Hybridization and stringency washes were performed according to Sambrook and Russell (2001). The 1.8 kb *Vst1* DNA probe was ^{32}P-labeled by the random priming method of Feinberg and Vogelstein (1983).

Resveratrol analysis by HPLC

The frozen youngest mature inoculated leaves (see induction of mRNA) of transgenic line Vst7, along with control plants, harvested at 0, 5, 24, 48, and 72 h, were used for resveratrol analysis. Resveratrol analysis was carried out according to Jeandet et al. (1997), using an HPLC device (Shimadzu) equipped with a reverse phase C18 HPLC column (Waters, YMC Basic S-5, 4.6×250 mm). Metabolites were extracted from 0.5 g leaves with 80% methanol. The leaf sample was ground and vortexed vigorously at 37°C for 2 h in a thermo mixer (Eppendorf model 5436). The extract was centrifuged to remove insoluble debris. The supernatant was concentrated to dryness then dissolved in 100% methanol and filtered for HPLC analysis.

Enzymatic hydrolysis of the resveratrol glucoside using β-D-glucosidase (Sigma) was carried out according to Jeandet et al. (1997) with slight modification. After removal of methanol, the residue was dissolved in 25 mM citric acid/phosphate buffer (pH 5.2) and enzyme was added to a final concentration of 1 mg/ml. The mixture was incubated at 37°C for 16 h. Samples were concentrated, re-dissolved in methanol, and filtered before HPLC analysis. The identity of free resveratrol was confirmed by co-elution with a resveratrol standard (Sigma) and by comparison of UV absorbance spectra. Resveratrol glycoside contents were determined by measuring the free resveratrol after enzyme conversion, using a calibration curve of standard resveratrol. The quantification was carried out in triplicate. The resveratrol glycoside contents were expressed in microgram/gram fresh weight of resveratrol produced.

P. palmivora zoospore extraction and leaf-disk inoculation assay

The zoospore suspension was prepared from two or three Petri plates of *P. palmivora* culture grown on 8%

V8 juice agar at 25°C for 7–8 days. Sterilized water (5–10 ml) was placed on the surface of cultures to be harvested, and a spatula was used to gently rub the agar surface to dislodge the sporangia. The suspension solutions from all plates were pooled. An aliquot of suspension was placed in an Eppendorf tube that was shaken vigorously on a vortex mixer to induce zoospore encystment. The non-mobile spores were counted on a haemocytometer. The suspension concentration was adjusted to 1×10^4–1×10^6 spores ml^{-1} and used immediately for inoculation.

The youngest fully expanded mature leaves from five transgenic papaya lines were harvested, washed, and dried on a paper tower. Leaf-disks (20 mm in diameter) were excised carefully with a cork borer to avoid major veins from the sampled leaves. The leaf-disks were immediately placed in Petri plates containing water agar. Spore suspension (20 μ; 1×10^6 spores ml^{-1}) was pipetted onto the center of each leaf-disk and the Petri plates were placed in a growth chamber maintained at 24°C, 12 h light, and 100% relative humidity. Water-soaked spots on the leaf discs could be observed after 24 h; necrotic lesions appeared and expanded after that. Diameters of lesions on the leaf-disk were measured 3 days after inoculation. For each transgenic line, six disks from the youngest fully expanded leaf from three individual plants were pooled as 18 leaf-disks per line for each assay. The data presented are the means of three independent experiments conducted on different dates.

Greenhouse plant assay

Based on laboratory assessments and the availability of an adequate number of individual plants, the transformed line Vst7 was selected to evaluate whole plant response to *P. palmivora* inoculation under greenhouse conditions. Two separate experiments were conducted in a completely randomized design with ten plants for each treatment (*P. palmivora* zoospore suspension versus water control). Treatment consisted of a 10 ml inoculation adjacent to the stem of 3-month-old plants with either water or a zoospore suspension (10^4 spores ml^{-1}) of *P. palmivora*. Seedling response was scored 14 days after inoculation. A visual disease rating of 0 to 4 was given to each plant based on the following symptoms: 0 = healthy plant with no visual symptoms, 1 = showing slight leaf wilt or stress, 2 = severe leaf wilt, 3 = leaf abscission and stem wilt, and 4 = dead plant.

Data analysis

Data from lesion size and disease rating were analyzed using the general linear model (GLM) procedure of the SAS (Statistical Analysis System, Cary, N.C.). When treatment effects were significant ($P<0.05$), means were separated using the least significant difference (LSD) ($P=0.05$).

Results

Inhibition of mycelium growth by resveratrol

Resveratrol was active against mycelium growth of both *P. capsici* and *P. palmivora* at 0.5 mM. Mycelium growth of both *Phytophthora* spp. was inhibited to about 50% of the growth in control medium by 1.0 mM, the highest concentration tested (Fig. 1). Resveratrol at 1.0 mM was only slightly effective against the other major papaya pathogen, *C. gloeosporiodes*.

Transformation of papaya with the stilbene synthase gene *Vst*1

Twenty-eight plates of embryogenic callus derived from papaya cv. Kapoho were used for co-transformation of

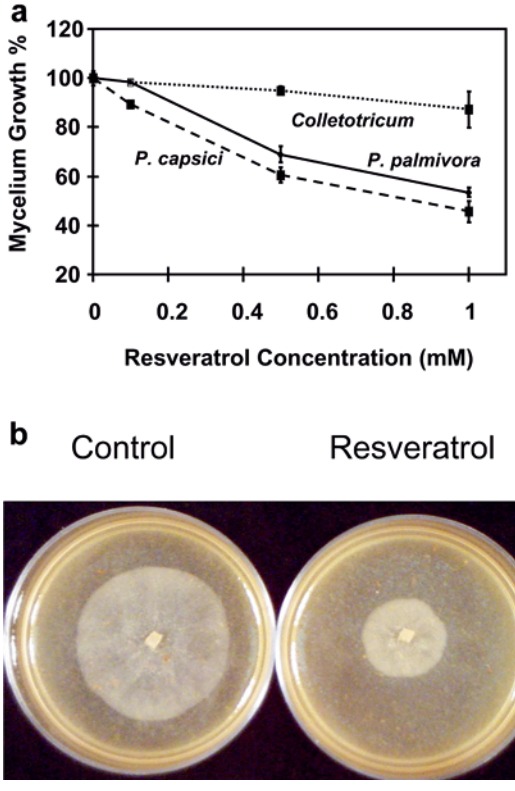

Fig. 1 **a** Mycelium growth of *Phytophthora palmivora*, *Colletotricum gloeosporiodes*, and *Phytophthora capisici* on culture medium containing trans-3,4′,5-trihydroxy-stilbene (resveratrol). Resveratrol was solublized in 1% ethanol (final concentration) in V8 juice agar. The control was supplemented with an equal amount of ethanol. The relative level of mycelium growth was calculated using control as 100%. Growth diameter value was the calculated average of three separate diameter measurements of the mycelium growth 4 days after inoculation. Data points are the means from two independent experiments. *Bars* 1 SD. **b** Representative *P. palmivora* mycelium growth on V8 juice agar medium containing resveratrol. The control plate medium contains 1% ethanol. The resveratrol plate contains 1.0 mM resveratrol in 1% ethanol. The photograph was taken 4 days after inoculation

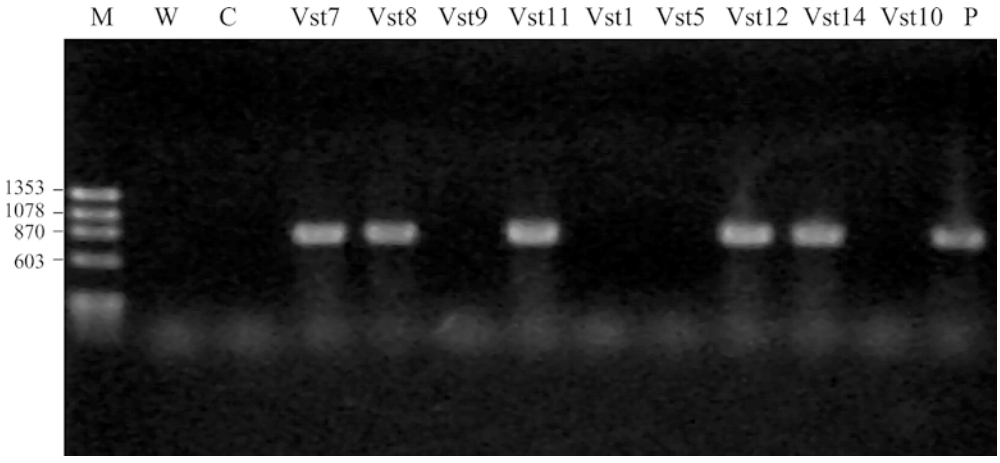

Fig. 2 Agarose gel electrophoresis of PCR amplification of the *Vst*1-specific fragment of DNA isolated from *Vst*1 transgenic papaya lines. Lanes: *M* Molecular size marker, *W* water, *C* non-transformed papaya, *P* plasmid control, *1–9* transgenic lines transformed with p *VST*1 constructs (1-Vst7, 2-Vst8, 3-Vst9, 4-Vst11, 5-Vst1, 6-Vst5, 7-Vst12, 8-Vst14, 9-Vst10)

the stilbene synthase gene *Vst*1 and the hygromycin resistance gene *hpt* through the biolistic delivery system. Thirty independent transgenic lines were selected by survival on hygromycin selection medium and confirmed by PCR using primers specific for the *hpt* gene. PCR amplified *hpt* gene was detected in all 30 selected lines. Therefore, for this experiment, the transformation rate for the hygromycin resistance gene is 1.1 lines per plate. Co-transformed stilbene synthase gene *Vst*1 was confirmed by PCR using *Vst*1 specific primers (Fig. 2) to amplify a unique 850 bp fragment. Out of 30 *hpt* positive lines, 16 are positive for the *Vst*1 gene, indicating the co-transformation rate for the stilbene synthase gene with the hygromycin gene is a little over 50%. Southern blot analyses showed that PCR positive plants contain between 1 and 10 copies of the *Vst1* transgene (Fig. 3). Two transformed lines, Vst12 and Vst14, derived from different bombarded plates, showed similar DNA restriction patterns; however, the intensity of the fragments was different, indicating that they might consist of differing numbers of tandem repeats inserted within the same locus.

Induction of stilbene synthase mRNA by inoculation with *P. palmivora*

Stilbene synthase mRNA was detected in the leaves of a Vst7 transgenic plant 5 h after inoculation with *P. palmivora* (Fig. 4). The accumulation of stilbene synthase mRNA reached a maximum after 24 h and declined thereafter to be barely discernible by 72 h.

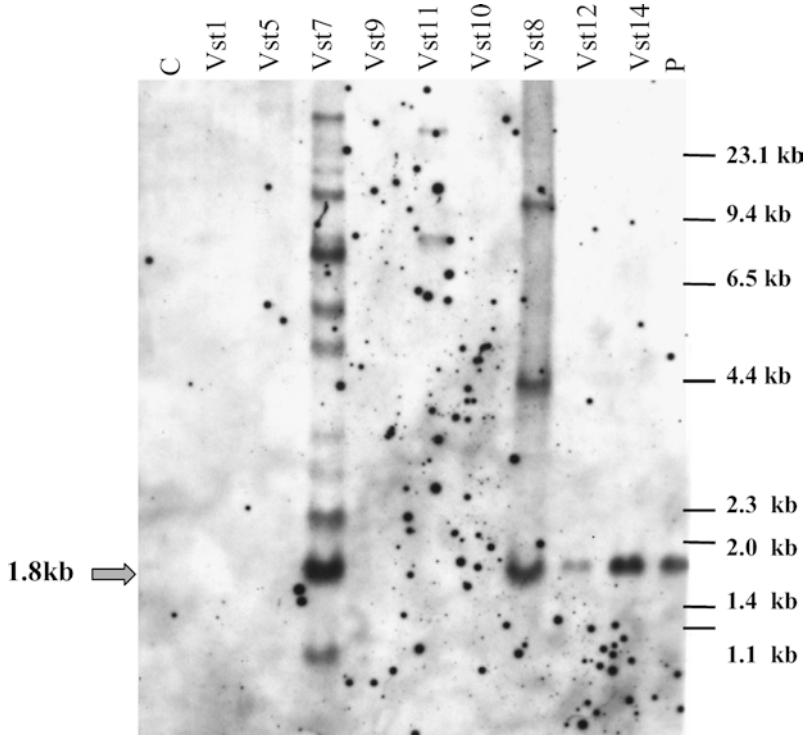

Fig. 3 Southern analysis of *Eco*RI-digested genomic DNA extracted from papaya leaves labeled with a 1.8 kb chemifluorescent stilbene synthase probe. Lanes: *C* Non-transformed control, *Vst1–Vst14* transgenic papaya lines transformed with the pVST1 construct, *P* plasmid DNA. Molecular sizes are indicated on the right

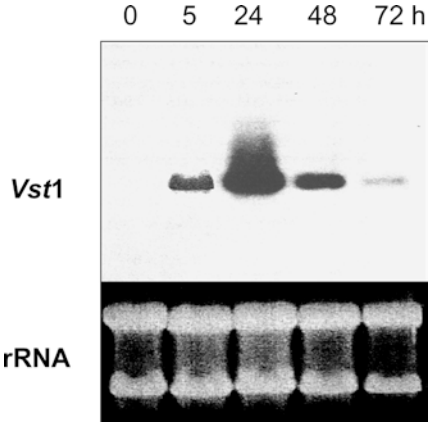

Fig. 4 Northern blot analysis showing stilbene synthase mRNA accumulation in leaves of a transgenic papaya line (Vst7) at 0, 5, 24, 48, and 72 h post-inoculation with spores of *P. palmivora*. For each time-point, leaves from three plants were pooled for RNA extraction with a total of 15 plants used for five time-points

Additional transgenic papaya plant lines, Vst8, Vst11, Vst12, and Vst14, showed a similar temporal response (data not shown) to *P. palmivora*, even though they have a different copy number of transgenes.

Resveratrol analysis

A sharp additional peak (retention time = 6.4 min) in the HPLC chromatogram was detected in the methanol extract from inoculated leaves of transgenic Vst7 plants, whereas extracts of the non-inoculated Vst7 and non-transformed control plants showed no additional peak. The retention time of the peak was earlier than that of the resveratrol standard (retention time = 11.5 min), suggesting the presence of resveratrol glucoside (Jeandet et al. 1997; Kobayashi et al. 2000). The enzymatic conversion of this peak to resveratrol, as confirmed by co-elution of standard resveratrol and UV absorption, confirmed that the additional peak is a resveratrol glucoside.

The time-course of resveratrol glucoside production after pathogen inoculation in transgenic Vst7, based on the amount of free resveratrol produced after enzymatic conversion is listed in Table 1. The resveratrol contents were detected in the plants 5 h after inoculation. Accumulation of resveratrol peaked at 54.2 ± 8.5 μg/g fresh weight 24 h after inoculation and declined after 48 and 72 h post inoculation. The resveratrol contents were correlated with the stilbene synthase mRNA accumulation induced by the pathogen (Fig. 4).

Table 1 Resveratrol (trans-3,4′,5-trihydroxy-stilbene) contents in the inoculated transgenic line Vst7 after enzymatic conversion from resveratrol glucosides. Resveratrol contents were determined by HPLC analysis showing resveratrol accumulation in leaves of a transgenic papaya line (Vst7) at 0, 5, 24, 48, and 72 h post-inoculation with spores of *Phytophthora palmivora*. For each time-point, leaves from 3 plants were pooled for RNA extraction with a total of 15 plants used for five time-points

Hours after inoculation	0	5	24	48	72
Resveratrol (mean ± SD) (μg/g fresh weight)	Not detected	23.5 ± 6.2	54. ± 8.5	31.2 ± 7.5	12.8 ± 6.3

Disease response to *P. palmivora* of *Vst*1 transformed papaya

Five independent transgenic lines, Vst7, Vst8, Vst11, Vst12, and Vst14, along with non-transformed control plants were selected for leaf-disk assay to assess tolerance of plants to *P. palmivora*. Spore suspensions (20 μl; 1×10^6 spores ml^{-1}) were pipetted onto the center of leaf disks cultured on MS medium in Petri plates. Plates were placed in a growth chamber maintained at 24°C with 12 h light and 100% relative humidity. Water-soaked spots were observed on the non-transformed controls within 24 h; necrotic lesions appeared later and proceeded to expand. Necrotic lesions were observed on all treated leaves except those inoculated with a water-extract from culture medium without the fungus. Diameters of lesions measured 3 days after inoculation were significantly smaller ($P < 0.05$) in all five *Vst*1 transformed lines than in the non-transformed controls (Fig. 5). On average, the lesions of the transgenic plants were reduced about 25–30% in diameter and about 40–50% in infection area. Cotton blue dye was used to stain the lesions and hand sections were made of the leaf tissue for microscopic confirmation that the lesions were associated with *P. palmivora* infection. Mycelium of *P. palmivora* were observed in every lesion.

Transgenic line Vst7 remained much healthier than did non-transformed control plants when challenged with *P. palmivora* zoospores in the potting mix (Table 2). The totals from two separate experiments showed 6/20 (30%) of the control plants developed root rot

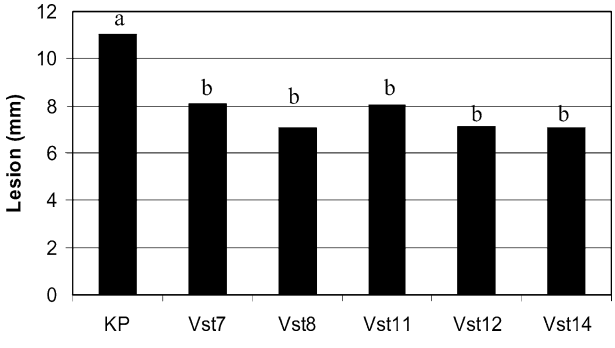

Fig. 5 Lesion size in diameter (mm) of *P. palmivora*-inoculated leaf disks of transgenic lines (Vst7, Vst8, Vst11, Vst12 and Vst14) and non-transformed control to evaluate tolerance to *P. palmivora*. Diameters of necrotic lesions were measured 3 days after inoculation. Data are means from three independent experiments. Values noted with different letters (a, b) are significantly different ($P \le 0.05$) by the least significant difference (LSD) test of SAS (Statistical Analysis System, Cary, N.C.)

Table 2 Disease rating of transgenic line Vst7 and non-transformed control papaya plants 14 days after *P. palmivora* inoculation. Vst7 and control lines were clonally multiplied, rooted, and maintained in the greenhouse. Evaluations were based on ten individual plants per replicate trial in a completely randomized design for each treatment (control and Vst7). Experiments were carried out in two separate trials. Inoculum consisted of *P. palmivora*zoospores suspensions (10 ml $\times 10^4$ zoospores ml^{-1}), applied to the plants as described in Materials and methods

Treatment	Trial	Number of plants in each disease rating[a]					Trial disease rating[b]	Treatment average[c]	Relative disease rating (%)
		4 dead	3 stem wilt	2 leaf wilt	1 slight wilt	0 healthy			
Control	1	4	0	1	2	3	2.0	1.95	100%
	2	2	2	1	3	2	1.9		
Transformed	1	0	1	1	4	4	0.9	0.7	36%
Vst7	2	0	0	1	3	6	0.5		

[a]Based on plant appearance, assigned 14 days after *P. palmivora* challenge:*0* no symptoms, healthy plant; *1* slight leaf wilt or stress; *2* leaf wilt; *3* leaf fall off and stem wilt, *4* dead

[b]Trial disease rating: number of plants at each rating × rating number/10 (the number of plants in that trial)

[c]Treatment average of disease incidence is the mean of two experimental trials

symptoms leading to death within 14 days, while none of the transgenic plants died during the same period. At the other extreme, 10/20 (50%) of the transgenic plants maintained a healthy appearance without any obvious root rot symptoms, while only 5/20 (25%) of the control plants remained healthy (Fig. 6). The average disease rating, on the 0–4 scale, of the Vst7 transgenic line was 0.7 compared to 1.95 for the non-transformed control. Over all disease ratings, the level of disease apparent in line Vst7 was only 35% of that of the controls.

Discussion

In vitro inhibition of pathogen mycelium growth by resveratrol was used to predict whether transgenic enhancement of resveratrol production by the *Vst*1 gene in papaya might have potential to increase resistance to the two important papaya fungal pathogens, *P. palmivora* and *C. gloeosporiodes*. At the highest concentration tested (1.0 mM), there was no inhibition of *Colletotricum* growth. The lack of inhibition of *Colletotricum* was similar to the low level reported against *A. solani* (Hipskind and Paiva 2000). On the other hand, growth inhibition of *P. palmivora* was 50% at the highest concentration of resveratrol. The fact that inhibition was not higher could be due to the low solubility of resveratrol, which resulted in its partial precipitation above 500 μM, even when 2% ethanol was used as the solvent (Adrian et al. 1997; Coutos-Thevenot et al. 2001). The 50% inhibition was similar to that at 1.0 mM concentration we found for inhibition of mycelium growth of the soil blight pathogen of tomato, eggplant and pepper, *P. capsici*, and as has been reported for the two grape pathogens *B. cinerea* and *Eutypa lata* (Coutos-Thevenot et al. 2001). Both grapevine and tobacco plants transformed with the *Vst*1 gene showed enhanced production of resveratrol and increased pathogen resistance (Hain et al. 1993; Thomzik et al. 1997). Our in vitro results coupled with published results on other plants indicated a good potential that transformation with the *Vst*1 gene might increase production of resveratrol in papaya sufficiently to inhibit the in vivo development of *P. palmivora* to achieve increased disease resistance. Because resveratrol showed such effective in vitro inhibition against *Phytophthora* and such low inhibition of *Colletotricum*, subsequent research focused on *Phytophthora*.

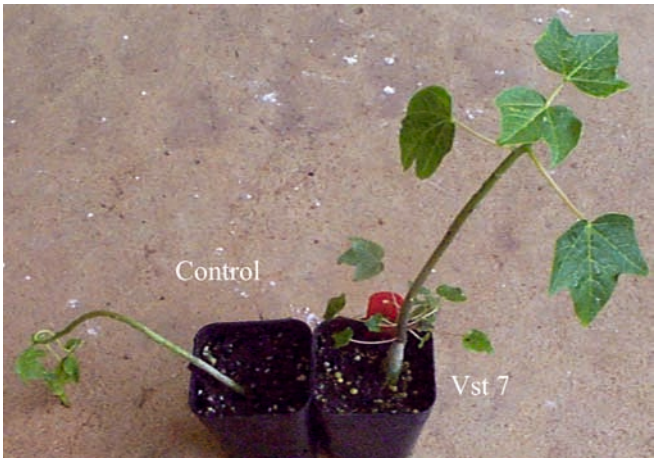

Fig. 6 Representative papaya plants challenged with root drenched *P. palmivora*. The control is a plant that went through the same tissue culture process as the transgenic plant but which was not bombarded for transformation. Vst7 is a papaya line transformed with the stilbene synthase gene. Inoculum consisted of *P. palmivora* zoospores suspensions (10 ml $\times 10^4$ zoospores ml^{-1}) as described in Materials and methods. Pictures were taken 14 days after *P. palmivora* challenge

Tobacco, tomato, and rice plants transformed with *Vst*1 under the control of its own promoter showed a rapid accumulation of both stilbene synthase mRNA and its product stilbene following inoculation with pathogenic fungi (Hain et al. 1990; Stark-Lorenzen et al. 1997; Thomzik et al. 1997). Likewise, our present results show that stilbene synthase mRNA was rapidly and transiently induced when *Vst*1-transformed papaya was challenged by the papaya pathogen *P. palmivora*. This temporal pattern indicates that the *Vst*1 promoter can

be activated by *P. palmivora*, and the transient nature of the expression implies that after the pathogen is suppressed or inhibited, the stilbene synthase mRNA returns to a pre-inoculation base level. No specific mRNA accumulation was detectable immediately after inoculation, indicating that there was little or no constitutive expression of stilbene synthase when *Vst*1 was under regulation by its own pathogen-inducible promoter. Papaya's lack of *Vst*1 mRNA at time zero confirms earlier studies that showed stilbene synthase mRNA accumulation had to be induced, whether by biotic stresses such as fungal attack, by the application of a fungal elicitor, or through abiotic stresses such as wounding or UV irradiation (Thomzik et al. 1997). It should be noted that elicitor application and UV irradiation produced only a relatively low accumulation of stilbene synthase mRNA compared to the quantity induced by wounding. In that work, the maximum increase was induced after inoculation with the pathogen. *Phytophthora infestans*. Our observation of the strong response of stilbene synthase mRNA to *P. palmivora* adds another case in which the promoter of *Vst*1 can be activated in a heterologous plant (papaya) by its particular fungal pathogen (*P. palmivora*). This suggests that the signal pathway of these defense-related genes may be conserved across a large group of plants. Because phytoalexins are toxic against a variety of pathogenic fungi, it is reasonable to assume that the described system might work in many untested host-pathogen interactions. However, since only certain fungal pathogens are inhibited by resveratrol it may be most efficient to first evaluate the in vitro effect of phytoalexins on pathogen growth before carrying out the more costly process of genetic transformation.

PCR amplification of the *hpt* gene in all 30 lines surviving selection on hygromycin-containing medium (data not shown) revealed a highly effective selection screen allowing no non-transformed escapes. Of the 30 lines testing positive for having the selection marker gene, 16 were also positive for the *Vst*1 gene. This indicates that the co-transformation rate through particle bombardment was about 50% in papaya, which is consistent with previously reported experiments on other plants (Chen et al. 1998). Southern blot analysis of the co-transformed lines showed that the majority of the multiple inserts were larger than the plasmid control. The occurrence of a smaller insert in the single line Vst 7 indicates a plasmid rearrangement to produce a smaller restriction fragment. The appearance of larger inserts in lines Vst 8, Vst 11, and Vst 17 could possibly result from incomplete digestion of the genomic DNA. However, our careful quantification of equal DNA gel loading and the similar, evenly-distributed digested DNA smear on the gel as visualized with ethidium bromide argues against this possibility. This leaves the suggestion that the presence of multiple copies of the transgene may have been the result of plasmid rearrangement prior to integration into the genome as is commonly reported with biolistic bombardment. Although the transgenic lines appear to have differing numbers of transgenes, they all showed a similar response in induction of *Vst*1 mRNA. This may indicate that some or even most of the insertions may not be functional.

The results from leaf disk assay indicated that *P. palmivora* is able to infect papaya leaf tissues and cause disease symptoms even though root rot and stem rot are the more common forms of expression in nature. The universal susceptibility revealed by this assay also indicates that the transgene product did not prevent fungal infection but only slowed the pathogen growth. This conclusion is consistent with the results of the in vitro pathogen inhibition assay in which the pathogen was not killed but only restricted in growth.

Resveratrol is a naturally occurring compound present in several consumed fruits and vegetables and thus should be acceptable in fruit of transformed papaya plants. Moreover, resveratrol may have beneficial effects on human health, such as inhibition of platelet aggregation (Bertelli 1998), vasorelaxing activity (Chan et al. 2000; Chen and Pace-Asciak 1996), and cancer chemopreventive activity (Jang et al. 1997).

On the other hand, overexpression of resveratrol has been reported to have detrimental effects on plants. In tobacco and petunia, overexpression of a transgenic stilbene synthase gene led to substrate competition between stilbene synthase and chalcone synthase and caused male sterility (Fischer et al. 1997). These problems might be overcome by transforming plants with a construct having a pathogen-inducible promoter so that stilbene synthase would be expressed only at a low basal level until there is a pathogen attack. Following a transitory rise, expression would be expected to return to a low level when the pathogen fails to establish. In our experiments, transcript of stilbene synthase rapidly accumulated 5 h after inoculation with *P. palmivora*, indicating that stilbene synthase expression is rather strong. It will be interesting to see whether stilbene synthase transformation will cause any detrimental effects on papaya development, especially under field conditions where various biotic and abiotic stresses might induce accumulation of stilbene synthase. A project is planned to self our present transgenic lines to produce T2 homozygous lines that will be evaluated for performance and disease reactions in field yield trials.

The resveratrol produced by transgenic stilbene synthase plants can be modified after synthesis by the addition of a hydrophilic moiety, possibly a sugar molecule, as has been described for *Vitis* (Waterhouse and Lamuela-Raventos 1994), kiwi (Kobayashi et al. 2000), alfalfa (Hipskind and Paiva 2000), and apple (Szankowski et al. 2003). Conflicting results were obtained when the biological activities of such glycosides were tested against pathogenic fungi in transgenic plants. In transgenic kiwi plants, the production of resveratrol or the glycoside trans-resveratrol-3-*O*-β-D-glycopyranoside (piceid) did not increase resistance against *B. cinerea*, probably due to the derivation of resveratrol (Kobayashi et al. 2000). In contrast, transgenic alfalfa accu-

mulating piceid after the introduction of a stilbene synthase showed a significant reduction in the size of necrotic lesions following infection with the pathogen *P. medicaginis* (Hipskind and Paiva 2000). In addition, agar plate assays indicated that the glycoside was also effective against this fungus (Hipskind and Paiva 2000). Our results showed that resveratrol produced in transgenic papaya was modified to a resveratrol-glycoside, possibly by the action of the endogenous glucosyltransferase. Although, we have not yet identified its structure, we have demonstrated production of resveratrol glucoside in transgenic plants inoculated with *P. palmivora* zoospores and correlated this production with mRNA levels of stilbene synthase. The results of leaf-disk and greenhouse inoculation assays indicate that transgenic papaya plants with induced reservatrol glucoside exhibit improved resistance to *P. palmivora*.

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