

Increased Disease Resistance in Papaya by Transforming a Pathogen-inducible Stilbene Synthase Gene

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Abstract

Phytoalexins have been shown to be important natural components in the defense of plants against fungal infection. Resveratrol is a major phytoalexin in grapevine. We conducted in vitro pathogen inhibition assays to show that Resveratrol inhibited fungal pathogens of papaya. Resveratrol at 1.0 mM in V8 agar culture medium inhibited mycelia growth of *P. palmivora* up to 50% of control. The compound was active against *P. palmivora* as low as 100 µM. Resveratrol was not as effective against the anthracnose pathogen, *Colletotrichum gloeosporioides*. We transformed papaya embryogenic cultures with the *stilbene synthase* gene cloned from grapevine and driven by its own inducible promoter along with the hygromycin resistance or kanamycin resistance gene under the control of a CaMV35S promoter. The presence of transgenes was confirmed by PCR and Southern blot. Twenty lines of transgenic plants were propagated from tissue cultures for greenhouse assays. Transgenic papaya plants were challenged with oomycete pathogen in greenhouse conditions. Data from greenhouse studies showed that the disease level in transgenic plants was reduced to 35% of the disease level in non-transformed control plants. Papaya lines produced with increased resistance to diseases became available for crossing with SunUp to produce hybrids that have resistance to both virus and fungal diseases.

INTRODUCTION

Papaya, one of the most important fruit crops in the tropics, is susceptible to a variety of pathogens including fungi, bacteria and viruses (Nishijima, 1994) that reduce yields and marketability of fruit. Since 1992, an epidemic of papaya ringspot virus (PRV) has severely curtailed production of papaya in Hawaii. However, genetic transformation of the papaya cultivar, 'Sunset', with the PRSV coat protein gene provided it and its hybrid, 'Rainbow', with a high level of resistance to the Hawaiian strain(s) of PRSV. PRSV resistant hybrid ('Rainbow') seeds have been produced and made available to Hawaii growers. Thus the critically immediate problem of PRSV susceptibility has been solved. However, the diseases caused by *Phytophthora* (damping off, root rot, fruit rot, stem rot) and *Colletotrichum* (anthracnose), *Oidium caricae* (powdery mildew) and more recently *Asperisporium caricae* (black leafspot) remain serious threats to production of papaya, especially for the transgenic SunUp, which is highly susceptible to these fungal pathogens. Simultaneous control of both PRSV and fungal diseases would decrease dependence on fungicides and significantly improve pre- and post-harvest fruit quality and increase productivity.

Phytoalexins have been shown to be important natural components in the defense of plants against fungal infection. Several crops, including grapevine and peanut synthesize the stilbene-type phytoalexin, Resveratrol, (trans-3,4', 5-trihydroxy-stilbene) when attacked by pathogens. Stilbene synthesis was induced in grapevine by inoculation with the pathogens *Botrytis cinerea* or *Plasmopara viticola* (Blaich, 1980). The level of

resistance to *P. viticola* was positively correlated with the capacity of *Vitis spp.* to synthesize stilbene (Dercks and Creasy, 1989).

Stilbene synthase genes were isolated from grapevine and transformed into tobacco (Hain et al., 1993), oil-seed rape (Thomzik, 1993), tomato (Thomzik et al., 1997), potato (Stahl et al., 1994), rice (Lorenzen et al., 1997), barley and wheat (Brauer et al., 1997). A significant increase in resistance to the pathogen *B. cinerea* was reported for transgenic tobacco plants expressing grapevine stilbene synthase genes (Hain et al., 1993). The effectiveness of stilbene synthase genes in increasing resistance in transgenic tomatoes was shown by a 65% reduction in disease incidence following inoculation with the pathogen *Phytophthora infestans* (Thomzik et al., 1997). Results from transgenic rice plants indicated that the stilbene synthase gene enhanced plant resistance against the rice blast fungal pathogen *Pyricularia oryzae* (Lorenzen et al., 1997). In transgenic barley and wheat plants, resistance against infections caused by *Botrytis cinerea*, *E. graminis* and *H. terres* were also enhanced (Brauer et al., 1997).

We produced transgenic papaya with a transformation construct that contains the *stilbene synthase* gene from grapevine under control of its own inducible promoter and a hygromycin- or kanamycin- resistance gene under the control of a CaMV35S-promoter (Hain et al., 1993). Transgenic papaya, Solo Kapoho variety was transformed using the biolistic delivery system. The transgenic papaya plants were shown to be improved broadly for resistance against papaya pathogenic fungi.

MATERIALS AND METHODS

Plant Materials and Transformation Procedure

Standard procedures (Fitch et al., 1992) were used for producing somatic embryogenic cultures, which were subjected to biolistic microprojectile transformation. The embryogenic tissues were co-transformed with a selection marker gene, hygromycin or kanamycin resistance gene, by particle bombardment using conditions optimized for papaya embryogenic callus (Fitch et al., 1993). After a 10-day recovery period for the callus in induction medium without antibiotic selection, tissues were transferred to the selection medium containing up to 100 mg/l kanamycin or 50 mg/l hygromycin, based on the papaya killing curves that were generated (Figs. 2 and 3). Bombarded papaya tissue cultures were cultured in the dark for 3 months with subcultures transferred at 3-week intervals or until hygromycin- or kanamycin- resistant callus could be selected. Antibiotic-resistant calli were regenerated into plants using the methods of Fitch et al. (1992).

Regeneration of Plants

Regenerated transformed plants containing the stilbene synthase transgene were micropropagated according to Fitch et al. (unpublished), then grown in the greenhouse until ready for pathology evaluations.

PCR and Southern Blot Analysis

The putative transformants were screened for the presence of the transgene by PCR and Southern blot analysis, according to protocols used routinely in our laboratory. Transformants positive for the stilbene synthase gene based on the PCR results were screened by Southern analysis to confirm proper integration of the transgene. Southern blot analyses were performed on genomic DNA from leaves of transgenic plants using a stilbene synthase gene fragment as a probe. The maximum number of stilbene synthase genes inserted into the papaya genome was estimated from the number of DNA bands unique to the transgenic plants in restriction digests of their genomic DNA.

In Vitro Pathogen Inoculation

Inhibition assays of spore germination and mycelia growth of these fungal pathogens were performed according to procedures reported in the literatures (review see

Kuc, 1995). In vitro antifungal assays for *Phytophthora* and anthracnose were carried out in a V8 agar medium.

Fungal Resistance Testing of Transgenic Plants in a Greenhouse

Following the laboratory assessments, selected transformed lines were further evaluated for resistance in greenhouse studies. Disease resistance assessments in the greenhouse were conducted using standard inoculation and disease evaluation procedures. For *Phytophthora*, inoculum consisted of *P. palmivora* zoospore suspensions (10^4 zoospores/ml). Seedling mortality was used to estimate resistance. For powdery mildew, papaya seedlings were inoculated by dusting conidia from diseased plants onto the test seedlings. The disease symptoms on these challenged seedlings will continue to be evaluated over a 3 to 6 month period.

RESULTS

In Vitro Pathogen Inoculation

We conducted in vitro pathogen inhibition experiments with Resveratrol in the culture medium to test its inhibition effects on papaya fungal pathogens (Fig. 1). Resveratrol at 1.0 mM in the V8 agar culture medium inhibited mycelia growth of *P. palmivora* to 50% of control. The compound was shown to be active against *P. palmivora* at concentrations as low as 0.1 mM. But Resveratrol was not as effective against the anthracnose pathogen, *Colletotrichum gloeosporioides*. At 1.0 mM concentration, Resveratrol inhibited *Colletotrichum* by only 10%. The growth of *Phytophthora capsici* was also significantly inhibited by Resveratrol.

Transformation of Papaya

The levels of the antibiotics, geneticin or hygromycin for killing non-transformed papaya embryogenic calli were determined by measuring fresh weight increase after two months growth on media containing antibiotics with monthly subcultures (Figs. 2 and 3). Between 50 and 100 mg/l geneticin or 20 to 50 mg/l hygromycin appeared to be effective for selection of transgenic lines.

Embryogenic tissues of Kamiya and Kapoho varieties were transformed with the stilbene synthase gene, along with the kanamycin or hygromycin resistance selectable marker gene by particle bombardment using conditions previously optimized for papaya embryogenic callus. Thirty independent transgenic lines were selected by survivals on kanamycin or hygromycin selection medium. Transformation was confirmed by PCR and by Southern analysis to confirm proper integration of the transgene. Southern blot analyses were performed on genomic DNA from leaves of transgenic plants using a stilbene synthase fragment as a probe. The transformed plants are being rooted and micropropagated for evaluation for fungal resistance in greenhouse and field.

Evaluation of Fungal Resistance of Transgenic Papaya

Twenty individual plants of each transgenic line with the stilbene synthase gene were produced to screen for levels of fungal resistance. Because of our low success in rooting and hardening of plants from tissue culture, we transplanted individual transgenic plants to the field and produced F1 seedlings for fungal resistance evaluation. Twenty plants from one of transgenic lines were produced and challenged with root drench inoculation with zoospores of *Phytophthora palmivora* and left for disease development. Results are shown in Figure 4 and Table 1.

Transgenic papaya plants transformed with stilbene synthase performed much better than control plants after being challenged with *P. palmivora* zoospores in the putting mix. Out of 20 plants (Table 1), 6 control plants developed root rot symptoms and died after 14 days, while none of transgenic plants died during the same period. 10 out of 20 transgenic plants remained as healthy plants without any obvious root rot symptoms, while only half of amount of control plants remained healthy. Overall, the disease

incident of transgenic plants is significantly reduced to 75% of the control plants.

DISCUSSION

Tobacco and tomato plants transformed with the grapevine stilbene synthase genes showed a rapid accumulation of both stilbene synthase mRNA and its product, stilbene, after inoculation with *B. cinerea* or *P. infestans*. The transfer of grapevine (dicot) stilbene synthase genes to rice (monocot) also resulted in a rapid accumulation of stilbene-synthase-specific mRNA after inoculation with a fungal pathogen. These results indicated that the stilbene synthase gene, under the control of its own promoter, behaves the same way in unrelated transformed plants just as in the host from which it was isolated. These results prove that stilbene synthase can be induced in heterologous plant systems to broadly increase resistance to fungal pathogens. Hence the signal pathway of these defense-related genes is apparently 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 would work in many host-pathogen interactions, such as papaya and its fungal pathogens.

Stilbene biosynthesis specifically depends on the product of the stilbene synthase gene since the precursor molecules for the formation of hydroxy-stilbenes, malonyl-CoA and *p*-coumaroyl-CoA, are both commonly present in plants. The end product of the action of the enzyme on these precursors is stilbene, a natural compound present in several consumed fruits and vegetables, which should be acceptable in papaya fruit. Furthermore, using a gene with a pathogen-inducible promoter means that stilbene synthase should be expressed only at a low basal level in transgenic plants until there is a pathogen attack. Following a transitory rise in expression, the expression is expected to return to a low level when the pathogen fails to establish. We are planning to carry out northern blot analyses on transgenic papaya with stilbene synthase gene under its own promoter to test the hypothesis of inducible expression of this resistance gene.

In summary, we reported that transgenic papaya plants with stilbene synthase gene have been produced. Greenhouse inoculation with *P. palmivora* indicated that transgenic papaya has reduced disease symptom by 75% reduction compared to control. Plants showing significantly increased resistance to fungal pathogens in greenhouse experiment will be selfed and crossed with PRV resistance cultivars used in other breeding programs.

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Tables

Table 1. Disease rating of transgenic plant inoculated with *P. palmivora*.

	Disease Rating				
	4 (dead)	3	2	1	0 (healthy)
Control	6	2	2	5	5
Transgenic	0	1	2	7	10

P. palmivora inoculum at 1 000 zoospores/ml x 5 ml per plant.

Disease rating was evaluated on the 14th day after inoculation.

Figures

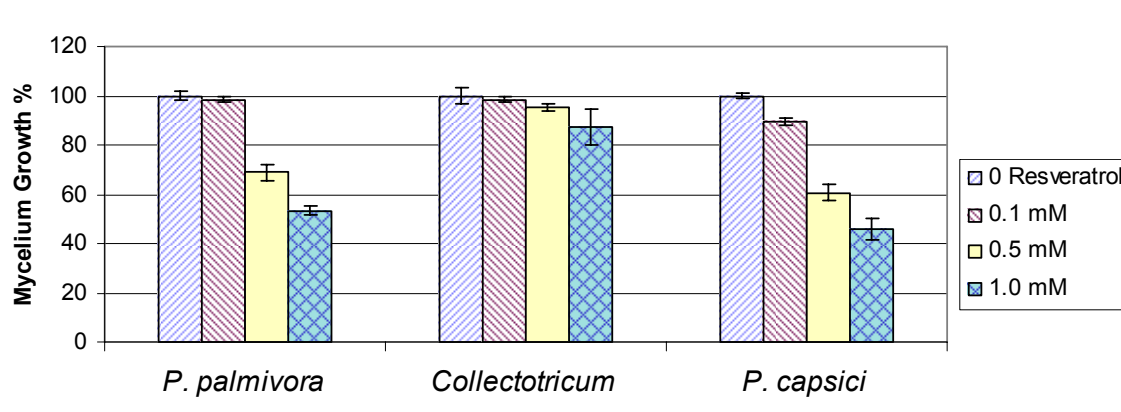


Fig. 1. Inhibition of mycelium growth by resveratrol.

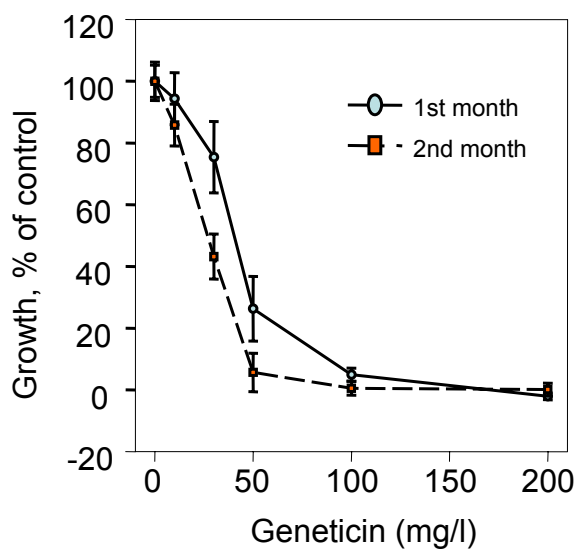


Fig. 2. Growth inhibition curves of papaya embryogenic calli on antibiotic geneticin medium.

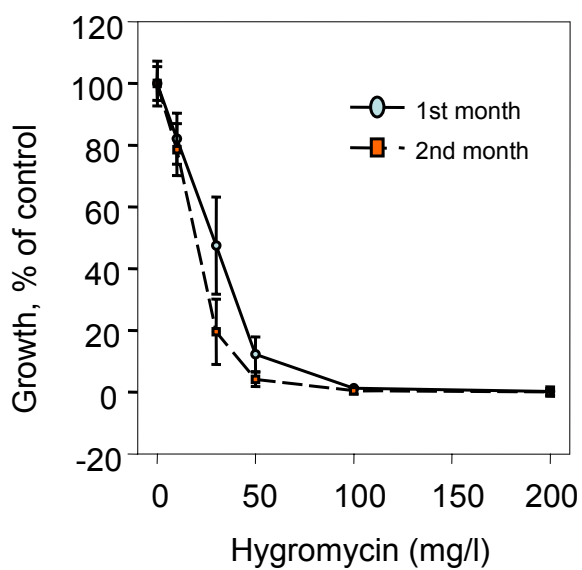


Fig. 3. Growth inhibition curves of papaya embryogenic calli on antibiotic hygromycin medium.

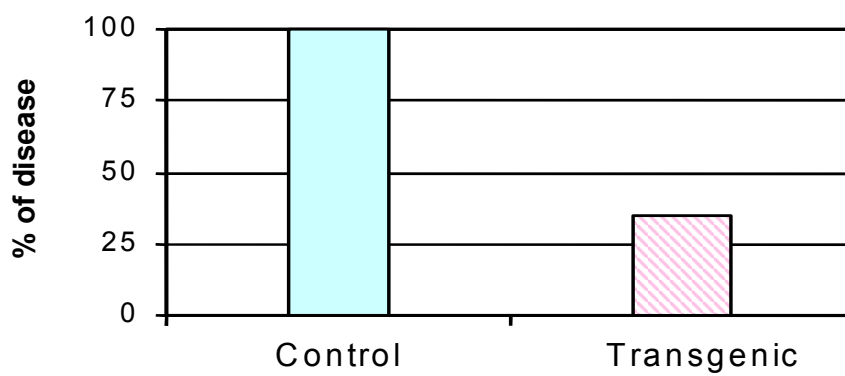


Fig. 4. Disease rating of transgenic papaya plant compared to untransformed control plants.