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Kiwifruits (*Actinidia deliciosa*) transformed with a *Vitis* stilbene synthase gene produce piceid (resveratrol-glucoside)

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Abstract A stilbene synthase gene was isolated from three *Vitis* spp. (*V. vinifera*, *V. labrusca* and *V. riparia*). These genes were placed under the control of the cauliflower mosaic virus 35S promoter and introduced into kiwifruit (*Actinidia deliciosa*) plants by *Agrobacterium*-mediated gene transfer. The introduced gene(s) were expressed and piceid (resveratrol-glucoside) rather than resveratrol was produced in the leaves of the transformants. Resveratrol produced by the action of the integrated gene(s) seems to be metabolized into piceid by an endogenous glycosyltransferase. Among the transformants obtained, the highest piceid content in the young leaves was 182 μg/g fresh weight. Although these transformants did not show resistance against *Botrytis cinerea*, which causes gray-mould disease, the fruits may have some beneficial effects on human health.

Key words Kiwifruit · Stilbene synthase · Transformant · Resveratrol · Piceid

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Introduction

Resveratrol (*trans*–3,5,4′-trihydroxystilbene) is a phytoalexin produced in several plants, including grapevine (Langcake and Pryce 1976), in response to fungal infection or UV irradiation. A correlation between the disease resistance of grapevine cultivars and their potential to produce this compound has been reported (Langcake and McCarthy 1979; Stein and Blaich 1985). Moreover, direct evidence of its biological activity has been shown by Adrian et al. (1997), who reported that resveratrol (60–140 μg/ml) reduced the germination of conidia of *Botrytis cinerea* and its mycelial growth. A clear relationship between resveratrol and disease resistance of the leaf has been demonstrated with transgenic tobacco as well (Hain et al. 1993). Resveratrol is synthesized by an enzyme, stilbene synthase, and the genes encoding this enzyme have been isolated from a grapevine cultivar (*Vitis vinifera* cv. Optima) and transferred into tobacco (Hain et al. 1993), rice (Stark-Lorenzen et al. 1997), barley (Leckband and Lörz 1998), and wheat (Leckband and Lörz 1998). The transgenic tobacco and barley showed an increase in resistance to *B. cinerea* (Hain et al. 1993; Leckband and Lörz 1998), and the transgenic rice also had enhanced resistance against *Pyricularia oryzae* (Stark-Lorenzen et al. 1997).

On the other hand, resveratrol and/or piceid (*trans*–3,5,4′-trihydroxystilbene-3-mono-D-glucoside) are considered to have some beneficial effects on human health. These compounds are the constituents of red wine which are known to reduce coronary heart-disease mortality, and possess activities preventing atherosclerosis, such as inhibition of platelet aggregation (Bertelli et al. 1995; Pace-Asciak et al. 1995; Shan et al. 1990), of low-density lipoprotein (LDL) oxidation (Frankel et al. 1993), and of eicosanoid synthesis (Kimura et al. 1985; Pace-Asciak et al. 1995). An anticancer effect of resveratrol has also been reported (Jang et al. 1997).

Resveratrol production was determined in transgenic tobacco, but it was not examined in the other transgenic plants. We report here that transgenic kiwifruit plants containing the stilbene synthase gene from grapevines produce piceid instead of resveratrol and do not show resistance against *B. cinerea*.

Materials and methods

Isolation of the stilbene synthase gene and vector construction

Total DNA was extracted from leaves of *V. vinifera* cv. Optima (resveratrol production: low), *V. labrusca* cv. Concord (resveratrol production: medium), and *V. riparia* (resveratrol production: high), as described by Kobayashi et al. (1996). Polymerase chain reaction (PCR) amplification was carried out using oligonucleotide primers designed for the 5′ untranslated region of pSV25 (a stilbene synthase cDNA clone isolated from *V. vinifera* cv. Optima; Melchior and Kindl 1990, 1991) adding a *Bam*HI site (5′-CCGGATCCGCTTCAATTTCATTACGT-3′) and its 3′ untranslated region with the addition of a *Sac*I site (5′-CCGAGCTCCTCCTATTTGATACATTA-3′). A reaction cycle of 94 °C for 30 sec, 55 °C for 30 s, and 72 °C for 2 min was repeated 35 times, followed by 72 °C for 10 min. Then, the PCR products were cloned into a pCR II vector (Invitrogen, Calif., USA). After confirmation of its sequence, the clones were called pVIN, pLAB, and pRIP, respectively. The stilbene synthase genes were then excised from the plasmids by *Bam*HI and *Sac*I digestion and integrated into a binary vector, pBI121 (Clontech, Calif., USA), from which the β-glucuronidase (GUS) gene sequence had been removed by the same digestion, to prepare a 35S promoter- stilbene synthase- nopaline synthase polyadenylation site construct. The recombinant plasmids were called pBSVIN, pBSLAB, and pBSRIP, respectively (Fig. 1).

Plant transformation

The chimeric genes (pBSVIN, pBSLAB, and pBSRIP) were transferred from *Escherichia coli* HB101 to the disarmed *Agrobacterium tumefaciens* strain LBA4404 by triparental mating using the conjugative *E. co1i* RK2013. Leaf disks and petioles of kiwifruit (*Actinidia deliciosa* cv. Hayward) were infected with the *Agrobacterium*, then transformants were selected on a solidified medium containing kanamycin (50 μg/ml), and plantlets were regenerated as described by Matsuta et al. (1993).

Southern blot analysis

Transgenic plants were first screened by the PCR method described above, and then total DNAs were isolated from the leaves of PCR-positive and control plants. Five micrograms of DNA was digested with *Bam*HI followed by *Eco*RI, and another 5 μg of DNA was single-digested with *Bam*HI at 37 °C for more than 5 h. Fragments were separated by electrophoresis in a 0.8 % agarose gel in TAE (40 mM Tris-acetate (pH 8.0), 1 mM EDTA) buffer and transferred to a nylon membrane. The stilbene synthase gene (1.5 kbp in size) from pVIN was used as a probe. The DNAs fixed on membranes were hybridized with the probe, which was labeled with digoxigenin (DIG) using a DIG labeling kit (Boehringer Mannheim, Germany). The hybridization lasted for 16 h at 68 °C in 5 × standard saline citrate (SSC), 0.5 % blocking reagent, 0.1 % *N*-lauroylsarcosine sodium salt, and 0.02 % sodium dodecyl sulfate (SDS). Membranes were washed twice in 2 × SSC, 0.1 % SDS at room temperature for 5 min each and then washed twice in 0.1 × SSC, 0.1 % SDS at 65 °C for 15 min each. Target DNA was detected as described by the manufacturer (Boehringer Mannheim, Germany).

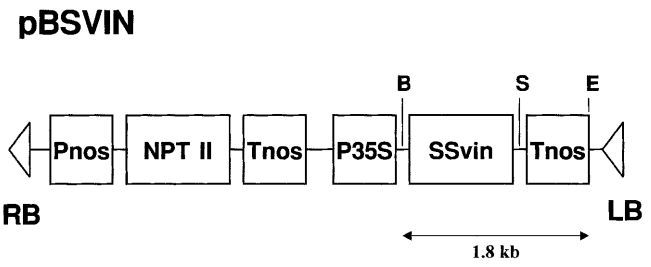

Fig. 1 Schematic map of the T-DNA region of pBSVIN (*RB* right border, *LB* left border, *Pnos* nopaline synthase promoter, *NPT II* neomycin phosphotransferase II, *Tnos* nopaline synthase terminator, *P35S* 35S promoter, *SSvin* stilbene synthase gene from *Vitis vinifera*, *B Bam*HI, *E Eco*RI, *S Sac*I)

Resveratrol analysis

Sample preparation and high-performance liquid chromatography (HPLC) analysis were carried out according to Jeandet et al. (1997), but a Shim-pack CLC-ODS column (Shimadzu, Kyoto, Japan) was used. One gram of young leaves of the same stage (5–7 cm in width) was collected from transformants and a non-transformed control plant, and stilbenes were extracted. Analysis was done in triplicate. The enzymatic hydrolysis of collected peaks and the quantification of piceid were also carried out as described by Jeandet et al. (1997).

Inoculation of *B. cinerea*

B. cinerea isolated from a grape berry was used as inoculum. Leaves from five selected transformants and a control plant were inoculated with spores of *B. cinerea* according to Nakamura et al. (1999). Ten inoculation sites were prepared using five to ten leaves in each plant and the experiment was repeated three times. Five days after inoculation, the diameter of the necrotic lesion was measured.

Northern blot analysis

Total RNA from the young leaves of selected transformants and the control plant was isolated using the SDS-phenol method (Sambrook et al. 1989) with modifications as described by Kobayashi et al. (1996). To recognize the expression of the introduced gene(s), 20 μg of total RNA was separated by electrophoresis in a 1.2 % agarose gel containing 0.66 M formaldehyde and then transferred to a nylon membrane. The RNA was hybridized with the same probe as used for Southern blot analysis. Hybridization lasted for 16 h at 42 °C in 5 × SSC, 50 % formamide, 50 mM sodium phosphate (pH 7.0), 2 % blocking reagent, 0.1 % *N*-lauroylsarcosine sodium salt, 7 % SDS, and 50 μg/ml salmon sperm DNA. Membranes were washed under the same conditions as the Southern blot analysis. Target RNA was detected as described by the manufacturer (Boehringer Mannheim, Germany).

Results and discussion

Isolation of stilbene synthase genes

Stilbene synthase genes are known to belong to a multi-gene family, and one of the genes, pSV25, has been shown by bacterial expression experiments to produce

a catalytically active stilbene synthase (Melchior and Kindl 1990). This gene is the most highly expressed among the six stilbene synthase genes tested (Wiese et al. 1994). Thus, we focused on pSV25 and tried to isolate the pSV25 homologous gene from three *Vitis* species differing in resveratrol production and disease resistance. The oligonucleotide primers designed for the 5′ and 3′ untranslated regions of pSV25 were used to amplify the pSV25 homologous gene from the *Vitis* species, and the cloned DNAs were sequenced. The nucleotide sequence of a cloned DNA (pVIN) from *V. vinifera* completely coincided with the pSV25 sequence, except for the fact that the pVIN contained an intron. From a comparison of pVIN and pSV25 sequences, the pVIN proved to have a 178 bp exon 1, a 358 bp intron, and a 998 bp exon 2. The sequences of the other DNA clones (pLAB from *V. labrusca* and pRIP from *V. riparia*) were highly homologous to the pVIN sequence (98 % in each case). The sequences of pLAB and pRIP differed from pVIN in 1 bp each within the exon 1, 10 or 12 bp in the intron (pRIP and pLAB: 359 bp), 15 or 16 bp in the exon 2, and 7 or 3 bp in the 3′ untranslated region, respectively. Deduced amino acid sequences were also highly homologous to each other, differing only in six amino acid residues between pVIN and pLAB and between pVIN and pRIP (Fig. 2). Wiese et al. (1994) had previously isolated a stilbene synthase gene, Vst1, from 'Optima' and determined its structure, including the intron. The deduced amino acid sequences of Vst1 and pSV25 were shown to differ slightly. A comparison of the sequence of pVIN intron with that of Vst1 intron (358 bp) indicated 88.2 % identity.

Transformation

Leaf disks and petioles of kiwifruit inoculated with *A. turmefaciens* were cultured on a selection medium. After approximately 2 weeks of culture, some calli formed at the cut ends of the segments, and adventitious buds differentiated from the calli after 1–2 months of culture. When the adventitious buds had developed into shoots of approximately 5 mm in length, they were cut off the calli and transferred to a medium for plant regeneration. One plant was selected from each segment and proliferated by subculturing on the same medium. Nineteen plants were regenerated from 141 treated segments inoculated with pBSVIN, 21 / 122 from pBSLAB, and 24 / 117 from pBSRIP, respectively.

DNA detection

The presence of the stilbene synthase gene sequence in the regenerated plants (lines) was checked by PCR. Eighty-three percent of the plants showed a clear band corresponding to the relevant sequence of the gene, but the control showed no band (data not shown). The plants identified to have the gene were transferred to soil to stimulate their growth, and further confirmation of the integration of the gene(s) was made by Southern blot analysis. When the DNAs from the plants were double-digested with *Bam*HI and *Eco*RI, a 1.8-kbp band corresponding to the fragment containing stilbene synthase gene + NOS terminator was detected in all of the plants but not in the control plant (Fig. 3A). To determine whether the observed

Fig. 2 The deduced amino acid sequences of stilbene synthase from *Vitis* spp. pVIN from *V. vinifera*, pLAB from *V. labrusca*, and pRIP from *V. riparia*

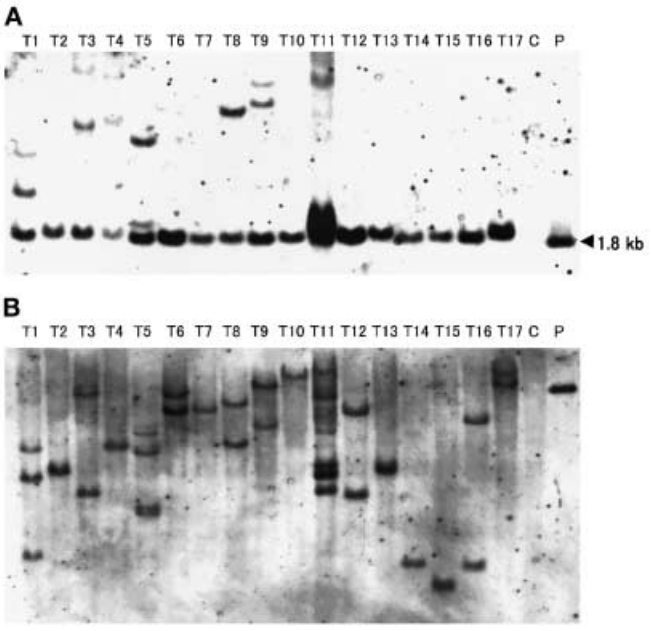

Fig. 3A,B Southern blot analysis of kiwifruits transformed with pBSVIN. [*lanes T1-T17* DNAs from transformants, *lane C* DNA from non-transformed control plant, *lane P* plasmid DNA (pBSVIN)]. **A** DNAs double-digested by *Bam*HI-*Eco*RI. A 1.8-kbp band corresponding to the fragment containing stilbene synthase+NOS terminator (Fig. 1) was detected in all of the plants but not in the control plant. **B** DNAs single-digested by *Bam*HI

bands were the result of the integration of the gene(s) into the plant genome rather than contamination by the bacteria, the DNAs were single-digested with *Bam*HI. None of the plants showed a band corresponding to the plasmid DNA, and all had several bands (from one to eight) which represented junctions between T-DNA(s) and adjacent plant DNA (Fig. 3B). These findings showed that the plants had integrated the gene(s) into the plant genome from one to eight copies.

Identification and quantification of piceid

A HPLC profile of leaf extract from a transformant is shown in Fig. 4A. Two major peaks, A and B, and some minor peaks were detected, whereas the extract from the non-transformed control plant showed no peaks. The two major peaks were not resveratrol, because the retention times of the peaks were shorter than that of resveratrol (Fig. 4D). Jeandet et al. (1997) presented the HPLC profiles of stilbene compounds and showed that *trans*- or *cis*-piceid had shorter retention times than *trans*- or *cis*-resveratrol. This suggests the possibility that the peaks are *trans*- or *cis*-piceid. Thus, we examined whether the peaks were derived from piceid. The peaks were collected from the extract of a transformant by HPLC, dried, redissolved in distilled water, hydrolyzed enzymatically, and then again analyzed by HPLC. The collected peak B substance (Fig. 4B) treated with β-D-glucosidase resulted in the occurrence of a new peak (Fig. 4C) corresponding to resveratrol (Fig. 4D); thus, peak B was identified as piceid. However, peak A could not be identified; treatment with β-D-glucosidase had no effect on the mobility of peak A. Therefore, quantification was made only with respect to peak B (piceid). Jeandet et al. (1997) proposed a hypothesis regarding the metabolism of resveratrol: resveratrol is synthesized by stilbene synthase and then rapidly glycosylated by a glycosyltransferase to piceid. Although transformants produced piceid instead of resveratrol, resveratrol produced by the action of the integrated stilbene synthase gene(s) might be metabolized into piceid as they suggested.

The piceid content of the transformants was quantified using calibration curves of resveratrol, as described by Jeandet et al. (1997). Hain et al. (1993) showed that young leaves contained higher amounts of resveratrol than older ones in transgenic tobacco integrating stilbene synthase genes. In this study, moreover, young leaves contained higher amounts of piceid than older leaves, and the content in the latter decreased to about one tenth of the former. Thus, leaves of the same stage, about 5–7 cm in width, were collected from the plants and analyzed. The piceid content differed among the individual plants, but there was no significant difference in mean concentration of piceid in the transformants among the vectors used (Table 1). This suggests that the amino acid differences in the genes do not affect their activity. In the transgenic tobacco, a correlation between resveratrol production and disease susceptibility was demonstrated (Hain et al. 1993). Although they determined resveratrol contents by an enzyme-linked

Table 1 Piceid contents in the young leaves of transformants

Vectors used	No. of plants analyzed	No. of plants producing indicated amounts of piceid				Piceid contents (mean ± SE)
		1–10	11–50	51–100	101–200[a]	
pBSVIN	17	3	8	4	2	48.3 ± 11.7
pBSLAB	18	2	9	5	2	48.4 ± 8.8
pBSRIP	18	3	11	3	1	37.7 ± 8.5

[a] μg/g fresh weight

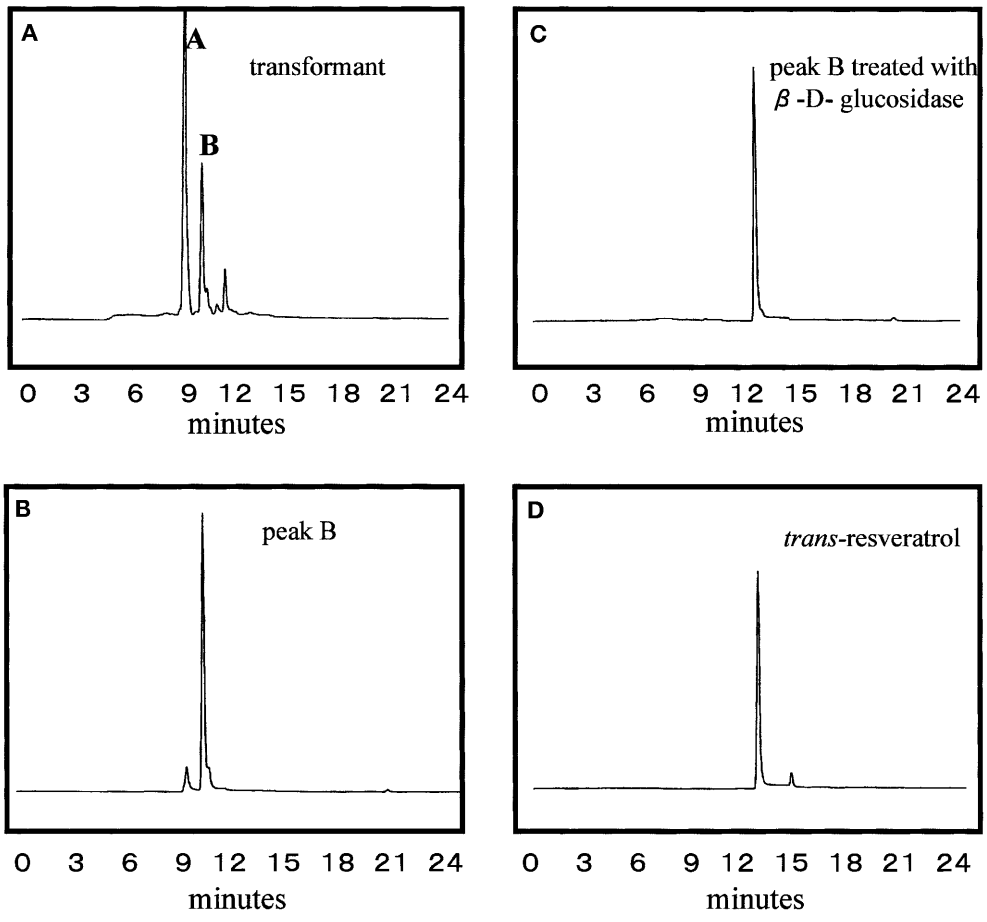

Fig. 4A-D Identification of piceid in the leaf extract from a transformant by HPLC. **A** An elution pattern of the extract from a transformant. No peak was detected in the extract from a non-transformed control plant. **B** An HPLC profile of peak B substance collected from the extract of the transformant by HPLC. **C** A profile of peak B substance after treatment with *β*-D-glucosidase; a new peak corresponds to *trans*-resveratrol (**D**). Treatment with*β*-D-had no effect on the mobility of peak A. **D** A profile of *trans*-resveratrol

immunosorbent assay (ELISA) using polyclonal antisera against resveratrol, the values of the resveratrol content probably did not contain resveratrol derivatives, including piceid. Using HPLC analysis and ELISA, they observed that the transgenic tobacco with a groundnut stilbene synthase produced resveratrol (Hain et al. 1990). In tobacco, it seems that an endogenous glycosyltransferase does not act rapidly. In other transgenic plants such as rice (Stark-Lorenzen et al. 1997), barley (Leckband and Lörz 1998), and wheat (Leckband and Lörz 1998), the resveratrol or piceid production was not examined.

The highest piceid content in the transformants was 182 μg/g fresh wt. in the young leaves and 20 μg/g fresh wt. in the old leaves. It has been reported that piceid inhibited platelet aggregation at concentrations of 6.7–107.2 μM (Shan et al. 1990) and also inhibited the synthesis of three eicosanoids from arachidonate by platelets (related to the process of aggregation) at concentrations of 55.3–251.7 μM (Kimura et al. 1985). Resveratrol has an inhibitory effect on platelet aggregation at concentrations of 129.9–164.7 μM (Pace-Asciak et al. 1995) or 3.56 μg/l (Bertelli et al. 1995), on eicosanoid formation at 0.683–2.72 μM (Kimura et al. 1985) or 6.09–76.9 μM (Pace-Asciak et al. 1995), and on LDL oxidation at 10 μM (Frankel et al. 1993). Furthermore, Jang et al. (1997) showed that resveratrol had cancer chemopreventive activity in three major stages of carcinogenesis (initiation, promotion and progression) and also inhibited tumorigenesis in a mouse skin-cancer model: application of 25 μmol resveratrol twice a week for 18 weeks reduced the number of skin tumors per mouse by 98%. Since human intestinal micro-organisms produce*β*-glucosidase (Bokkenheuser et al. 1987), dietary piceid would probably be converted to resveratrol in the intestine. If we assume that piceid accumulates in the fruits (unfortunately, fruits have not yet been set) to the same concentration as observed in old leaves of the transformant producing the highest amount of piceid, then about 2 mg piceid should be produced in an average-sized fruit (about 100 g). This amount of piceid may have a positive biological effect on human health.

Disease susceptibility and gene expression

The leaves from five transformants producing higher amounts of piceid (over 100 μg/g fresh wt.) and control plants were inoculated with spores of *B. cinerea*. In all plants, the disease symptom, a dark-brown lesion, was observed 3 days after inoculation. The

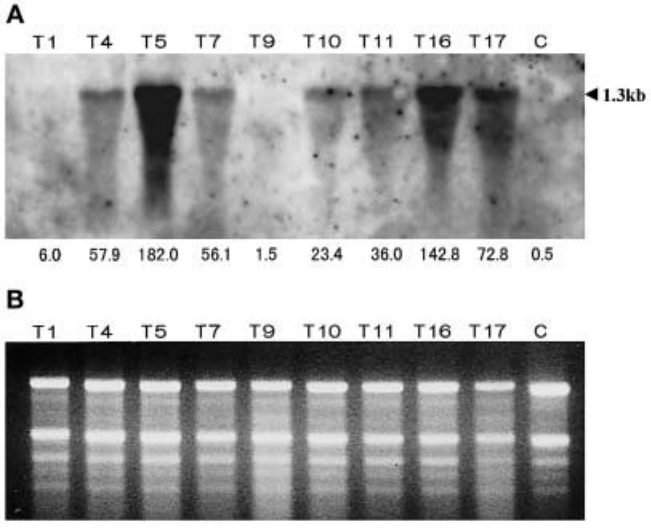

Fig. 5A,B Northern blot analysis of kiwifruits transformed with pBSVIN (*lanes T1-T17* total RNAs from transformants, *lane C* total RNA from non-transformed control plant). **A** Hybridized with DIG-labeled pVIN fragment (*numerals* show the piceid content in μg/g fresh wt.) of the transformants: a close correlation between mRNA expression and piceid content of the plants was observed. Transformant 'T11' with eight copies of the gene (Fig. 3) does not have a high gene expression. **B** Staining by ethidium bromide

diameter of the necrotic lesion of the transformants was not different from that of the control plants (data not shown). Stilbene synthase genes have been introduced into several plants, and the resulting transgenic plants showed increased disease resistance (Hain et al. 1993; Leckband and Lörz 1998; Stark-Lorenzen et al. 1997). In our experiment, however, transgenic kiwifruits did not show resistance to *B. cinerea*. This may be due to the difference in products or plant species used: transgenic tobacco produced resveratrol up to 400 μg/g fresh wt. (this value was obtained by an ELISA), whereas transgenic kiwifruits produced piceid at a maximum concentration of 182 μg/g fresh wt. The production of resveratrol or piceid was not determined in other transgenic plants. Resveratrol has been known to reduce the germination of conidia of *B. cinerea* and its mycelial growth at concentrations of 60–140 μg/ml (Adrian et al. 1997) and is produced in infected or UV-irradiated leaves of Vitaceae members from 50 to 400 μg/g fresh wt. (Langcake and Pryce 1976). Unfortunately, it is not known whether piceid has an anti-fungal activity. We were not able to examine its activity because we could not obtain pure piceid. If piceid has the same anti-fungal activity as resveratrol, however, a transgenic kiwifruit producing the highest amount of piceid (182 μg/g) can be expected to show some signs of resistance against *B. cinerea*. Since the transformant did not show any sign of resistance, piceid apparently does not have the same anti-fungal activity as resveratrol.

Expression of the stilbene synthase gene was examined by Northern blot analysis. The transformants, except T1 and T9, showed a band which was lacking in the control plant (Fig. 5), confirming that the introduced gene is transcribed into mRNA in the transformants. The strength of the band, which reflected the level of mRNA expression, differed among the individual transformants, and a close correlation between the expression and piceid content of a plant was observed; no correlation was recognized between the number of integrated genes and gene expression (Figs. 3B, 5).

In conclusion, we isolated pSV25 homologous stilbene synthase genes from three *Vitis* spp. and introduced them into kiwifruit. Transgenic kiwifruits did not show resistance against *B. cinerea* but produced a high amount of piceid. Thus, fruits of the transformants may have some beneficial effects on human health.

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