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Expression of soybean β-1,3-endoglucanase cDNA and effect on disease tolerance in kiwifruit plants

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Abstract Kiwifruit was transformed with a soybean β-1,3-endoglucanase (EC 3.2.1.39) cDNA under the control of the cauliflower mosaic virus (CaMV) 35S RNA promoter. The introduced gene was expressed in young leaves of the transformants. Assays of protein extracts from young leaves showed an increase in enzyme activity in many transformants, the transformant with the highest level of enzyme activity having an about sixfold increase over the control plants. When leaves from control and three transformants were inoculated with *Botrytis cinerea*, which causes gray mold disease, the disease lesion areas for two transformants were smaller than on control plants.

Key words *Agrobacterium* · Kiwifruit · β-1,3-Endoglucanase · Transformation · Disease tolerance

Abbreviations *CaMV* Cauliflower mosaic virus · *DIG* Digoxigenin · *SSC* Standard saline citrate

Introduction

There are varieties of fungal diseases that cause serious damage to kiwifruit production in Japan, such as fruit ripe rot caused by *Botryosphaeria* sp. and/or *Phomopsis* sp. and gray mold caused by *Botrytis cinerea*. Improved tolerance is thus desired in kiwifruit; however, the genetic improvement of disease tolerance by traditional cross-breeding has been hindered by the gynodioecious characteristics, genetic heterogeneity, and long life cycle of kiwifruit. The introduction of gene(s) by DNA transfer offers an alternative method for such improvement, and transformation of kiwifruit has been described (Matsuta et al. 1990, 1993; Uematsu et al. 1991; Janssen and Gardner 1993; Kobayashi et al. 1996).

β-1,3-Endoglucanase catalyzes the hydrolysis of β-1,3-glucan which is a major component of the cell wall of most fungi. Enzyme activity is usually low in a healthy plant but it increases during fungal infection. It has been proposed that induction of enzyme activity is related to the plant defense reaction. Keen and Yoshikawa (1983) studied interactions between soybean and fungal pathogens and showed that while the soybean β-1,3-endoglucanase did not directly inhibit fungal growth, the enzyme solubilized the elicitors from the fungal cell walls which induced production of antifungal phytoalexins. The cDNA of soybean β-1,3-endoglucanase has been cloned and characterized (Takeuchi et al. 1990), and transgenic tobacco expressing the cDNA under control of the cauliflower mosaic virus (CaMV) 35S promoter has been shown to exhibit complete resistance to fungal diseases (Yoshikawa et al. 1993). Based on these results, we attempted to improve the disease tolerance of kiwifruit plants through expression of the soybean β-1,3-endoglucanase cDNA.

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Materials and methods

Agrobacterium strain

Disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) harboring a binary vector pROKla-EG (Yoshikawa et al. 1993) which originated in BIN 19 (Bevan 1984) was used. The plasmid pROKla-EG contains the *npt*II gene linked to the nopaline synthase gene (*nos*) promoter and the soybean β-1,3-endoglucanase cDNA linked to the CaMV 35S (35S) promoter in the T-DNA region (Fig. 1).

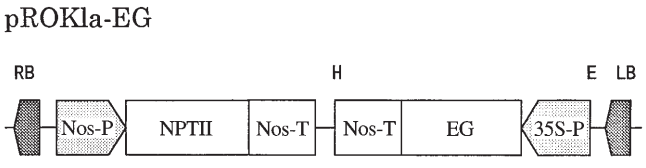

Fig. 1 Diagram of T-DNA region of pROKla-EG (*RB* right border, *LB* left border, *Nos-P* Nopaline synthase gene promoter; *NPT*II Neomycin phosphotransferase gene, *Nos*-T Nopaline synthase gene terminator, *35S-P* 35S promoter, *EG* Soybean β-1,3-endoglucanase cDNA, *H Hin*dIII, *E Eco*RI)

Plant transformation

Leaf disks, petiole, and stem segments of the kiwifruit (*Actinidia chinensis*) 'Hayward' were infected with LBA4404/pROKla-EG. The transformants were selected on a solidified medium containing kanamycin (50 µg/ml) and plantlets regenerated as described by Matsuta et al. (1990, 1993).

PCR analysis

PCR was used to check for the presence of the introduced transgene in the regenerated shoots at an early stage. DNA was isolated from leaves of in-vitro-regenerated shoots by the method of Honda and Hirai (1990) with modifications as described by Nakamura et al. (1998). PCR amplification was carried out as described by Kaneyoshi et al. (1994). Oligonucleotide primers were designed to correspond with the CaMV 35S promoter and the soybean β-1,3-endoglucanase cDNA and were expected to amplify a 830-bp fragment spanning the junction of these elements; the sequences were 5′GAT GTG ATA TCT CCA CTG ACG TAA G3′ and 5′AAC CAT CTT GCA CTA CCA CCG AAG G3′, respectively. The reaction cycle of 94 °C for 1 min, 40 °C for 2 min, and 72 °C for 3 min was repeated 45 times, followed by 72 °C for 7 min.

Southern blot analysis

Genomic DNA was isolated from leaves of ten randomly selected PCR-positive regenerated plants and an untransformed control plant according to the method of Rogers and Bendich (1985) with modifications as described by Nakamura et al. (1998). DNA concentrations of extracts were determined by measuring absorbance at 260 nm. Five micrograms of DNA was double digested with *Hin*dIII and *Eco*RI, and another 5 µg was digested with only *Hin*dIII or *Eco*RI. Products were separated by electrophoresis on a 0.8% agarose gel and transferred to a nylon membrane. The soybean β-1,3-endoglucanase cDNA (1.3 kb), labeled with digoxigenin (DIG) using a DIG-labeling kit (Boehringer Mannheim, Germany), was used as a probe. Hybridization was for 16 h at 68 °C in 5× standard saline citrate (SSC), 0.5% blocking reagent (Boehringer Mannheim, Germany), 0.1% N-lauroylsarcosine sodium salt, and 0.02% SDS. Membranes were washed twice in 2×SSC, 0.1% SDS at room temperature for 5 min each, 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).

Northern blot analysis

Total RNA from the young leaves of the same ten transformants and control plant analyzed using Southern blots was isolated using the SDS-phenol method (Sambrook et al. 1989) with modifications as described by Kobayashi et al. (1996). RNA concentrations of extracts were determined by measuring absorbance at 260 nm. Twenty micrograms of total RNA was separated by electrophoresis on a 1.17% 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 was for 16 h at 68 °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 testes DNA. Membranes were washed under the same conditions as the Southern blot analysis. Target RNA was detected as described by the manufacturer (Boehringer Mannheim).

Western blot analysis and assay of endoglucanase activity

Total protein of the ten transformants and control plant was extracted from young leaves. Approximately 500 mg of leaf tissue was collected for each transformant, frozen in liquid nitrogen, ground into a powder, and 0.5 mg insoluble-PVP was added. This mixture was then suspended in 10 ml extraction buffer [100 mM Tris-HCl (pH 7.3), 0.5 M EDTA, 10 mM 2-mercaptoethanol, 1 mM dithiothreitol]. The extract was centrifuged at 14,000 rpm at 4 °C for 20 min. Ammonium sulfate was added to the supernatant until the final concentration was 80% and it was kept at 4 °C for more than 2 h. The mixture was centrifuged at 14,000 rpm at 4 °C for 20 min and the supernatant discarded. Pellets were then resuspended in 0.5 ml 50 mM sodium acetate buffer (pH 5.5) and centrifuged to remove the debris. The supernatant was desalted using Pharmacia NAP-5 columns (Pharmacia Biotech) and saved as crude protein extracts. Protein content of these crude extracts was assayed according to Bradford (1976), using the Protein Assay Dye Reagent (Bio-Rad, USA) and bovine serum albumin as standard.

Western blot analysis was conducted using an antiserum specific to the soybean β-1,3-endoglucanase and alkaline-phosphatase-conjugated goat anti-rabbit IgG. Ten micrograms of total soluble protein was fractionated by SDS-PAGE and transferred to a PVDF membrane and then reacted with the soybean β-1,3-endoglucanase antiserum. After subsequent washing, β-1,3-endoglucanase was detected according to the manufacturer (Tropix, USA).

β-1,3-Endoglucanase activity in crude protein extracts was assayed by measuring the rate of reducing sugar production with laminarin as substrate, as described by Keen and Yoshikawa (1983). Twenty microliters of the crude protein extract was added to 480 µl of 0.5 mg/ml laminarin in 50 mM sodium acetic acid (pH 5.5) and the mixture was incubated at 35 °C for 30 min. Then, 500 µl of Nelson alkaline copper reagent was added and reducing sugars were measured by the Nelson method (Ashwell 1957). Results were expressed as nmol glucose equivalents released per minute and standardized per milligram of protein content of used extract. The assay was performed at least three times.

Inoculation of *Botrytis cinerea* to transformants

B. cinerea isolated from grape was used as inoculum. Leaves from three transformants (T2, T8, T10) as well as control plants were inoculated with mycelial disks (5 mm diameter) of *B. cinerea* grown on potato-dextrose agar medium for 3 days. Untransformed plants regenerated from leaf disks, which were grown under the same conditions as transformants, were used as controls. The mycelial disks were put on the upper side of leaves and kept in a moist container in the dark at 20 °C. Twelve inoculation sites were prepared using three to six leaves per transformant and the experiment was repeated three times. Three days after inoculation, the diameter of the necrotic lesion was measured. Leaves were also inoculated with spores according to Tabei et al. (1998). The spores were suspended in 2.5% glucose, 1 mM inosine and 1.5% agar at a concentration of 1×10^6 spores/ml and then the 10 ml suspension was solidified in a 9-cm-diameter petri dish. Agar disks, 5 mm in diameter, containing spore suspension were placed on the reverse side of leaves of transformants and control plant, and kept in a moist container in the dark at 20 °C. Four leaves with one inoculation site were prepared per transformant. Four days after the inoculation, the diameter of the necrotic lesion was measured.

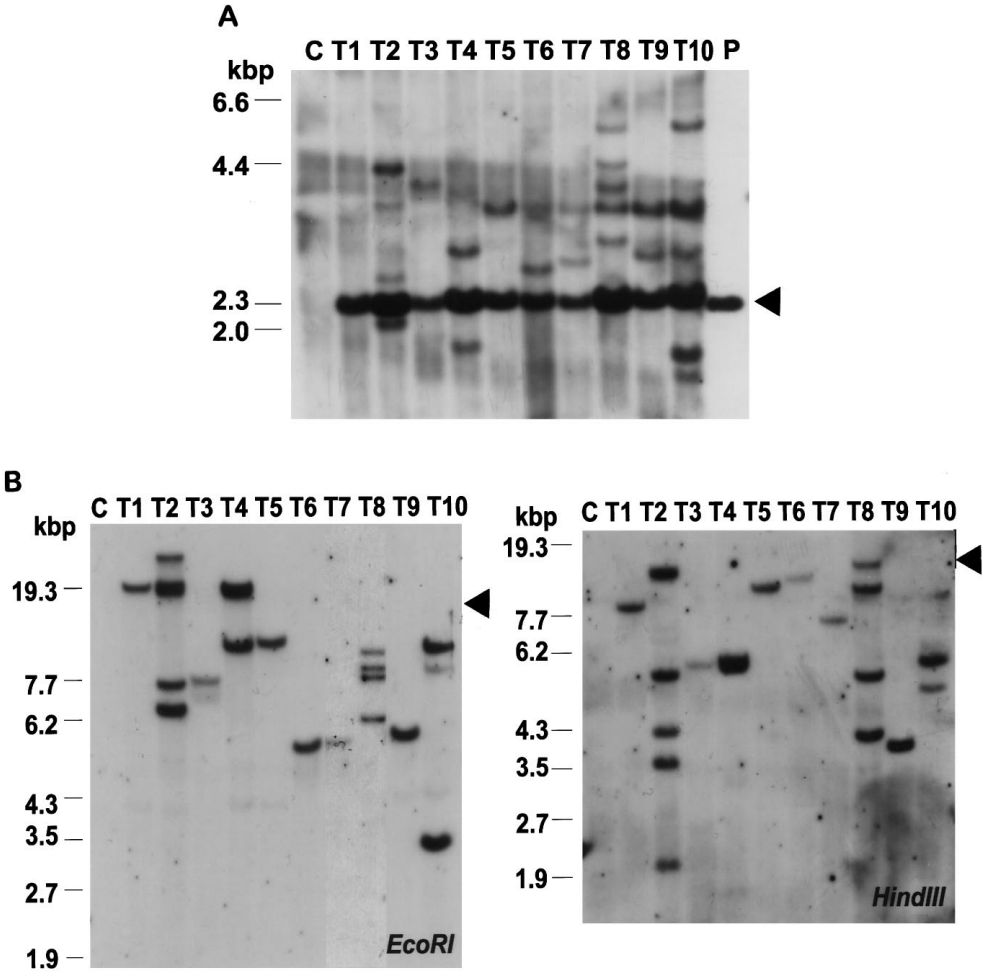

Fig. 2 A, B Southern blot analysis of transformed kiwifruits with soybean β-1,3-endoglucanase cDNA. [*lane C* DNA from non-transformed control plant, *lanes T1–T10* DNAs from transformants, *lane P* plasmid DNA (pROKla-EG), ▲ fragment size of pROKla-EG], **A** DNAs double-digested by *Hin*dIII-*Eco*RI. **B** DNAs single-digested by *Eco*RI (*left*) and *Hin*dIII (*right*)

Result and discussion

Transformation

Segments inoculated with the *Agrobacterium* were cultured on selection medium. Some calli formed at the cut ends of the segments and adventitious buds differentiated from the calli. The adventitious buds were subsequently transferred to a medium for plant regeneration.

The presence of the soybean β-1,3-endoglucanase cDNA sequence in the regenerated shoots was checked by PCR at an early stage. Twenty-six kanamycin-resistant regenerated shoot lines were examined: 17 (65.4%) showed amplification of the expected 830-bp band and the remainder and control showed no band (data not shown). Shoots in which the cDNA sequence was identified by PCR were rooted on the medium containing 1 mg/ml indole-3-butyric acid and developed into whole plants [hereafter called PCR(+) plants].

DNA and RNA detection

The presence of the soybean β-1,3-endoglucanase cDNA in the PCR(+) plants was examined by Southern blot analysis. When the DNAs were double digested with *Hin*dIII and *Eco*RI, the expected 2.3-kbp band (corresponding to the fragment containing the CaMV 35 S promoter, the soybean β-1,3-endoglucanase cDNA and the *nos* terminator) was detected in all of the PCR(+) plants (Fig. 2 A, lanes T1 to T10), but not in the control plant (Fig. 2 A, lane C). To confirm that the observed 2.3-kbp band was not due to remaining bacteria, the DNAs were single-digested with *Hin*dIII or *Eco*RI. The intact pROKla-EG plasmid has a single restriction site for *Hin*dIII or EcoRI with a size of 15.0 kbp (Fig. 1). None of the PCR(+) plants showed the 15.0-kb band, indicating that the bacteria were not present. Each PCR(+) plant showed several bands (from one to five, varying in size) which represented junctions between T-DNA(s) and adjacent plant DNA (Fig. 2 B). These findings showed that these PCR(+) plants had integrated the cDNA fragment at one to five sites in the plant genome.

Accumulation of the soybean β-1,3-endoglucanase mRNA was examined by Northern blot analysis. All of the transformants examined showed the expected 1.3-kb band which was lacking in the control plant (Fig. 3), confirming that the soybean β-1,3-endoglucanase cDNA is transcribed into mRNA in transformants. The strength of the band, which reflected the level of mRNA expression, dif-

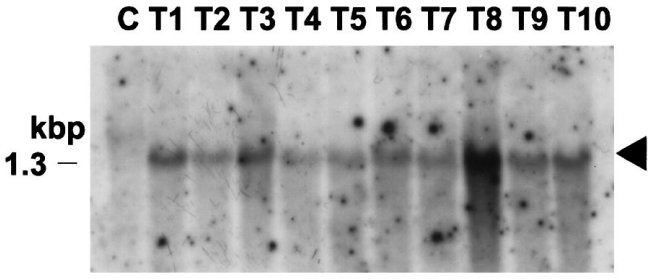

Fig. 3 Northern blot analysis of kiwifruits transformed with soybean β-1,3-endoglucanase cDNA. (*lane C* total RNA from non-transformed control plant, *lanes T1–T10* total RNAs from transformants, ▲ size of mRNA from soybean β-1,3-endoglucanase cDNA)

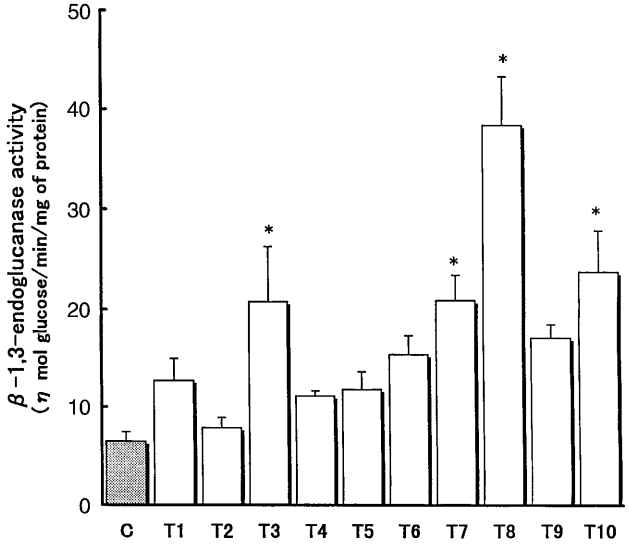

Fig. 4 β-1,3-Endoglucanase activity in young leaves of transformed kiwifruits (*C* non-transformed control plant, *T1–T10* transformants, *Vertical bars* represent SE of means and *asterisks* indicate a significant difference from the control at the 0.05 level by Dunnett's one-way *T*-test

fered among the individual transformants. Transformant T8 showed an especially strong hybridization signal, indicating a high level of expression.

Protein detection and enzyme assay

The production of the soybean β-1,3-endoglucanase protein was examined by Western blot analysis. The specific band was detected in all the transformants, but not in the control plant (data not shown). The molecular mass of the band was estimated to be 33.5 kDa, which is the size of the native soybean protein. This result indicates that soybean β-1,3-endoglucanase was produced in all transformants. Consistent with the results obtained from Northern blotting, transformant T8 had an especially strong band, while the remaining transformants had a faint band.

The β-1,3-endoglucanase enzyme activity was assayed by measuring the rate of reduction of sugar production with laminarin as substrate. The enzyme activity differed among transformants (Fig. 4). Transformant T8 showed the highest activity, about six-fold over the control plants. T3, T7, and T10 showed activity three- to four-fold over the controls and the remaining transformants showed less than a three-fold increase. Levels of enzyme activity in T3, T7, T8, and T10 were significantly greater than that of the control plant whereas in the remaining transformants they were not. Transformant T8 showed the highest level of mRNA expression, protein production, and enzyme activity.

Table 1 Means of diameter of the lesion formed on leaves of transformants and control plant by inoculation with *Botrytis cinerea*. *Different letters* indicate significant difference at the 0.05 level by Tukey's standardized-range test

	Lesion diameter (mm ± SE)	
	Inoculation with mycelial disks (after 3 days)	Inoculation with spores (after 4 days)
Control	29.2 ± 0.4467 a	18.7 ± 0.5105 a
T2	29.6 ± 0.6234 a	20.9 ± 2.1277 a
T8	26.1 ± 0.3533 b	10.6 ± 0.3752 b
T10	27.2 ± 0.4120 b	12.2 ± 0.7947 b

Disease tolerance

Three transformants were inoculated with *B. cinerea*, two of which had shown a significant increase in enzyme activity (T8, about six-fold and T10, about four-fold over the control plants) and one of which had not (T2). The leaves from transformants and those from the control plant were inoculated with the mycelial disks. Leaves from all plants showed the characteristic dark-brown necrotic lesion 3 days after inoculation; however, the area of the lesions in transformants T8 and T10 was significantly smaller than that of the control plant (Table 1). The sizes of lesions in T2 were not different from those in the control plant. Thus, the growth of invading mycelium may be limited by some effect of the enzyme in transformants with high levels of glucanase activity. There appears to be a greater effect when spores instead of mycelial disks are used as inoculum (Table 1, Fig. 5). This might be due to the difference in mycelium density between mycelial disk and spore disk, because there was no difference in rate of germination of spores. Additional inoculation with spores was performed on the control and T8 transformant; a similar phenomenon was observed (data not shown).

Yoshikawa et al. (1993) reported that tobacco plants transformed with soybean β-1,3-endoglucanase cDNA showed up to four-fold higher β-1,3-endoglucanase activity than control plants. There was a high correlation between glucanase activity and disease resistance, and the transformed tobacco plants with high glucanase activity showed complete resistance to *Phytophthora parasitica* var. *nicotianae* and *Alternaria alternata*. A similar phenomenon was reported by Ito et al. (1994, 1995) using transformed eggplants with soybean β-1,3-endoglucanase

Fig. 5 Disease symptoms formed on leaves of the control plant (*left*) and the transformant T8 (*right*) by inoculation with spores of *B. cinerea*

cDNA. The transformed eggplants with high glucanase activity showed a statistically significant degree of disease resistance to *P. capsici*. In our experiments, transformed kiwifruit did not show complete resistance to *B. cinerea*; however, the area of lesions was smaller in transformants having increased enzyme activity. Thus, the growth of invading fungal pathogens may be limited by the defense reaction induced by the enzyme. It has been reported that while soybean β-1,3-endoglucanase does not directly inhibit fungal growth, the enzyme solubilizes elicitors from the fungal cell walls which induce production of antifungal phytoalexins (Keen and Yoshikawa 1983). It has also been reported that leaves of tobacco transformed with soybean β-1,3-endoglucanase cDNA indeed induced the transcription of a plant defense gene, phenylalanine ammonialyase, in response to fungal attack to a greater extent than untransformed leaves (Yoshikawa et al. 1993). The mechanism of tolerance in the transformed kiwifruits described here is not clear and further studies are necessary. In this report, transformants were only assayed for resistance to *B. cinerea* in leaves. There are other important fungal pathogens, *Botryosphaeria* sp. and *Phomopsis* sp; which cause fruit ripe rot disease, however these pathogens attack only fruits. Since the transformants described here have not yet set fruit, such analyses will have to be carried out at a future date.

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