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Short Communication

Resistance to insects in transgenic *Solanum* plants expressing a peroxidase gene from horseradish

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Abstract To engineer insect-resistant *Solanum* plants, a peroxidase C2 gene (*prxC2*) from horseradish was introduced into *Solanum integrifolium* Poir. cv. Hiranasu. We produced 77 transgenic Hiranasu plants. Peroxidase expression was confirmed at the transcriptional and translational levels by northern blot analysis and by measuring peroxidase activity, respectively. Feeding test results show that transformant no. 180 is more resistant to corn earworm (*Heliothis armigera*) and common cutworm (*Spodoptera litura*) than the wild-type plants. We also found a correlation between insect resistance and lignin content in the transgenic plants. In particular, the lignin content of transformant no. 180 was 30% higher than that of wild-type plants. These results further confirm that peroxidase is functionally expressed in transgenic plants and suggest that the increased lignin content is a basis for the insect resistance in transgenic Hiranasu plants.

Key words: Insect resistance, lignin, peroxidase, *Solanum integrifolium*.

Various defense responses are induced in plants by wound stresses caused by pathogens or invading insects. A group of proteins called pathogenesis-related (PR) proteins are actively synthesized in accordance with defense responses. Expression of PR protein genes is induced not only by pathogenic infection, but also by the wound itself. The PR protein genes are classified into 17 families, from PR-1 to PR-17. Among these, the PR-9 gene family has peroxidase properties (Van Loon et al. 1994), including the lignin-forming peroxidase of tobacco (Lagrimini et al. 1987).

Peroxidase, in plants, includes various isoenzymes and isoforms. The catalytic reactions of peroxidase are diverse, and peroxidase plays an important role in plants. Peroxidase is associated with lignin biosynthesis in the cell wall (Lagrimini 1991), removal of hydrogen peroxide (Grisebach 1981), oxidation of reduced toxic compounds (Gasper et al. 1982), oxidation of 3-indoleacetic acid (IAA) (Hinnman and Lan 1965), and growth during the plant's life cycle (Horton 1993). Wound-inducible peroxidase functions in the defense reaction against pathogens and insects by producing lignin, which helps thicken the plant cell wall. An example is the induction of tobacco plant resistance to wildfire illness and *Phytophthora* rot by introducing a wound-inducible peroxidase gene from rice (Hiraga et al. 1998). However, few reports have described the introduction of peroxidase genes as means to produce insect- and pathogen-resistant plants. The peroxidase gene *prxC2* from horseradish encodes a basic peroxidase isoenzyme (Fujiyama et al. 1990). Expression of *prxC2* is induced by wounding, and the promoter region of *prxC2* was therefore used as a model to study the mechanisms of wound-inducible transcription (Kawaoka et al. 1994a). Here, we introduced the *prxC2* gene into *Solanum* plants to breed insect-resistant plants. The expression of *prxC2* in transgenic plants was confirmed and the levels of insect resistance of the transformants, as well as the lignin content, were analyzed.

Agrobacterium tumefaciens EHA101 harboring binary plasmid pBI121 containing *prxC2* gene under the control of the CaMV35S promoter was used to infect hypocotyls of *Solanum integrifolium* Poir. cv. Hiranasu. After two days of co-cultivation, hypocotyls were cultured in selection medium (Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 100 mg l^{-1} kanamycin, 400 mg l^{-1} carbenicillin, 3% sucrose, 0.1 mg l^{-1} IAA, and 0.3% gellan gum). The rooting medium used was MS medium with 50 mg l^{-1} kanamycin, 400 mg l^{-1} carbenicillin, 3% sucrose, and 0.3% gellan gum. We

Abbreviations: CaMV, cauliflower mosaic virus; IAA, 3-indoleacetic acid; PR, pathogenesis-related; *prxC2*, gene of horseradish peroxidase C2.
This article can be found at http://www.jspcmb.jp/

obtained 130 regenerated adventitious shoots, and the presence of the transgene was confirmed by genomic DNA PCR. Total DNA was extracted from the leaves according to methods described in Edwards et al. (1991). The two primers, C2-5′ (5′-GCATTCCTCTTCCAGTTTGA-3′) and C2-3′ (5′-GGAGCTGGCAAAGTCATTAG-3′), were designed to amplify a fragment of the *prxC2* gene. Amplification was carried out in a PerkinElmer GeneAmp PCR System 2400 (PerkinElmer Inc., Milan, Italy) using the following parameters: preheating at 94°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min, and a final heating at 72°C for 7 min. The reaction mixture was analyzed by 1.5% agarose gel electrophoresis. The resulting 980 bp fragment of the *prxC2* gene was amplified from 77 shoots (1.5% of the shoots generated).

Expression at the transcriptional level was examined by northern blot analysis for four Hiranasu transformants. Total RNA was prepared from transformants' leaves using the ATA method (Nagy et al. 1988). We analyzed RNA (10 μg) using 1.5% formalin agarose. The RNA was transferred to a nylon membrane (Hybond N, Amersham Biosciences Corp.) and hybridized with [α-^{32}P]dCTP-labeled 980 kb DNA fragment of *prxC2*. mRNA of *prxC2* was detected in the transformants (nos. 136, 180, 184, and 201) (Figure 1). These results showed that the introduced *prxC2* was expressed in these transformants.

We measured the peroxidase activity of six Hiranasu transformants to confirm expression at the translational level. Fresh leaves from each transformant were homogenized in 50 mM sodium phosphate buffer (pH 7.0). The crude leaf extract was allowed to react with o-aminophenol at 25°C for 3 min. Peroxidase activity was calculated by measuring the increased absorption of the visible region at 480 nm, for the isophenoxazine created by the reaction (Kawaoka et al. 1994b; Yamada et al. 1987). The results are shown in Table 1. Notably, transformant no. 180 demonstrated 20 times the peroxidase activity of the control.

Larvae of *Heliothis armigera* (corn earworm) were used in feeding experiments to test the insect tolerance of the six transgenic plants confirmed to have high peroxidase activity. The percent mortality of corn earworms fed on leaves of five of the transgenic plants was equivalent to or lower than that of earworms fed on non-transgenic leaves. However, the corn earworms

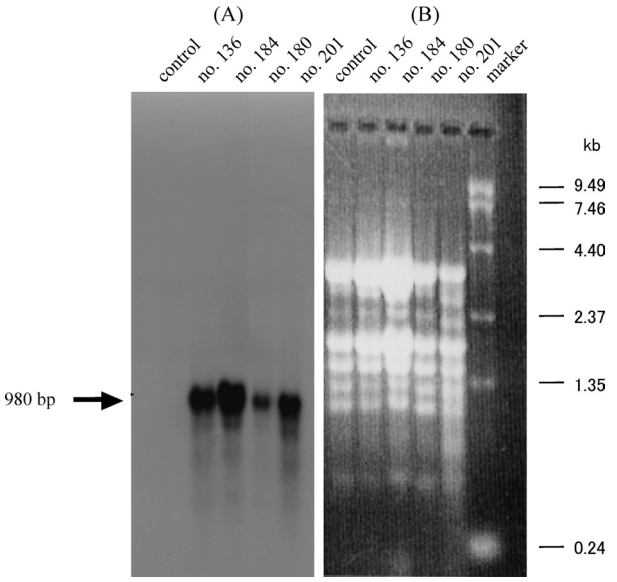

Figure 1. Northern blot confirming expression of *prxC2* in transgenic Hiranasu. (A) Northern blot, and (B) agarose gel stained with ethidium bromide. The control is from a non-transformed plant, and the arrow indicates the position of *prxC2* mRNA.

Table 1. Increased peroxidase activity and lignin content confer insect resistance in transgenic Hiranasu plants for corn earworm and common cutworm.

Insects	Plants	Feeding rate of the first leaf (%)	Mortality of insect (%)	Peroxidase activity[2] (Umg-potein^{-1})	Klason lignin (%)[3]	
					First leaf	Second leaf
Corn earworm	Control	42.5	20.0	0.01	16.8±1.9[4]	20.2±1.7
	No. 136	100.0	0.0	0.28	19.5±1.5	21.1±2.1
	No. 180	±[1]	100.0	0.20	21.9±1.8	23.6±2.5
	No. 184	71.6	20.0	0.49	18.4±1.5	17.4±2.4
	No. 201	41.5	20.0	0.25	nd[5]	nd
	No. 204	37.5	20.0	0.60	nd	nd
	No. 249	54.8	0.0	0.29	nd	nd
Common cutworm	Control	22.9	0.0	0.01	16.8±1.9	20.2±1.7
	No. 180	0.2	90.0	0.20	21.9±1.8	23.6±2.5
	No. 184	24.5	0.0	0.49	18.4±1.5	17.4±2.4

Five corn earworms (second instar larva) or ten common cutworms (first instar larva) were set in the plastic container.
Leaves and larvae were examined 3 days from the start of the experiment.
[1] The degree of which a very small area of the leaf surface was consumed.
[2] Peroxidase activity was calculated by measuring the increased absorption of the 480 nm of isophenoxazine.
[3] Klason lignin (%)=W/S×100 (W: residue weight (g), S: dry weight (g) of the sample).
[4] Values represent the mean±SD.
[5] No data.

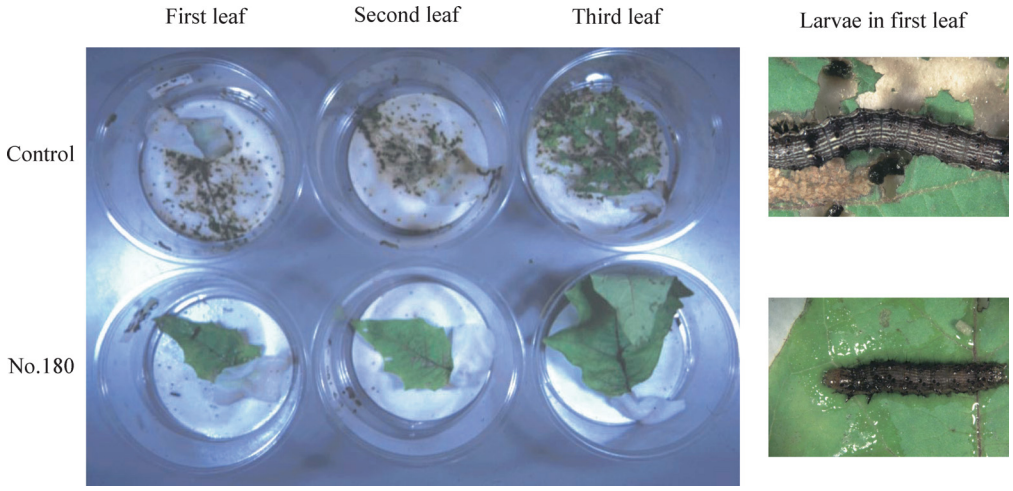

Figure 2. Feeding test to evaluate the levels of insect tolerance of transgenic leaves. The first, second, or third leaves of the control (wild-type) or the transgenic Hiranasu no. 180 were placed in plastic containers containing water-soaked filter paper. Five corn earworms were placed with the leaves for 3 days at 25°C, after which these photos were taken.

could consume only the tissues on the leaf surface of transformant no. 180; subsequently, all the larvae died (Figure 2). In addition, two transformants (nos. 180 and 184) were used in feeding experiments to test the tolerance toward *Spodoptera litura* (common cutworm). Only 0.2% of the leaf of transformant no. 180 was consumed by the common cutworm. Moreover, 9 of 10 (90%) of the common cutworms died. In contrast, the level of insect resistance of transformant no. 184 was equivalent to that of the non-transgenic control (Table 1). These results demonstrate that transformant no. 180 acquired resistance toward *Heliothis armigera* and *Spodoptera litura*.

We next measured the lignin content of the leaves of transformants nos. 136, 180, and 184, as well as the non-transgenic control. The lignin weight was determined using the Klason method (Dence et al. 1992). Powdered samples (1 g) of leaves that were degreased after drying were treated in 15 ml of 72% sulfuric acid for 4 h at room temperature. Sulfate ester and cellodextrin were hydrolyzed in boiled sulfuric acid (about 3%) for 4 h. The lignin content in the first and second leaves of transformant no. 180 was increased about 30% and 17%, respectively, compared to the control. The lignin content of the transformants showing no insect resistance was less than that of transformant no. 180 (Table 1). Some transformants that contain more lignin than the non-transformants did not exhibit insect resistance. Since only transformant no. 180 obtained insect resistance, our results suggest that lignin content over 21.9% may be effective for insect resistance. Although a plant's second leaf naturally contains high amount of lignin, the first leaf of transformant no. 180 contained even more lignin than the second leaf of the control plant. Furthermore, insect damage to the cell wall is known to increase the lignin content in plant tissues (Buendgen et al. 1990; Ostrander and Coors 1997).

Transformant no. 180, which had 20-fold greater peroxidase activity than the control, demonstrated resistance against Lepidoptera larvae. Nevertheless, transformant plants with nearly equivalent peroxidase activity (nos. 136, 201, and 249) and transformant plants with greater peroxidase activity (nos. 184 and 204) did not show insect resistance. The lignin content of transformant no. 180 was 30% higher than that of the non-transformants; however, this increase was lower than anticipated based on its peroxidase activity. Lignin biosynthesized by the peroxidase should accumulate gradually in proportion to plant growth, except in cases of injury and other stresses. In transformants, there was no correlation between peroxidase activity and lignin content. Though peroxidase is directly associated with the process of lignin production, the lignin content in transgenic plants overexpressing peroxidase did not greatly exceed that of the standard value. This is due to existing suppression mechanisms that control the amount of lignin in plant tissues; too much lignin will negatively impact plant growth.

Wound-inducible expression of *prxC2* was used as a model to study the mechanisms of induced transcription in response to wound stress (Kawaoka et al. 1994a). This study confirmed the model in *Solanum* plants. Here, overexpressed *prxC2* contributed to defense against insect-caused wound stress. The use of a tissue-specific or period-specific promoter will be important in the future breeding of advanced plant varieties.

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