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Enhanced Resistance against a Fungal Pathogen *Sphaerotheca humuli* in Transgenic Strawberry Expressing a Rice Chitinase Gene

Hiroshi ASAO[1,4], Yoko NISHIZAWA[2], Shigeru ARAI[1], Takanori SATO[3], Masashi HIRAI[3], Kazuya YOSHIDA[4], Atsuhiko SHINMYO[4] and Tadaaki HIBI[5]

[1] *Nara Agricultural Experiment Station, Kashihara, Nara 634, Japan*
[2] *National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305, Japan*
[3] *National Research Institute of Vegetable, Ornamental Plants and Tea, Ano, Mie 514-23, Japan*
[4] *Nara Institute of Science and Technology, Ikoma, Nara 630-01, Japan*
[5] *The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan*

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Abstract

Fragaria × *Ananassa* Duch. cv. Toyonoka is a main variety of strawberry in Japan, but it is susceptible to a pathogenic fungus, *Sphaerotheca humuli*. Rice chitinase gene under the control of cauliflower mosaic virus (CaMV) 35S promoter was introduced into the strawberry plants using *Agrobacterium tumefaciens*. The transgenic plants showed an increased resistance to the powdery mildew, *S. humuli*.

1. Introduction

In Japan, conventional plant breeding and selection has been successful for many years facilitating the development of important strawberry cultivars (*Fragaria*×*Ananassa* Duch. cv. Toyonoka, Nyohou, and Houkouwase). However, they are susceptible to host-selective pathogens ; *Sphaerotheca humuli, Colletotrichum fragariae* and *Fusarium oxysporum*, respectively. Especially, in spite of its vulnerability to *S. humuli*, a causal pathogen of powdery mildew disease, the strawberry cultivar Toyonoka is the most popular in Japan. To overcome this disadvantage many conventional selective breeding trials have been carried out, but it appears difficult to introduce only the resistant trait because of the high heterozygosity and ploidy of the strawberry plants. Therefore we have started to develop techniques for genetic manipulation of strawberry. Prerequisite protocols for regeneration of strawberry tissue [1] and transformation of strawberry with *Agrobacterium tumefaciens* [2] have been already developed. Three important cultivars of strawberry were transformed with *A.tumefaciens* carring plasmid pBI121 which contains a kanamycin resistant gene and a β-glucuronidase gene. Among them, Nyohou was the most efficient, while Toyonoka and Houkouwase showed lower transformation efficiencies.

Chitinase catalyzes the hydrolysis of chitin (β-1, 4-linked polymer of *N*-acetyl-D-glucosamine) which is a major component of the cell wall of most fungi [3]. Since chitinase has been shown to be active *in vitro* against various pathogens [4], it has been proposed that induction of chitinase activity is one part of plant defense response [5]. Therefore the introduction of chitinase gene into the plant would be the effective strategy to enhance the resistance against fungi. Nishizawa and Hibi [6], and Nishizawa *et al.* [7] have cloned three class I chitinase genes from rice.

In this report enhanced resistance against *S. humuli* in transgenic strawberries transformed with the rice chitinase gene is described.

2. Materials and Methods

2. 1 Transformation procedures

Excised leaf-discs and petioles of *Fragaria*× *Ananassa* Duch. cv. Toyonoka that were aseptially grown, were pretreated for one day by the shaking culture (100 rpm) with MS liquid medium [8] containing 2 mg/*l* 6-benzylaminopurine (BA), 0. 2 mg/*l* 2,4-dichlorophenoxyacetic acid (2,4-D) and 3 % sucrose. Then the explants were inoculated with *A. tumefaciens* LBA4404 carrying a pBI121-RCC2. The pBI121-RCC2 was a modified plasmid of pBI121 (Clontech Laboratories) that contained NPT II gene, a CaMV 35S promoter and a rice chitinase cDNA RCC2 [7] replacing β-glucuronidase gene (**Fig. 1**). The cDNA RCC2 (The accession number ; X56787) was about 1. 1 Kb long, including a 9 bp 5′ flanking sequence and a 137 bp 3′ flanking sequence. Transformants were selected on the same MS agar medium with 50 mg/*l* kanamycin and 100 mg/*l* carbenicillin at 25°C under 16 hrs/day light at 50μmol $m^{-2}s^{-1}$. Regenerated plants were transferred to soil and

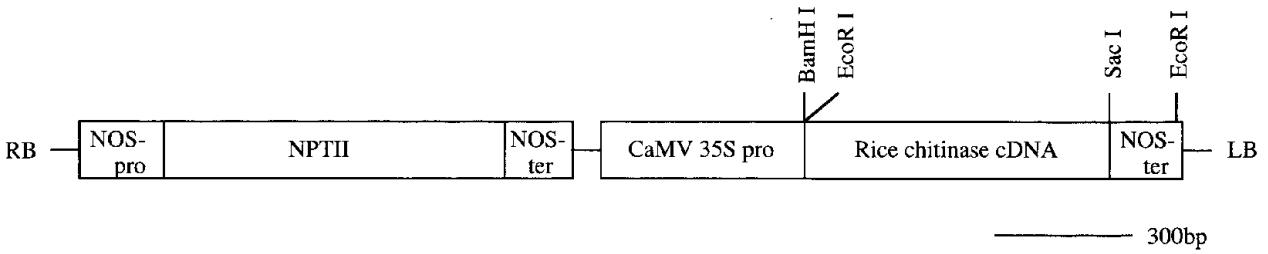

Fig. 1 Structure of T-DNA region in pBI121-RCC2.

grown in greenhouse under the conditions mentioned in ref. 2.

2. 2 DNA analysis

The rice chitinase gene in the strawberry transformants was detected by polymerase chain reaction (PCR). Total DNA was extracted from the leaves according to the method of Edwards *et al.* [9] The PCR mixture (10μl) was composed of 10 mM Tris-HCl (pH8. 9), 80 mM KCl, 1. 5 mM $MgCl_2$, 0. 1 % sodium cholate, 0. 1 % Triton X-100, 0. 2 mM each of dATP, dGTP, dCTP and dTTP, 0. 2 mM primers, 10 to 30 ng template DNA, and 0. 5 units AmpliTaq DNA Polymerase (Perkin Elmer). The two primers, 5′-TGGATCCAGCGGCTCGTCGGTTG-3′ for 3′ proximal coding region of the chitinase gene (added with an artificial *Bam*H I site ; underlined) and 5′-GTATAATTGCGGGACTCTAAT -3′ for NOS terminator region, were used for detecting the integrated gene. Amplification was carried out in a DNA Thermal Cycler (Perkin Elmer) with preheating at 94°C for 2 min., and with 45 cycles of 94°C for 30 sec., 60°C for 2 min., 72°C for 3 min., and then with postheating at 72°C for 7 min. The reaction mixture (10μl) was analyzed by 1. 2 % agarose gel electrophoresis. The amplified DNAs were transferred to a nylon membrane and hybridized with the ^{32}P-labeled 300 bp DNA fragment of the plasmid DNA (pBI121-RCC2) as the probe.

2. 3 Assay of chitinase activity

Fresh leaves 0. 5 g of each transgenic plant were homogenized in 0. 02 M citric acid/0. 04 M Na_2HPO_4 buffer (pH6. 8) and centrifuged at 15, 000 rpm for 15 min. The supernatant was dialyzed against the same buffer for 18 hrs at 4°C and used as a crude enzyme solution. For chitinase reaction, 2 ml of 0. 05 M citric acid/0. 1 M Na_2HPO_4 (pH6. 8) containing 20 mg of carboxymethyl chitin (Ichimaru Pharcos, Japan) was mixed with 1 ml of the crude enzyme solution, incubated with shaking at 37°C for 1 hr, and stopped by the addition of 1 ml of trichloroacetic acid. After centrifugation at 15, 000 rpm for 10 min., the concentration of reducing sugars in the supernatant was measured by a modified Schales' method [10]. One unit (U) was defined as the amount of chitinase which produces 1 μmol of reducing sugars as *N*-acetyl-D-glucosamine per min. under these condition.

2.4 Inoculation of S. humuli

S. humuli (10^5 spores/ml) was inoculated onto leaves of transgenic plants in the isolated greenhouse. Thirty days after inoculation, disease development in surface area of leaves was measured and the percentage of colony area/total leaf area was calculated.

3. Results and Discussion

Leaf-discs (4, 000 explants) and petioles (4, 128 explants) were co-cultivated with *A. tumefaciens* carrying the modified pBI121 containing a rice chitinase gene, and were screened for their ability to form callus on selection medium containing 50 mg/l of kanamycin. All 544 explants showing active callus formation on kanamycin agar plate were selected after 10 weeks. Frequencies of transformation were 6. 2 % and 7. 2 % in leaf-discs and petioles, respectively (**Table 1**). 123 out of 544 calli formed green shoots (22. 6 %) within 10 weeks after transfer to shoot regeneration medium. All the regenerated plants were grown in an isolated greenhouse.

When PCR amplification was done using the template DNAs extracted from the transgenic strawberry leaves and the primers described in Materials and methods, a 300 bp fragment was amplified in transformants, as well as when the plasmid pBI121-RCC2 was used for the template as the positive control. The identity of the PCR amplified chitinase gene was confirmed by Southern hybridization (**Fig. 2**). This suggested that at least a part of the rice chitinase gene was present in the transgenic strawberry. PCR using more upstream primers for the rice chitinase gene was not successful because of the extremely high GC content (74 %) of the gene, which was also observed when pBI121-RCC2 was used as a template.

The chitinase activity and the average colony area of *S. humuli* in leaves of transformed plants were measured. The average chitinase activity of twenty-five transformants had a significantly higher level compare to equivalent non-transformants (**Table 2**). When the transformed strawberries were infected with *S. humuli*, disease development was substantially

Table 1.
Formation of the callus and shoot from explants co-cultured with *Agrobacterium tumefaciens* on the selection medium.

Explant	No. of explants examined	No. of explants formed calli	No. of explants regenerated shoots
Leaf discs	4000	246(6.2 %)*	36(0.9 %)
Petioles	4128	298(7.2 %)	87(2.1 %)
Total	8128	544(6.7 %)	123(1.5 %)

* The values within parentheses indicates the percentage of No. of explants examined.

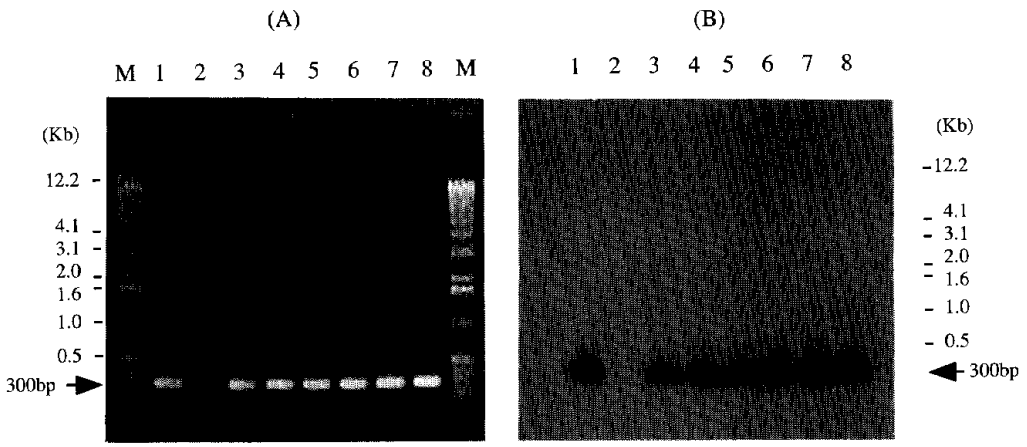

Fig. 2 Amplification of rice chitinase gene fragment by PCR(A) and Southern hybridization analysis(B) in transformed strawberry.
Lane 1, amplification of the 300 bp fragment from pBI121-RCC2 plasmid; lane 2, control (non-transformedplant) ; lanes 3-8; independent transgenic plants; and lane M, DNA 1Kb ladder.

Table 2.
Chitinase activity in transformant leaves and disease development of *S. humuli*.

Plant	GluNAc* (mU/g fresh weight)	Disease development (lesion area/leaf,%)
Transgenic plants	44.0±5.7	22.0±2.5
Control plants	13.8±1.0	40.0±2.7

* *N*-Acetyl-D-glucosamine.
Values represent the means ±SE from twenty-five determinations.

reduced, compared to equivalent non-transformants. The average colony area of transformed plants was 22.0 ± 2.5 % (2.5 %-53.2 %), that of control plants was 40.0±2.7 % (15.5 %-64.7 %) (**Table 2**). There was a correlation between chitinase activity and percentage of areas presenting lesions. As is shown in **Fig. 3**, especially plant C showed a remarkable resistance to *S. humuli* among all the transformants. Our results clearly indicated that the over expression of the chitinase gene caused an enhanced resistance against *S. humuli*.

Effects of the transgene of chitinase on the resistance against the fungal infection might be explained by fungal cell wall lysis by the enzyme or by releasing bioactive cell wall degradation products that elicit the induction of plant defense mechanisms. The first report of success with the transgene of chitinase in disease breeding was the introduction of a bean vacuolar chitinase gene into tobacco and *Brassica napus*, which resulted in partial protection against *Rhizoctonia solani* [11]. Recently Zhu *et al.* [12] have described that the genes encoding the rice chitinase and the alfalfa glucanase caused an enhanced resistance against *Cercospora nicotianae* in transgenic tobacco. Lin *et al.* [13] have also indicated the enhanced resistance against *Rhizoctonia solani* in transgenic Indica rice plants with a rice chitinase gene. Ikeda *et al.* [14] reported that the haustoria of *S. humuli* in strawberry leaves were digested by the treatment with exogenous microbial chitinase. In this report we have shown that introduction of a foreign chitinase gene into strawberry is effective to enhance resistance against *S. humuli*. Assay of the disease-resistance in the clonally propagated progeny of the transformants is now in progress. There would be great interest in the disease breeding of the straw-

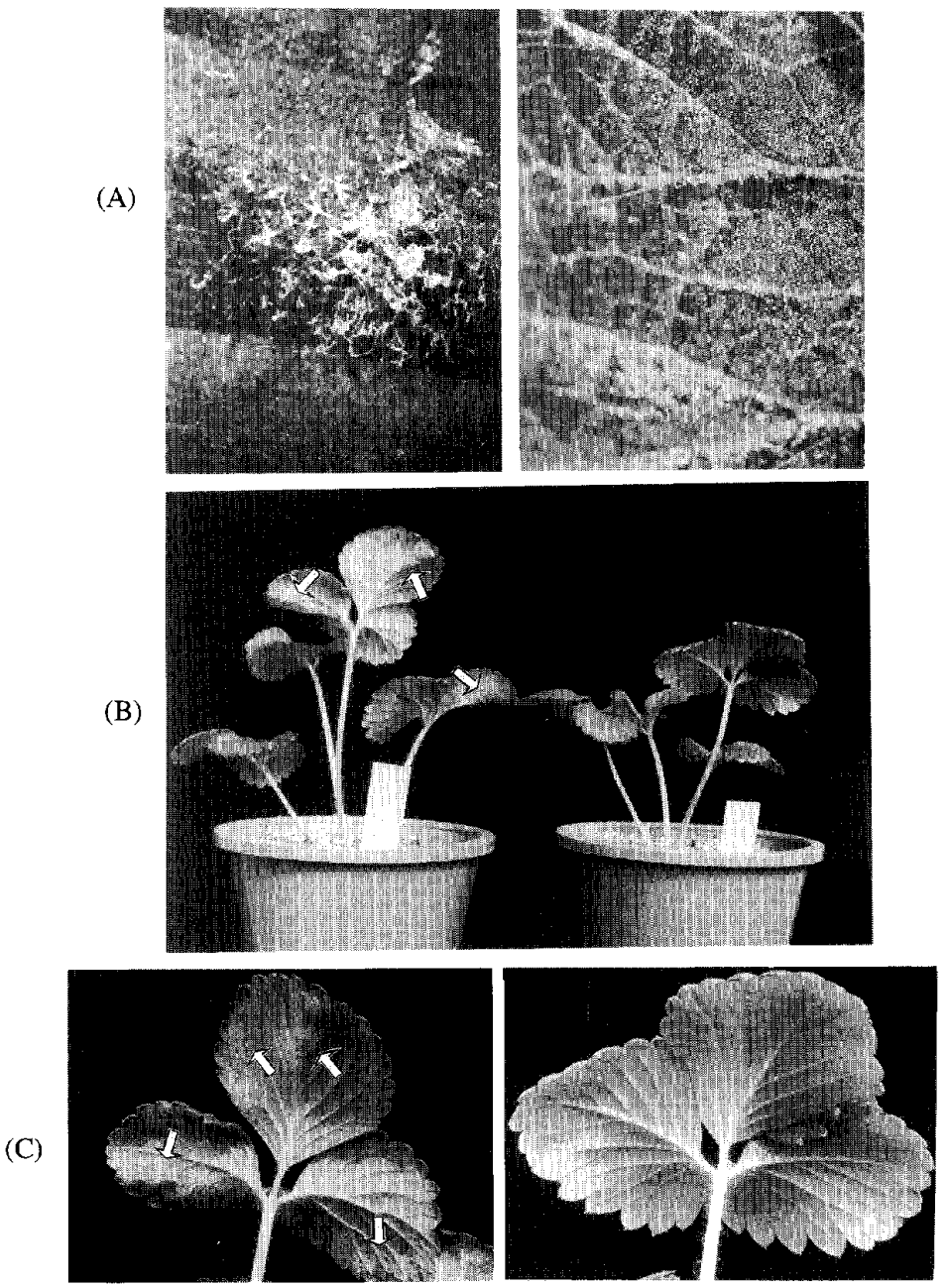

Fig. 3 Resistance of the transformed strawberry against *S. humuli*.
Comparison of control plant (left) and transformant plant C (right). Ten days after inoculation (A) and thirty days after inoculation (B, C).
'⇨'indicate the lesion area.

berry to various fungi through gene manipulations using the chitinase gene.

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