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

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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* carrying plasmid pBII21 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 aseptically 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 pBII21–RCC2. The pBII21–RCC2 was a modified plasmid of pBII21 (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 50 μmol m⁻²s⁻¹. Regenerated plants were transferred to soil and
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 (10 μl) was composed of 10 mM Tris-HCl (pH 8.3), 80 mM KCl, 1.5 mM MgCl₂, 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 AmpliTag DNA Polymerase (Perkin Elmer). The two primers, 5′-TGGATCCAGCGGCTCGGTGTTG-3′ for 3′ proximal coding region of the chitinase gene (added with an artificial BamHI 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 (10 μl) was analyzed by 1.2% agarose gel electrophoresis. The amplified DNAs were transferred to a nylon membrane and hybridized with the 32P-labeled 300 bp DNA fragment of the plasmid DNA (pB1121-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 Na₂HPO₄ 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 Na₂HPO₄ (pH 6.8) containing 20 mg of carboxymethyl chitin (Ichimarу 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 13,000 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 conditions.

2.4 Inoculation of S. humuli

S. humuli (10⁶ 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 pB1121-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 pB1121-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.

<table>
<thead>
<tr>
<th>Explant</th>
<th>No. of explants examined</th>
<th>No. of explants formed calli</th>
<th>No. of explants regenerated shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf discs</td>
<td>4000</td>
<td>246(6.2 %)*</td>
<td>360(0.9 %)</td>
</tr>
<tr>
<td>Petioles</td>
<td>4128</td>
<td>298(7.2 %)</td>
<td>87(2.1 %)</td>
</tr>
<tr>
<td>Total</td>
<td>8128</td>
<td>544(6.7 %)</td>
<td>123(1.5 %)</td>
</tr>
</tbody>
</table>

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

![Fig. 2](image-url) 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 pBI21-RCC2 plasmid; lane 2, control (non-transformed plant); lanes 3-8, independent transgenic plants; and lane M, DNA 1 Kb ladder.

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

<table>
<thead>
<tr>
<th>Plant</th>
<th>GluNAc* (mU/g fresh weight)</th>
<th>Disease development (lesion area/leaf, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transgenic plants</td>
<td>44.0±5.7</td>
<td>22.0±2.5</td>
</tr>
<tr>
<td>Control plants</td>
<td>13.8±1.0</td>
<td>40.0±2.7</td>
</tr>
</tbody>
</table>

* 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 nicotiana 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 thehaustoria 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. laronii*.
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.

References


