Preliminary Communication

Plant Chitinase as a Possible Biocontrol Agent for Use Instead of Chemical Fungicides

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We investigated whether a plant chitinase can be used as a biocontrol agent instead of chemical fungicides by spraying chitinase E (family 19; class IV) from a yam (Dioscorea opposita Thunb) alone or together with β-1,3-glucanase directly onto the surface of a powdery mildew infecting strawberry berries and leaves. Results were observed by eye and with a scanning electron microscope. The powdery mildew infecting the strawberries was degraded, mainly by the chitinase, and the disease did not appear again for more than 2 weeks. These results indicated that this kind of plant chitinase might be safe and biodegradable biocontrol agent for use instead of conventional fungicides.

Key words: biocontrol agent; class IV chitinase; family 19 chitinase; powdery mildew; yam

Nowadays we face a population explosion and environmental pollution, and starvation is occurring in many regions. For production of enough food, some plant diseases are being controlled with chemical fungicides. Unfortunately these chemicals have sometimes caused environmental and health problems. In this study we investigated whether it is possible to use a plant chitinase as a biocontrol agent instead of chemical fungicides.

During their evolution since the beginning of the Devonian period, many plant taxa have evolved sophisticated defense mechanisms that use an array of defense compounds such as pathogenesis-related proteins and phytoalexins. An aggressive form of plant defense involves the production of lytic enzymes such as chitinase, which is classified as a pathogenesis-related protein-3.1) Chitinase (EC 3.2.1.14) is an endo-type enzyme that hydrolyzes chitin, a structural component of the cell walls of fungi, shells of crustaceans, and integument and peritrophic membranes of insects. Chitinases are classified into two families, 18 and 19, on the basis of their amino acid sequences.2) Plants and some microorganisms such as Streptomyces produce both family 18 and 19 chitinases, while the other organisms produce only family 18 chitinases. Plant chitinases have been classified into more than four classes.3) Plant chitinases are constitutively present in certain tissues or reproductive organs such as seeds, tubers, and flowers, and are induced by plant pathogens and insect pests.3) We have purified and characterized several chitinase isozymes from yam tuber.4) Yam chitinase E, in family 19 and class IV, occurs constitutively in the tuber and is induced by elicitors including plant pathogens such as Fusarium oxysporum.5) This enzyme is lytic against this species,6) and is both stable and biodegradable. Another group found that a transgenic strawberry carrying a rice class I chitinase gene has a resistance to powdery mildew.7) However, we have not found a class I chitinase in yams. Both class I and class IV chitinases have a chitin-binding domain in the N-terminal region, so yam class IV chitinase also may act against strawberry powdery mildew. Therefore, we selected chitinase E for study here.

Chitinase E was purified from tubers of yam Dioscorea opposita Thunb by the method of Arakane et al.6) Zymolyase 20T, which is mainly β-1,3-glucanase (EC 3.2.1.39), was purchased (Seikagaku Corp., Tokyo, Japan). Young offshoots of strawberry, Fragaria ananassa Duch., Toyonoka, were individually planted in vinyl pots 12 cm across and 10 cm deep, and pots were placed in a greenhouse without temperature or light control. Three months later, the plants were used for the experiments. Spores of strawberry powdery mildew, Sphaerotheca humuli (de Candolle) Burrill, were collected from infected leaves by brushing. A spore suspension (4.1 × 10⁶ cfu/ml) containing 0.02% Kumiten (Kumiai Chemical Industry Co., Ltd., Tokyo, Japan), a nonionic detergent widely used in Japan as an adhesive agent for crops, and contains mainly polyoxyethylene(n)monylphenyl ether, was

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sprayed onto all parts of healthy strawberry plants. The concentration of Kumiten used was confirmed not to inhibit chitinase activity at all. The inoculated strawberry seedlings were kept under saturated humidity at 27°C for 12 h, and were then grown in a greenhouse at a temperature cycle of 20°C for 18 h and 23°C for 6 h for about 1 month. All enzyme solutions used (0.3 and 3 μM chitinase E, 0.6% Zymolyase 20T and a mixture of 3 μM chitinase E and 0.6% Zymolyase 20T, final concentrations) contained 0.02% Kumiten. A nonenzymatic solution of 0.02% Kumiten was used as the control. Fifty milliliters of one of these enzyme solutions or the control solution was sprayed by hand on each infected strawberry plant. The volume of 50 ml was enough to wet uniformly all parts of the plants with the sample solution. The powdery mildew infecting the berries was observed by scanning electron microscopy (SEM) just before and one week after the single treatment with the enzyme or control solution. The infected parts of strawberry plants were sliced with a knife into pieces measuring 5 × 5 mm and 1 to 2 mm thick. The slices were treated with 2.5% glutaraldehyde for 1 h, dehydrated in an ascending series of ethanol concentration (50, 70, 80, 90, 99, and 100%) twice, for 20 min at each concentration and with t-butyl alcohol for 20 min twice, and then lyophilized for 2 h in a freeze-drying device (JEOL JFD-300). The samples were coated with 20 nm of gold with an ion sputtering device (JEOL JFC-1500) and observed under a SEM (JEOL JSM-6100). The experiments were done repeatedly using strawberry plants infected to similar degrees and different degrees. For the clearest possible comparison of the effects of treatment, we evaluated the data from experiments done with heavily infected strawberry plants.

First we investigated whether the combination of yam chitinase E and Zymolyase 20T was effective on strawberry powdery mildew. We succeeded in causing degradation or lysis of Fusarium oxysporum with a mixture of 1.5 μM yam chitinase E and 3% Zymolyase 20T (final concentrations). Here, we tried using double concentrations of both enzymes. However, treatment of strawberry plants with 6% Zymolyase 20T alone caused browning of the leaves. A one tenth concentration did not brown leaves, so we used a mixture of 3 μM yam chitinase E and 0.6% Zymolyase 20T (final concentrations) for treatment. Most of the white powder on the infected parts of the berries disappeared immediately after being sprayed with this solution. One week later, the disease had disappeared completely, and did not appear again at least for two weeks; infection of the leaves stopped spreading, and the infected spots gradually turned from white to brown (Fig. 1-1). When treatment was with the control solution, the white powder spread over the surface of berries and leaves with time after first mostly disappearing when sprayed (Fig. 1-2). One week after the treatment with the mixture of yam chitinase E and Zymolyase 20T, SEM at low magnification showed that the hyphae of the powdery mildew looked unhealthy, without complete conidia, and being recumbent. Higher magnification showed that the surfaces of the hyphae were severely damaged, with holes in the surfaces, unlike the results with the control solution. These findings suggested that this combination of chitinase E and the β-1,3-glucanase included in Zymolyase 20T hydrolyzed cell-wall components of the hyphae and conidia.

Both visual and SEM observations indicated that the 3 μM yam chitinase E degraded the powdery mildew, but that 0.6% Zymolyase 20T did not. SEM showed that the yam chitinase E damaged both hyphae and conidia severely (Fig. 1-3), but 0.6% Zymolyase 20T did not damage them (Fig. 1-4). Comparison of recovering effect with yam chitinase E alone and the combination of yam chitinase E and Zymolyase 20T showed a synergistic effect of the two preparations, even though Zymolyase 20T alone did not give a recovering effect. These results also suggest that the cell walls of this powdery mildew consist mainly of chitin, with glucan as a minor component.

We investigated if a lower concentration of yam chitinase E caused a recovering effect by changing the concentration from 3 to 0.3 μM. Even this one tenth concentration of yam chitinase E damaged the hyphae and conidia (not shown) although the effects were less than with 3 μM yam chitinase E (Fig. 1-3). No enzyme solution used in this study other than the 6% Zymolyase 20T caused undesired effects in any parts of the strawberry plants.

As a method for crop protection without use of conventional fungicides and pesticides, or with their reduced use, the introduction of a certain chitinase gene into plants has been tried, with some successes. However, transgenic plants, especially transgenic crops, are not being well received by the public. We need to find still other methods that do not involve chemical fungicides, either. Our results showed that yam chitinase E was effective when used together with another pathogenesis-related protein, β-1,3-glucanase (PR-2), to restore health to mildew-infected strawberry plants and prevent the disease for two weeks. As far as we know, this is the first report on direct use of an enzyme, chitinase, as a biocontrol agent instead of chemical fungicides.

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Fig. 1. Effects of Yam Chitinase on Powdery Mildew Infecting Strawberries.
Infected strawberry plants were sprayed with a mixture of 3 μM chitinase E and 0.6% Zymolyase 20T (panel 1), a nonenzymatic solution as the control (panel 2), 3 μM chitinase E alone (panel 3), or 0.6% Zymolyase 20T alone (panel 4). Pathogens on the surface of the berries and leaves were observed by eye (A) just before and 1 day and 1 week after the single treatment, and in detail by SEM (B) one week after the treatment. A, Arrows indicate the infected spots. B, a: surface of a berry; b: overall view of the pathogen; c: conidia; d: surface of the hyphae.
References


