Prevention of the hypersensitive reaction of potato cells to infection with an incompatible race of *Phytophthora infestans* by constituents of the zoospores

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The hypersensitive reaction induced in potato tuber or petiole tissue by infection by an incompatible race was prevented or delayed by prior treatment with a high molecular weight fraction obtained from supernatant of zoospore homogenates of *Phytophthora infestans*. Experiments were carried out with the potato varieties Rishiri (resistant gene *R*), Eniwa (resistant gene *R*), Pentland Ace (resistant gene *R*), and two races of *P. infestans*, race 0 and race 1.

Browning of tissues induced by infection by the incompatible race was reduced by prior treatment with the high molecular weight fraction which was obtained in a void volume of a Sephadex G-25 column. The proportion of unbrowned cells infected by the incompatible fungus increased with increasing concentrations of the fraction. Hypersensitive death of cells treated with the fraction was prevented without significant effects on either the penetration of the host cells by an incompatible race of the fungus or growth of the intracellular hyphae at an early stage of infection. These protective effects of the high molecular weight fraction of zoospore homogenates seemed in part to be host specific for the cultivars and races used.

**INTRODUCTION**

A potato plant cell infected with an incompatible race of *Phytophthora infestans* may die hypersensitively within about 30 min [5, 9]. With a compatible race, on the contrary, the infected potato cell survives far longer in coexistence with the parasite, possibly for 2 to 3 days. This marked difference between compatibility and incompatibility in the host–parasite relationship of potato late blight is controlled by independent Mendelian type genes [2, 3]. However, the question of why a host cell reacts differently to different races has not been answered satisfactorily. Possibly some unknown principle of a pathogen specifically causes the hypersensitive death of an incompatible host cell or some unknown system specifically retards hypersensitive death of the compatible host cell.

Tomiyama reported that a potato cell which has previously been infected by a compatible race loses its capacity to react hypersensitively to infection by an incompatible race [8]. He suggested that reduced metabolic activity of the host cell, caused by contact with the compatible parasite, is responsible for its inability to react rapidly to infection by the incompatible race. However, potato cells in which protein synthesis has been inhibited by prior treatment with blasticidin S retain their ability to react hypersensitively to infection by an incompatible race [1]. Therefore, loss of the ability to react hypersensitively by potato cells previously infected with a
compatible race cannot necessarily be explained solely on the basis of metabolic activity. There is a possibility that compatible races may have some system which delays or prevents the mechanism of hypersensitivity.

As one of a series of attempts to elucidate the mechanism of host–parasite specificity, the present study considers whether some substance(s) in the zoospores affects the hypersensitivity of potato cells to infection by an incompatible race of *P. infestans*.

**MATERIALS AND METHODS**

*Preparation of potato tuber and petiole tissues*

Potato cultivars Rishiri, Eniwa and Pentland Ace, carrying the *R₁*, *R₂* and *R₃* genes for resistance, respectively, were used in this experiment. Tubers were stored in a cold room at 4 °C until used. Tissue cylinders, 18 mm in diameter, were cut out from central parenchymatous tissue with a cork borer. Disks, 3 mm thick, were cut from the cylinders and were washed thoroughly with a large volume of distilled water in order to remove the contents of destroyed cells.

Petioles were obtained from plants grown for about 2 months in a glasshouse. The petioles were cut longitudinally with a razor blade and washed with distilled water. These prepared disks and petioles were incubated in a moist chamber at 18 °C.

*Preparation of zoospore suspensions*

Two races of *P. infestans* (0 and 1) were used. Race 0 is incompatible with Rishiri, Eniwa and Pentland Ace. Race 1 is compatible with Rishiri and Eniwa, but incompatible with Pentland Ace.

The zoosporangial suspensions were obtained from mycelial mats growing on the cut surfaces of fresh tubers (Irish Cobbler, cv., bearing the *r* gene which confers compatibility to all races of *P. infestans*) at 18 °C, about 7 days after inoculation. Zoosporangia were well-washed with distilled water and collected over filter paper (Toyo No. 2, Toyoroshi Co. Ltd). A suspension of zoospores was prepared by incubating the zoosporangia in distilled water at 10 to 12 °C for about 2 h, then centrifuging at 250 g for 2 min to eliminate ungerminated zoosporangia. The concentration of zoospores was adjusted by dilution after counting in a haemacytometer.

*Extraction of zoospore constituents*

Zoospore suspensions stored at −30 °C were thawed, combined with 0·1 m-Tris–HCl buffer, pH 7·4, to make a final concentration of 0·01 m-buffer, and homogenized in a Teflon–glass homogenizer in an ice bath for 5 min. The homogenate was centrifuged at 10 000 g for 30 min at 4 °C. The supernatant was passed through a Sephadex G 25 column (1·4 × 27 cm) equilibrated with 0·01 m-Tris–HCl buffer, pH 7·4, in a cold room at 4 °C. A high molecular weight fraction in the void volume was usually used for assay. The concentration of this fraction was tentatively represented as optical density at 260 nm because the fraction has a maximum absorption at this wavelength.
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Assay

The high molecular weight fractions, henceforth termed extracts, were assayed for their ability to prevent the hypersensitive reaction of potato cells. Fifty μl samples of solution of extracts were applied to the surfaces of tuber disks in a moist chamber at 18 °C, or alternatively the surface of cut petioles was brought into contact with cotton cloths containing appropriate amounts of the solution in Petri dishes at 18 °C. These treatments were carried out more than 5 h after tissues were cut. The treated tissues were inoculated using the same method by which extracts were applied, with zoospore suspension of an incompatible race of *P. infestans* at a concentration of $1.2 \times 10^5/\text{ml}$, 23 to 24 h after the tissues were cut.

Two methods of assay were employed; one by microscopic observation of unbrowned cells of tuber tissues infected by an incompatible race 24 h after inoculation, the other by microscopic determination of the amount of cell death in petiole tissues using loss of vital staining ability with $10^{-4} \text{M-neutral red}$ and the absence of protoplasmic streaming in infected cells as criteria. The sections for microscopic observation were made by hand with a razor blade.

RESULTS

Reduction of browning of tissues

Cut surfaces of tuber tissues of Rishiri, Eniwa and Pentland Ace were treated with extracts from race 1 or race 0 18 h before inoculation with race 0. Table 1 shows the intensity of browning of the tissues caused by infection by the incompatible race.

**Table 1**

<table>
<thead>
<tr>
<th>Prior treatment</th>
<th>Rishiri ($R_1$)</th>
<th>Eniwa ($R_2$)</th>
<th>Pentland Ace ($R_3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.01 M-Tris-HCl buffer, pH 7.4)</td>
<td>+++++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Extract from race 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Extract from race 0</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Disks, 18 mm in diameter, were inoculated with 50 μl of $1.2 \times 10^6$ zoospores/ml.
* ++ + + + Strongest hypersensitive reaction.
* Concentration, 0.4 (O.D.) at 260 nm.

In the case of treatments within the compatible combinations, Rishiri and race 1 and Eniwa and race 1, a reduction of browning was observed.

Disks of Rishiri tuber tissue were inoculated with zoospores of incompatible race either at the same time as treatment with extracts of compatible zoospores, or, alternatively, 18 h after application of the extracts. Browning was markedly decreased after pre-treatment with extracts, but not by simultaneous application of extracts and inoculum (Plate 1).

Unbrowned cells infected by an incompatible race

The observation of browning of tissue did not necessarily provide an accurate determination of the hypersensitive reaction because there was a difference in the
activity of oxidative metabolism such as phenolic metabolism between the cultivars. Therefore, a method to observe microscopically infected unbrowned cells, which still exhibited protoplasmic streaming, was examined and, also, browning of cells in the tissue treated only with extracts was observed. There was a linear correlation between the proportion of host cells, which had browned in response to infection by an incompatible race and the concentration of zoospores in the inoculum up to \( 8 \times 10^4 \) zoospores/cm² of tissue (Fig. 1). Extracts and infection by the compatible race caused little browning of cells (Fig. 1).

![Graph](image)

**Fig. 1.** Effects of zoospore concentration in the inoculum and as a source of extracts on cellular browning. Tissue disks, 18 mm in diameter, were inoculated by 50 μl of zoospore suspension at the concentration shown or treated with 50 μl of extract derived from a zoospore suspension of similar concentration. About 1000 cells, in total, from two different experiments were observed under a microscope 24 h after inoculation or treatment. (----) Inoculated; (-----) treated; (●), race 0; (○) race 1.

Disks of Rishiri tuber tissue were treated with various concentrations of extract of race 1. The treated disks were then inoculated with \( 1.2 \times 10^8 \) race 0 zoospores/ml. Figure 2 shows that the proportion of unbrowned infected cells increased with the concentration of extract applied. It was also observed that some uninfected cells next to infected unbrowned cells began to show browning 48 h after inoculation. Furthermore, no mycelial mats grew on the tissue treated with extracts.

The percentage of unbrowned infected cells in Rishiri tissue, treated with extract from the incompatible race 0, was compared with that in tissue treated with extract from the compatible race 1. Fifty μl of extract was applied to disks at a concentration of 0.4 (OD) at 260 nm. The disks were inoculated with \( 8 \times 10^6 \) race 0 zoospores/cm² 18 h later, and unbrowned infected cells were observed 24 h after inoculation. The proportion of infected cells which did not become brown was similar in several
PLATE 1. Effect of treatment with extract of compatible race 1 applied 18 h before inoculation with incompatible race 0 on hypersensitive reaction of potato (Rishiri, $R_1$). The extract was obtained in the void volume of a Sephadex G-25 column (1.4 x 27 cm) from 5 ml of zoospore suspension ($5 \times 10^6$/ml) in 0.01 M-Tris-HCl buffer (pH 7.4). Tuber disks were inoculated with 50 μl of zoospore suspension ($1.2 \times 10^6$/ml). The upper two rows of disks were inoculated 18 h after treatment with extract, the lower two rows of disks simultaneously with extract. A, Control; the upper two disks treated with extract only, the lower two disks inoculated without extract. B, C and D were treated with extracts from replicate preparations.
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experiments using extracts from a compatible race, but it was lower and more variable in experiments using extracts from an incompatible race (Table 2). A decrease in the hypersensitive reaction of cells was thus strongly and consistently induced by treatment with extract from a compatible race.

![Graph](image)

**Fig. 2.** Effect of different concentration of extract from compatible zoospores (race 1) on the development of the hypersensitive reaction of potato tuber tissues (Rishiri, $R_R$). Tuber disks, 18 mm in diameter, were inoculated with 50 μl of $1\times10^8$ zoospores/ml of race 0 18 h after treatment with 50 μl of extract of race 1 at various concentrations. The concentration of extract was expressed on the basis of optical density at 260 nm.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% of unbrowned cells infected by an incompatible race $R_0$</th>
<th>% of unbrowned cells infected by an incompatible race $R_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Race 0</td>
<td>Race 1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>64.0</td>
<td>77.5</td>
</tr>
<tr>
<td>2</td>
<td>32.5</td>
<td>68.2</td>
</tr>
<tr>
<td>3</td>
<td>18.5</td>
<td>77.3</td>
</tr>
<tr>
<td>Mean</td>
<td>38.3</td>
<td>74.3</td>
</tr>
</tbody>
</table>

\[a\] About 300 cells were observed.

\[b\] Tuber disks were treated with extract at a concentration (o.d. = 0.4) 5 h after being cut, and they were inoculated by 50 μl of $1\times10^8$ zoospores/ml of race 0 18 h after treatment.

**Prevention of hypersensitive cell death**

The incompatible fungus normally caused necrosis of cells at the invasion site about 10 to 15 h after infection. Decreases in the rate of this hypersensitive reaction to race 0 were studied using petiole tissue at an early stage of infection by observing
penetration of host cells by the fungus, growth of intracellular hyphae and cell death as judged by loss of stainability with neutral red and cessation of protoplasmic streaming. Figure 3(a) shows that treatment with extract of race 1 had no significant effect on penetration. Figure 3(b) shows that there was also no significant effect on the growth of intracellular hyphae. Figure 3(c) shows the marked effect of pre-treatment with the fungal extract on the rate of hypersensitive cell death. Most

infected non-treated cells died within 5 h after inoculation, but more than 30% of the infected treated cells survived up to 7 h. Furthermore, about 30% of infected treated cells survived for 24 h after inoculation without browning. These results indicate that many cells pretreated with extract from compatible zoospores lose their ability to react hypersensitively to an incompatible fungus without a significant effect on penetration of host cells and elongation of intracellular hyphae.

DISCUSSION
It has been reported that the hypersensitive reaction of potato cells to an incompatible race of *P. infestans* was decreased by various means, such as a pre-infectional heat treatment [7], post-infectional treatment with 2,4-dinitrophenol [6] or sodium azide [9], and prior infection with a compatible race of *P. infestans* [8]. In addition, it is
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evident from the present results that high molecular weight components in the supernatant of zoosporial homogenates of *P. infestans* diminishes the hypersensitive reaction (Plate 1, Figs 2 and 3, Tables 1 and 2). The preventive effect of the extract was time (Plate 1) and concentration (Fig. 2) dependent, and it seemed to be restricted to the cells directly treated with the extracts.

The mechanism of the decrease of the hypersensitive reaction is still unknown. The changes in the hypersensitive reaction previously reported by Tomiyama [2, 6, 8, 9] has led to the suggestion that the higher the metabolic activity the more rapid is hypersensitive cell death. It was, however, found recently that cells in which protein synthesis is strongly inhibited by blasticidin S respond normally, with rapid cell death, to infection by an incompatible race [1]. Therefore, hypersensitivity of cells may not be necessarily related to their metabolic activity. Loss of capacity of cells to react hypersensitively after treatment with a compatible race may result from the retardation or masking of the mechanism of cell death. The prevention of the hypersensitive reaction by constituents of *P. infestans* may be caused by a similar process. Some components in extracts of zoospore may be involved in maintenance of compatibility with host cells.

As to the problems of specificity and function of these fractions in the host–parasite relationship, further work is necessary. Some properties of the protective factors will be reported in future papers.

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