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Research Notes

Changes in Protein Patterns Resulting from Infection of Rice Leaves with *Xanthomonas oryzae* pv. *oryzae*

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***Xanthomonas oryzae* pv. *oryzae* causes bacterial leaf blight disease in rice (*Oryza sativa*). To understand symptom expression and host-regulated responses at the molecular level in infected rice plants, we analyzed changes in soluble protein patterns by two-dimensional polyacrylamide gel electrophoresis. Total soluble proteins were isolated from leaves over a time course of 4, 18, 24, 48, and 72 hr after bacterial infection. In host plants that responded to infection, 21 polypeptides showed changes. Ten polypeptides increased in response to infection. One polypeptide decreased during infection. A class of 10 new polypeptides was induced by infection. Particularly noteworthy were two polypeptides that dramatically increased in level during infection. These polypeptides had low molecular sizes (20 kDa) and acidic pI values characteristic of "pathogenesis-related" proteins (PR proteins), which play an important role in plant-microbe interactions.**

Additional keyword: bacterial leaf blight disease.

Xanthomonas oryzae pv. *oryzae* (Ishiyama) Dye is the causal agent of bacterial leaf blight of rice, which is a widely distributed rice disease in Asia (Ou 1972). Bacterial leaf blight reduces rice yield by 20–30%, and in extreme cases losses may approach 50% (Reddy *et al.* 1979). This disease has been studied mostly from a morphological and physiological point of view (Barton-Willis *et al.* 1989; Guo and Leach 1989; Horino 1984; Horino *et al.* 1982; Jones *et al.* 1989; Mew 1987; Mew *et al.* 1982,1984), and molecular mechanisms of resistance to bacterial leaf blight in rice have not been investigated to date. The defense reactions of plants to infections of viruses, viroids, bacteria, or fungi are active processes that depend on the rapid, coordinated induction of a series of host genes (Bell *et al.* 1986). The development of disease resistance is correlated with the accumulation of host-synthesized polypeptides (Broglie *et al.* 1986). Proteins that accumulate in the pathogen-infected plant tissue in response to the infection are termed "pathogenesis-related" (PR) proteins (Antoniw *et al.* 1980). Several PR proteins have recently been identified in different plants (Fritzemeier *et al.* 1987; Redolfi 1983). Typically, PR proteins are acid-extractable, low molecular size proteins that accumulate in the intercellular spaces.

We are interested in understanding the molecular bases of symptom expression and host-regulated responses in bacterial leaf blight of rice. Because of reports that PR proteins accumulate in infected plants, our initial investigations have focused on an analysis of the changes in total soluble protein patterns that occur in response to infection of rice by *X. o.* pv. *oryzae*. In this study, we have used two-dimensional polyacrylamide gel electrophoresis (PAGE) to identify quantitative and qualitative changes that occur in the soluble proteins of pathogen-infected rice leaf tissues.

Seeds of the rice (*Oryza sativa* L.) cultivar Sum-Jin were germinated in vermiculite in a greenhouse environment under natural light. Seedlings were transplanted to pots containing soil supplemented with Complete Fertilizer 645 (Miwon Co., Seoul, Korea), and the plants were grown in a greenhouse for 70 days. *X. o.* pv. *oryzae* strain K2-115 was obtained from the Agricultural Sciences Institute, Suweon, Korea. Bacteria were cultured on Wakimoto's medium (Wakimoto 1955) for 2 days, washed with distilled water, and harvested by centrifugation at 4,000 × *g* for 20 min. The inoculum was suspended in distilled water

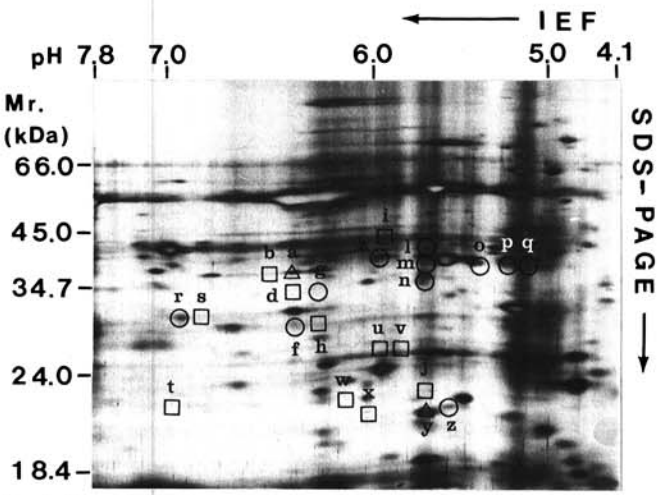

Fig. 1. Two-dimensional polyacrylamide gel analysis (PAGE) of total soluble proteins extracted from leaves of intact, control rice plants. Symbols: □ = induced; ○ = increased; △ = decreased protein level by water treatment or bacterial infection compared to the control. IEF = isoelectric focusing.

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to an OD of 1.0 at 600 nm (about 10^9 cells per milliliter) (Mew and Vera Cruz 1979). Inoculation (two to four sites per leaf) was done by the modified single needle-prick method (Hsieh and Buddenhagen 1974). Bacteria-infected or water-treated (wounded) control leaves were harvested at 4, 18, 24, 48, and 72 hr after treatment. For each experiment, 1 g of leaf tissue, from which the infection sites were removed, was homogenized in 1–1.5 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 2% Triton-100). The homogenates were centrifuged at 15,000 × *g* for 30 min at 4° C, and the resulting supernatants were dialyzed in 30% sucrose solution for 2 hr at 4° C. Protein was measured according to the method of Lowry *et al.* (1951). Polypeptides were separated by two-dimensional PAGE as described by O'Farrell (1975). For the first dimension, the isoelectric-focusing (IEF) gel contained 4% acrylamide, 9.0 M urea, 2% NP-40, and 2% carrier ampholytes consisting of a mixture of 4:5 Ampholites (pH 5–7) and 1:5 Ampholites (pH 3.5–10) (LKB, Bromma, Sweden). The lower reservoir was filled with 0.01 M H_3PO_4, and the upper reservoir was filled with 0.02 M NaOH. IEF was performed for 20 min at 200 V, 30 min at 300 V, 30 min at 400 V, and 16.5 hr at 800 V. The IEF gel was extruded into 0.0625 M Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 5% mercaptoethanol. The gel was shaken in this buffer solution at room temperature for 30 min. For the second dimension, the SDS gel contained 12% acrylamide, 0.375 M Tris-HCl (pH 8.8), and 0.1% SDS. The 3% stacking gel contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS. Electrophoresis was performed at 50 V for 1 hr and then at 100 V until the bromophenol blue reached the bottom of the gel. The migration buffer was 0.192 M glycine, 0.025 M Tris-HCl (pH 8.3), and 0.1% SDS. The following protein molecular mass markers were used: bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), pepsin (34.7 kDa), trypsinogen (24 kDa), and β-lactoglobulin (18.4 kDa). After electrophoresis in the second dimension, the gel was fixed in a solution

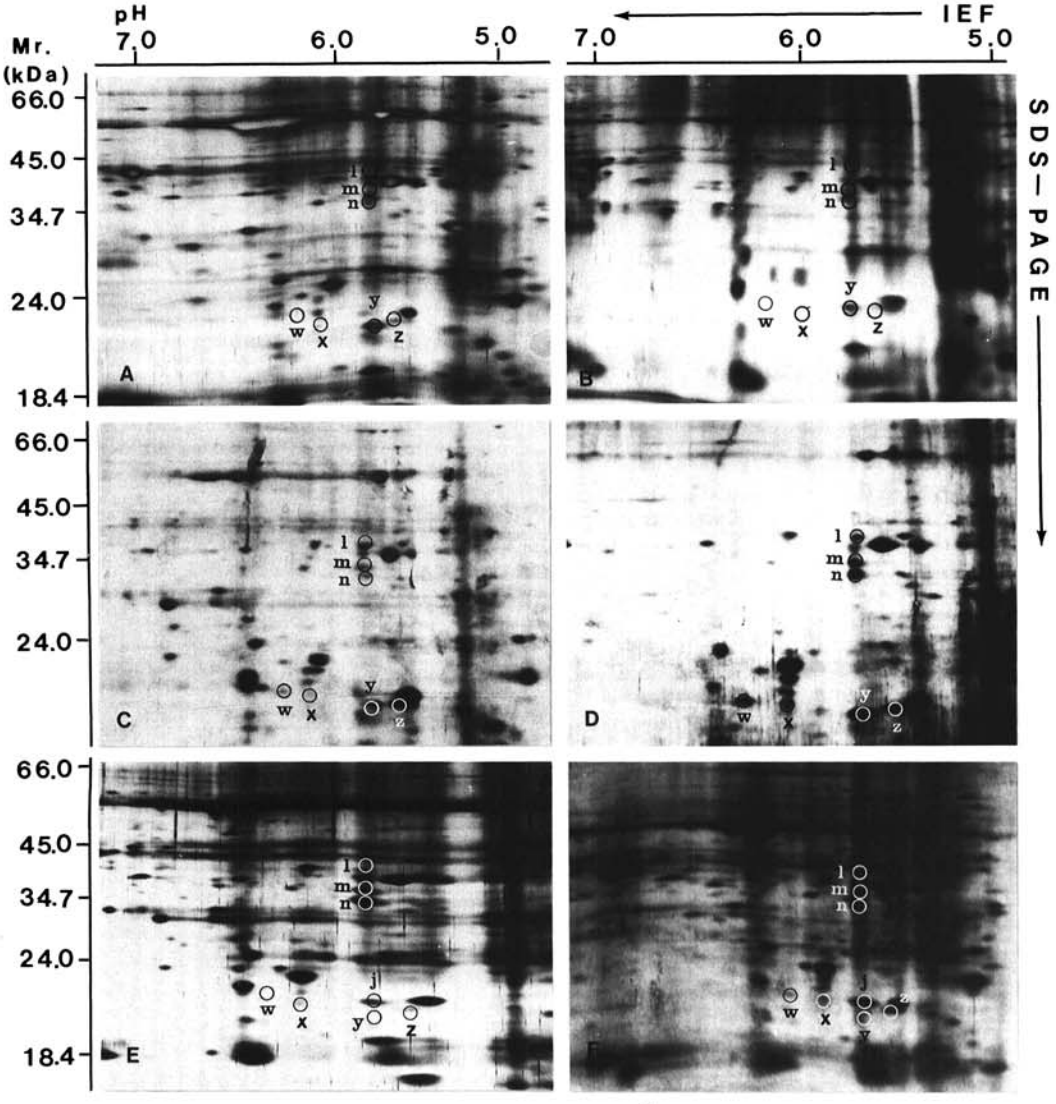

Fig. 2. Time course of changes in the patterns of soluble proteins from leaves of water-treated rice plants. **A,** Control; **B,** 4 hr after water treatment; **C,** 18 hr after water treatment; **D,** 24 hr after water treatment; **E,** 48 hr after water treatment; and **F,** 72 hr after water treatment. Polypeptides marked with circles were those in which changes were observed during the water treatment. IEF = isoelectric focusing.

containing 50% methanol and 12% acetic acid. Silver-staining was performed according to Merril (1981).

Biological symptoms of infection. Inoculation of rice with *X. o.* pv. *oryzae* strain K2-115, which is highly virulent, resulted in typical leaf blight symptoms. The site of injury turned yellow within 1–2 days after inoculation. These symptoms spread out along the veins of the leaves and were observed on whole leaves at 7–10 days post-inoculation. To determine if any changes in protein pattern were associated with disease development in infected plants, we extracted total soluble proteins from the leaf tissues at different times after treatment and analyzed the extracts by two-dimensional PAGE.

Changes in soluble proteins resulting from wounding of healthy plants. The pattern of soluble proteins extracted from wounded tissues treated with distilled water differed from that of untreated control tissues when analyzed by two-dimensional PAGE. The total number of soluble proteins detected in extracts of control tissues was about 200. The molecular sizes of the polypeptides ranged from 18.4 to 66.0 kDa, and pIs ranged from pH 4.1 to 7.8. Figure 1 shows the pattern of soluble proteins in the extracts from control plants. The protein patterns of wounded leaves differed both qualitatively and quantitatively from the patterns obtained with proteins extracted from untreated control leaves. Figure 2 shows the time course events, which were analyzed at 4, 18, 24, 48, and 72 hr after physical damage to rice leaves. Seventeen polypeptides displayed changes as a result of wounding. Seven polypeptides quantitatively increased, one polypeptide decreased, and nine polypeptides were detectable only after leaves were wounded (Table 1). For example, quantitative increases were observed in polypeptides l, m, n, and z. These polypeptides were hardly detected in control tissues, but in wounded tissues they gradually increased to higher levels. The change in polypeptide z was particularly striking; it reached maximum levels at 24 hr after treatment. Polypeptides w and x were not detected in control tissues, but appeared in wounded tissues at 24 hr after treatment. Maximum changes in the protein patterns of wounded leaves, compared with those of the control leaves, were detected at 72 hr after leaves were wounded. The wounding stress resulted in dramatic changes in polypeptides j (22.0 kDa) and y (19.0 kDa), both with a pI of 5.7. Polypeptide j, which was not detected in the control, was increased highly in wounded leaves analyzed 72 hr after leaves were wounded. Conversely, polypeptide y, which was present in control leaves, was hardly detected in wounded leaves analyzed 72 hr after leaves were wounded.

Changes in soluble proteins resulting from infection by *X. o.* pv. *oryzae*. Rice leaves infected with *X. o.* pv. *oryzae* strain K2-115 were harvested at 4, 18, 24, 48, and 72 hr after treatment, and the patterns of soluble proteins were analyzed by two-dimensional PAGE. Figure 3 shows the time course of events that occurred in rice leaves as a result of infection with *X. o.* pv. *oryzae*. Twenty-one polypeptides displayed changes during infection. Ten polypeptides increased in level, one polypeptide decreased, and 10 new polypeptides were detected after infection (Table 1). The overall protein patterns were similar to those observed in wounded leaves, but some changes were characteristic of infected tissues and were not observed in wounded tissues treated with water. For example, polypeptide a decreased in level, and polypeptides i and t were newly detected in response to infection. Polypeptides l, m, and n also increased after infection and after wounding. We were particularly interested in the polypeptides of 20.0 kDa (polypeptides w and z) and 41.0 kDa (polypeptides o, p, and q), which were significantly changed in infected leaves. Polypeptide w was synthesized immediately after bacterial infection and increased to maximum levels at 24 hr. After that time, the polypeptide level decreased. On the other hand, polypeptide w was synthesized at 18 hr after being wounded and reached maximum levels at 24 hr. Thus, the time of synthesis of polypeptide w was advanced by infection. Polypeptide z increased to high levels at 24 hr both after wounding and infection, but it was hardly detected after this time. Polypeptides o, p, and q increased within 4 hr after infection and reached a plateau in level after 18 hr.

In the investigation reported here, we have observed that several cytoplasmic soluble proteins displayed characteristic changes in wounded and infected leaves. The level of some proteins increased; other proteins decreased in response to infection. Another class of polypeptides, which had not been detected in healthy rice leaves, was induced by infection. Most of these changes were also observed in wounded rice plants treated with water. However, there were differences between infection-inducible and wound-inducible proteins. Two sets of proteins were distinguished. One set is composed of proteins that were synthesized at high relative levels at the onset of the infection but hardly detected in wounded tissues (i, o, p, and q). The other

Table 1. Changes in polypeptides extracted from rice leaves wounded with water treatment or infected with *Xanthomonas oryzae* pv. *oryzae* compared with polypeptides of the control leaves

Polypeptides	Size (kDa)	pI	Changes[a]	
			Wounding	Infection
a	40	6.3	NC	↓
b	44	6.5	+	+
d	43	6.3	+	+
f	30	6.5	↑	↑
g	33	6.2	↑	NC
h	30	6.3	+	+
i	44	5.9	NC	+
j	22	5.7	+	NC
k	41	5.9	NC	↑
l	42	5.7	↑	↑
m	41	5.7	↑	↑
n	36	5.7	↑	↑
o	41	5.4	NC	↑
p	41	5.3	NC	↑
q	41	5.2	NC	↑
r	32	6.9	↑	↑
s	32	6.8	+	+
t	22	6.9	NC	+
u	27	5.9	+	+
v	27	5.8	+	+
w	20	6.1	+	+
x	19	6.0	+	+
y	19	5.7	↓	NC
z	20	5.5	↑	↑

[a] + Indicates polypeptide induced by the indicated treatment. Arrows represent increase (↑) or decrease(↓). NC, not changed.

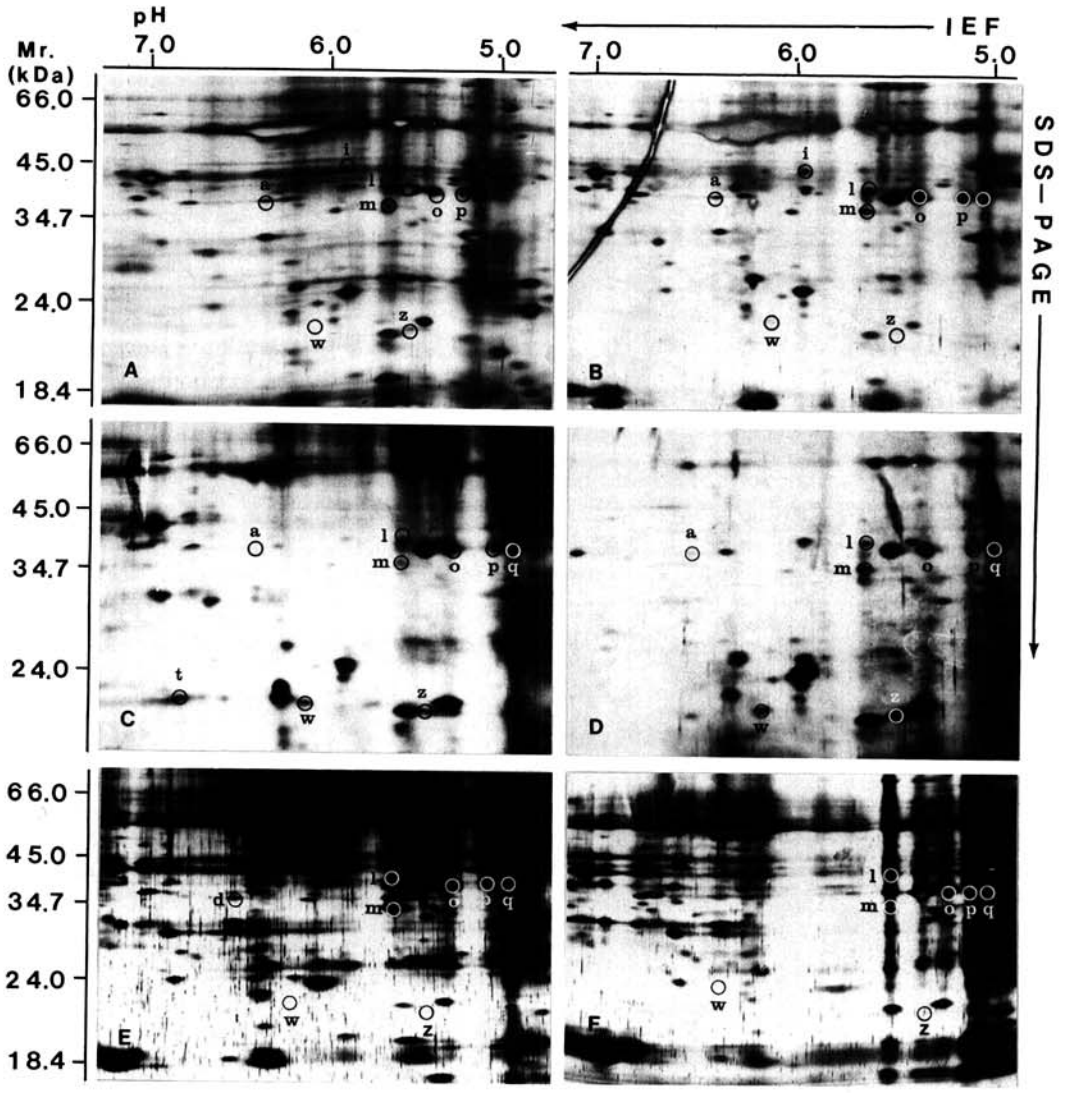

Fig. 3. Time course of changes in the patterns of soluble proteins from rice leaves inoculated with *Xanthomonas oryzae* pv. *oryzae*. **A,** Control; **B,** 4 hr after bacterial infection; **C,** 18 hr after bacterial infection; **D,** 24 hr after bacterial infection; **E,** 48 hr after bacterial infection; and **F,** 72 hr after bacterial infection. Polypeptides marked with circles were those in which changes were observed during the bacterial infection. IEF = isoelectric focusing.

set of proteins was detected in both infected and wounded leaves, but synthesis of polypeptides was advanced by infection (l and w). Some of the 21 polypeptides whose levels were influenced by infection with *X. o.* pv. *oryzae* could be candidates for PR proteins; PR proteins have functions specific to the host-pathogen interaction. Polypeptides o, p, and q are particularly strong candidates; they have molecular sizes (41.0 kDa) similar to those of β-1,3-glucanase (36.0 kDa), which accumulated in intercellular spaces after inoculation with *Phytopthora infestans* (Mont.) de Bary in potato plants (Kombrink *et al.* 1988). Polypeptides w and z also may be classified as wound-inducible PR proteins, because they are acidic and have low molecular sizes (20.0 kDa), which are characteristics of PR proteins (Van Loon 1985).

This is the first report that examines PR proteins produced in rice in response to infection with *X. o.* pv. *oryzae*. Further studies will focus on determining the function of these PR proteins in rice.

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