Initiation of Induced Nonhost Resistance of Oat Leaves to Rust Infection

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ABSTRACT

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Cross-protection experiments were made by first inoculating the abaxial surfaces of primary leaves of oats (cultivar Shokan 1) with oat-noncompatible rust fungi (*Puccinia coronata festucae, P. coronata lolii,* or *P. graminis tritici*) and later inoculating the adaxial surfaces with compatible race 203 of *P. coronata avenae*. The oat nonpathogens were heat-killed at various times after the first inoculation, and the subsequent inhibition of development of the challenge pathogen inside the leaf was used to assay induction of nonhost resistance. Elongation of intercellular hyphae and haustorium formation by race 203 were reduced when the second inoculation was made 12 hr or more, but not 8 hr, after the first inoculation. When stomatal penetration by *P. graminis tritici* was controlled by exposing plants to light at various times after the first inoculation, it was found that reduction of development of challenge race 203 occurred only after the oat nonpathogen had penetrated and produced substomatal vesicles. The initiation of nonhost resistance was not related to postinoculation time or formation of appressoria over stomates, but it was dependent on the formation of substomatal vesicles.

Additional key words: Avena sativa, crown rust, cross-protection, stomatal penetration, light radiation.

Several reports (6,8,9,11) indicate that appressorium formation by rust fungi is controlled by factors constitutively present on plant surfaces, which suggests that nonhost resistance at the prepenetration stage of rust infection requires no activation of defense mechansisms. On the contrary, restricted development of rust fungi within nonhost tissues after penetration is considered to be due to induced defense reactions. For example, Leath and Rowell (5) suggested the possible involvement of phytoalexins in the immune reaction of corn leaves to *Puccinia graminis*. Heath (2,3) compared morphological changes of plant tissues following infection with various rust fungi of different genera and proposed that several active mechanisms are involved in nonhost resistance. Yarwood (12), Johnston and Huffman (4), and Littlefield (7) demonstrated that the development of rust diseases on normally susceptible plants was reduced by previous inoculation with rust fungi which were pathogenic on other plant species. Such cross-protection phenomena are conceived at present to be induced resistant reactions.

In a previous paper (9), we indicated that the resistance of oat leaves to rust fungi pathogenic on gramineous plants other than oats was inducible and possibly linked with the synthesis of ribonucleic acid and protein which was activated subsequent to stomatal penetration. The present study was designed to detect the postinoculation time at which the nonhost resistance was initiated against the infection with the rust fungi by assaying the growth and development of a challenging compatible rust race as an assay.

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MATERIALS AND METHODS

Unless otherwise specified, the materials and methods used in this study were the same as those used in earlier studies (9,10). Seedlings of oat (*Avena sativa* L.) cultivar Shokan 1 were grown throughout the experiments at 23 C under continuous fluorescent illumination of 8,000 lux at leaf level. When neccessary, seedlings were placed in the dark or under illumination at 2,000 to 12,000 lux.

Double inoculation methods were employed for determining the initiation time of the response. All inoculations were done by applying uredospores with a soft hair brush. Numbers of uredospores were controlled to give appressoria over stomates at about 50 and 10% of total stomates on inoculated area for the first and second inoculations, respectively. The first inoculation was made onto the abaxial surface of primary leaves of 7-day-old seedlings with uredospores of *Puccinia coronata* Cda. f. sp. *avenae* Fraser & Led. (oat crown rust) race 226 (incompatible with the host cultivar Shokan 1), *P. coronata* Cda. f. sp. *festucae* Eriks. & Henn. (tall fescue crown rust), *P. coronata* Cda. f. sp. *lolii* Eriks. & Henn. (Italian ryegrass crown rust), or *P. graminis* (Pers.) f. sp. *tritici* Eriks. & Henn. race 56 (wheat stem rust). At various times after inoculation, the inoculated leaves were dipped into deionized water

at 50 C for 15 sec to kill the inocula, and subsequently uredospores of race 203 (compatible with host cultivar Shokan 1) were reinoculated onto the adaxial surface. The development of race 203 was not affected significantly by the heat treatment before inoculation as shown in Tables 1, 2, and 3.

For the observations of infection structures of the first inocula and penetrated stomata, leaf epidermis from inoculated sides was peeled off before the heat treatment and stained with cotton blue in lactophenol. The peeled leaves were not used for further inoculation. For the estimation of fungal development of the challange race 203 within leaf tissues, leaf segments were sampled 48 hr after the second inoculation and stained with a whole-leaf clearing method (10). Infected sites, each of which was invaded by a single uredospore, were randomly selected for the measurement of hyphal length and number of haustoria by optical microscopy. One hypha is regarded in this paper as all hyphae, including branches, originating from one substomatal vesicle, and number of haustoria indicates those formed on these hyphae.

TABLE 1. Development of race 203 of *Puccinia coronata avenae* on Shokan 1 oat leaves which previously had been inoculated with race 226 of *P. coronata avenae*, *Puccinia coronata festucae*, *Puccinia coronata lolii*, or *Puccinia graminis tritici*[a]

First inoculum	Interval between inoculations (hr)	Development of the challenge race 203: Length of hyphae (μm)	Haustoria[b] (no.)
None (nonheated)[c]	···	239.2 AB	2.24 AB
None (heated)[c]	···	281.9 A	2.45 A
P. coronata avenae race 226	8	268.2 AB	2.69 A
	12	110.2 C	0.58 CD
	16	97.4 C	0.48 D
P. coronata festucae	8	238.3 AB	2.08 B
	12	102.9 C	0.55 CD
	16	88.3 C	0.53 CD
P. coronata lolii	8	292.3 A	2.33 AB
	12	107.6 C	0.80 C
	16	75.1 C	0.48 D
P. graminis tritici	8	211.9 B	1.75 B
	12	129.3 C	0.78 C
	16	114.4 C	0.61 C

[a] Leaves were inoculated with the first inoculum, incubated at 8,000 lux for 8–16 hr, dipped into hot water and reinoculated with race 203. Development of race 203 was measured 48 hr after the second inoculation. Data are the means of two replications, with five seedlings per replicate and 10 infected sites per seedling. Different letters after each datum in the same column indicate significant differences by Duncan's multiple range test ($P = 0.05$).
[b] Number of haustoria per total hyphae observed.
[c] Leaves of seedlings grown under continuous illumination of 8,000 lux were inoculated with race 203 either without or following the heat treatment.

TABLE 2. Development of race 203 of *Puccinia coronata avenae* on Shokan 1 oat leaves which had been previously inoculated with race 226 of *P. coronata avenae*, *Puccinia coronata festucae*, *Puccinia coronata lolii*, or *Puccinia graminis tritici* and incubated in the dark[a]

First inoculum	Stomatal penetration from the first inoculum (%)[b]	Development of the challenge race 203: Length of hyphae (μm)	Haustoria (no.)[c]
None (non-heated)[d]	···	228.1 A	2.58 A
None (heated)[d]	···	253.8 A	2.58 A
P. coronata avenae race 226	44.5 A	105.9 B	0.85 B
P. coronata festucae	51.3 A	114.2 B	0.99 B
P. coronata lolli	64.6 A	110.5 B	0.71 B
P. graminis tritici	1.9 B	225.8 A	2.48 A

[a] Leaves were inoculated with the first inoculum, incubated in the dark for 12 hr, dipped into hot water, and reinoculated with race 203. Stomatal penetration was measured before the hot water treatment and development of race 203 was measured 48 hr after the second inoculation. Data are the means of two replications, with five seedings per replicate and 10 infected sites per seedling. Different letters after each datum in the same column indicate significant differences by Duncan's multiple range test ($P = 0.05$).
[b] Stomates under which substomatal vesicles of the first inoculum were produced as a percentage of total stomates observed.
[c] Number of haustoria per total hyphae observed.
[d] Leaves of seedlings grown under continuous illumination of 8,000 lux were inoculated with race 203 either without or following the heat treatment.

TABLE 3. Development of race 203 of *Puccinia coronata avenae* on Shokan 1 oat leaves which previously had been inoculated with *Puccinia graminis tritici* and incubated in the dark or under the light[a]

Incubation: Dark (hr)	Incubation: Light (lux)	Stomatal penetration from the first inoculum (%)[b]	Interval between inoculations (hr)	Development of the challenge race 203: Length of hyphae (μm)	Haustoria[c] (no.)
Second inoculation alone (non-heated)[d]				235.5 A	2.47 A
(heated)[d]				280.6 A	2.13 A
12	0	2.5 A	12	234.9 A	2.06 A
	2,000	13.6 B	16	187.3 B	1.42 B
	4,000	18.8 C	16	126.8 C	0.68 D
	12,000	27.8 D	16	132.2 C	0.76 CD
16	0	3.2 A	16	235.2 A	2.01 A
	4,000	27.6 D	20	127.7 C	0.79 CD
	12,000	42.3 E	20	133.7 C	0.94 C
20	0	4.8 A	20	241.5 A	2.18 A
	4,000	18.8 C	24	132.3 C	0.65 D
	12,000	31.8 D	24	134.2 C	0.99 C

[a] Leaves were inoculated with *P. graminis tritici*, incubated in the dark for 12–20 hr, placed under different light levels for 4 hr, dipped into hot water and reinoculated with race 203. Stomatal penetration was measured before the hot water treatment and development of race 203 was measured 48 hr after the second inoculation. Data are the means of two replications, with five seedlings per replicate and 10 infected sites per seedling. Different letters after each datum in the same column indicate significant differences by Duncan's multiple range test ($P = 0.05$).
[b] Stomates under which substomatal vesicles of the first inoculum were produced as a percentage of total stomates observed.
[c] Number of haustoria per total hyphae observed.
[d] Leaves of seedlings grown under continuous illumination of 8,000 lux were inoculated with race 203 either without or following the heat treatment.

RESULTS AND DISCUSSION

Period of time to induce resistance. Leaves were inoculated with each of three rust fungi nonpathogenic on oats, incubated at 8,000 lux for 8, 12, or 16 hr, dipped into hot water and then reinoculated with race 203. Race 226 of *P. coronata avenae* also was used as the first inoculum to compare the nonhost reaction with the incompatible host-pathogen reaction (Table 1). Hyphal elongation and haustorium formation of the second inoculum were not affected by the first inoculation when the leaves were heated and reinoculated 8 hr after the first inoculation. When preinoculated leaves were heated and reinoculated after 12 or 16 hr, however, the development of race 203 was reduced in all leaves to one half or less of the normal amount of growth. No substantial difference in the reduction in the development of race 203 was found between the leaves inoculated with race 226 and the other rust fungi. These results were similar to our previous observations on double inoculations with incompatible and compatible races for cultivar-specific resistance (10) and suggest that the nonhost resistance also was induced between 8 and 12 hr after inoculation.

Light requirement of *P. graminis tritici* for penetration of oats. Since substomatal vesicles of all fungi used in the present study were produced in stomatal cavities between 8 and 12 hr after inoculation (cf. Fig. 1), the results in Table 1 suggest the possibility that the observations were related to the process of stomatal penetration. To evaluate this possibility, further experiments were done using *P. graminis tritici* as a first inoculum. The use of this fungus was based on the possibility that stomatal penetration of oat leaves is dependent on light radiation, which has been demonstrated for wheat leaves (1,13). If so, the time and frequency of stomatal penetration could be controlled experimentally.

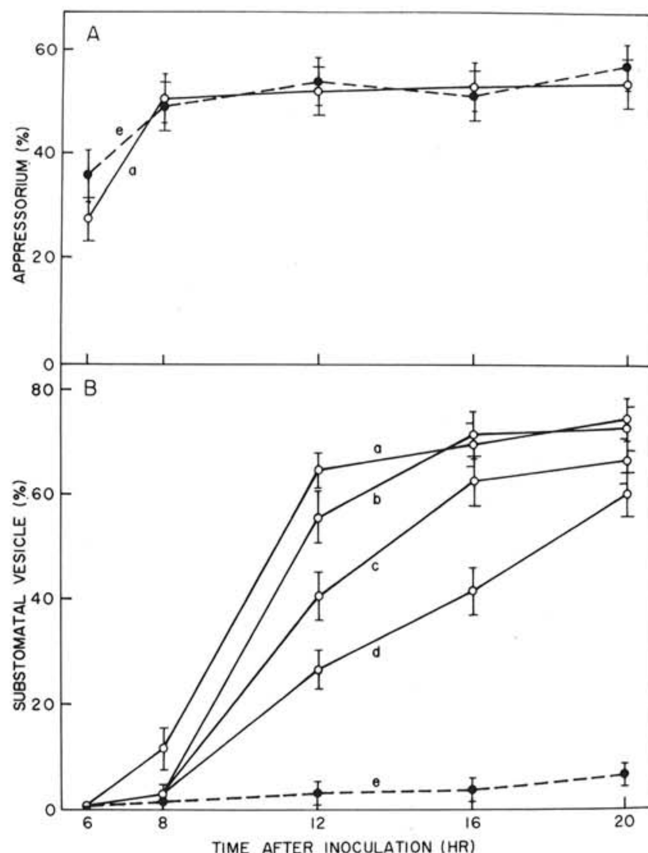

Fig. 1. Differentiation of infection structures of *Puccinia graminis tritici* on oat leaves under various light intensities. **A,** Appressoria over stomates as percentage of total germ tubes observed. **B,** Substomatal vesicles in stomatal cavity as percentage of appressoria observed. Curves a, b, c, and d: continuous light radiation of 12,000, 8,000, 4,000, and 2,000 lux, respectively. Curve e: continuous darkness. Each point represents the mean of two replications, with five seedlings per replicate and (A) 100 germ tubes or (B) 50 appressoria per seedling. The vertical bars indicate the total variation.

Seedlings inoculated with *P. graminis tritici* were placed under continuous illumination of 2,000 to 12,000 lux or in the dark. Frequency of appressorium formation over stomates by germ tubes and differentiation of substomatal vesicles beneath stomates were measured 8, 12, 16, and 20 hr after inoculation. Appressorium formation in the dark was equal to that under 12,000 lux (Fig. 1A), but formation of substomatal vesicles was clearly dependent on the light intensity (Fig. 1B). At 8,000 and 12,000 lux, vesicle formation was mostly completed between 8 and 12 hr after inoculation, but it was delayed as the light intensity decreased. In the dark, only a few appressoria produced substomatal vesicles. On the other hand, more than 70% of appressoria of race 226 of *P. coronata avenae, P. coronata festucae,* and *P. coronata lolii* produced substomatal vesicles in both the dark and light.

Stomatal penetration and determination of resistance. Two experiments were done. In the first experiment, leaves inoculated with race 226 or other rust fungi were incubated in the dark for 12 hr, dipped into hot water, and reinoculated with race 203 (Table 2). Race 226 of *P. coronata avenae,* and isolates *P. coronata festucae,* and *P. coronata lolii* penetrated stomates at high frequency, but *P. graminis tritici* seldom produced substomatal vesicles because of the failure of stomatal penetration. In leaves inoculated with the former three fungi, the development of race 203 was largely reduced, but in leaves inoculated with *P. graminis tritici* it was similar to that in the uninoculated control leaves.

In the second experiment, leaves inoculated with *P. graminis tritici* were incubated in the dark for 12 to 20 hr and then exposed to the light for 4 hr. The leaves were heated after the light radiation, reinoculated with race 203 onto the opposite leaf surface and incubated at 8,000 lux (Table 3). In leaves incubated in the dark for 12–20 hr after the first inoculation, frequency of stomatal penetration was less than 5% and no appreciable effect on the development of challenge race 203 was observed. The development of race 203 was moderately reduced in leaves where 13.6% of stomates was invaded by the first inoculation under the light radiation of 2,000 lux from 12 hr after the first inoculation. The development was largely reduced when about 20% or more of stomates were invaded by the first inoculum under the radiation of 4,000 lux, regardless of whether the challenge inoculation was made 16, 20, or 24 hr after the first inoculation. No significant difference in the reduction between the radiation of 4,000 and 12,000 lux, although different degrees of stomatal penetration had occurred.

The results in this section suggest that a certain degree of stomatal penetration is required for inducing the effect. The results also suggest (see also Table 2) that neither light radiation nor the time interval between the first and second inoculations is correlated with the restricted development of the challenge race 203.

We propose, by the results of the present study, that the initial events which determine the nonhost resistance of oat leaves to rust fungi pathogenic on gramineous plants other than oats are induced by stomatal penetration, as has been indicated for the cultivar-specific resistance in the Shokan 1-race 226 system (10).

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