Irreversible Recognition Demonstrated in the Hypersensitive Response of Oat Leaves Against the Crown Rust Fungus

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ABSTRACT

Expression of resistance in primary leaves of *Avena sativa* inoculated with an incompatible race of *Puccinia coronata avenae* was decreased by subsequent inoculation with a compatible race. This decrease was diminished as the time interval between inoculations was prolonged, and the reinoculation with a compatible race at 12 or more hours after the initial inoculation did not appreciably affect the expression of resistance. Heat-treated leaves inoculated with a uredospore mixture of incompatible and compatible races (95 : 5, w/w) and subsequently inoculated with a compatible race expressed a significant increase of hypersensitive necrosis and a marked decrease of uredosorus formation compared to leaves inoculated with only the uredospore mixture. It therefore appeared that, in this case, the compatible race was recognized as incompatible and the resistance reaction was amplified. The host cellular conditioning towards the resistance response seemed to be completed sometime between 8 and 12 hours after inoculation with an incompatible race.

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Additional key word: cross-protection.

Initial inoculation of plants with nonpathogenic organisms or incompatible races of pathogens frequently results in resistance to subsequent inoculation with compatible pathogens. Such cross-protection responses presumably arise from an invocation of plant defense reactions which are effective against the second, normally compatible, pathogen (3, 6). Rust and powdery mildew diseases have been a major subject of cross-protection research (2, 4, 5, 7, 8, 9, 10, 13, 14, 17, 20, 22, 23, 24) and investigators have speculated that the protection mechanism involves the production of antifungal substances either by the plants (2, 7, 8) or possibly the pathogen (5, 20, 22).

In late blight of potatoes and anthracnose of beans, Kuć and his colleagues (1, 15, 16, 19) demonstrated that the initial event determining cultivar incompatibility or compatibility is the differential recognition of invading pathogens by the host cells. Skipp and Deverall (18) also concluded that initiation of resistance in beans to anthracnose involves the specific initiation of a physiological change leading to the resistant reaction in the host. Ouchi et al. (13, 14), working with powdery mildew of barley, proposed the "induced accessibility" hypothesis, wherein, once initiated, both incompatible and compatible host responses are irreversible and independent of subsequent inoculation with reciprocal compatible or incompatible pathogen races.

In view of these hypotheses regarding the mechanisms of induced resistance and susceptibility in plants to pathogens, we have studied the primary recognition of incompatible and compatible races of *Puccinia coronata avenae* by oat leaves.

MATERIALS AND METHODS.—*Fungi and plants.—Puccinia coronata* Cda. f. sp. *avenae* Fraser & Led. races 226 and 203 were used in this work, and were maintained on the susceptible oat (*Avena sativa* L. 'Victoria 226-S') (21). Fresh uredospores were prepared by the method of Naito et al. (11). The oat cultivar Shokan 1 was used experimentally and is compatible (viz. susceptible type-4 host response) to race 203 and incompatible (viz. hypersensitive resistant type-0 host response) to race 226. Seeds were germinated on moist filter paper for 2 days. Germinated seeds of uniform size were grown on Vermiculite in uncovered petri dishes (9 cm in diameter) and grown before and after inoculation at 25 C under continuous fluorescent illumination (6,000-8,000 lux). The seedlings in each petri dish were supplied daily with 10 ml of Kasugai nutrient solution containing, per liter of water: 66 mg NH_4NO_3, 38 mg KH_2PO_4, 43 mg KCl, 245 mg $MgSO_4 \cdot 7H_2O$, 189 mg $Ca(NO_3)_2 \cdot 4H_2O$, and 2 mg $FeCl_3$, pH 7.0. After 7 days, 20 uniform seedlings per petri dish were selected for inoculation.

Inoculation.—Unless otherwise indicated, the first inoculation was made on the abaxial surface of leaves and the second inoculation, when necessary, on the adaxial surface. Spores were applied with a soft hair brush. Care was taken so that 30% or more of the stomata were blocked by rust infection structures. Inoculated plants were incubated in a saturated relative humidity chamber for 20 hours at 25 C. Uredospores of races 226 and 203 were simultaneously inoculated onto Shokan 1 in some experiments by two methods. In the first, uredospores of the two races were mixed and inoculated onto the abaxial leaf surface. In the other way, race 226 was inoculated onto the abaxial surface and race 203 onto the adaxial surface.

Heat treatment.—The culture solution in which seedlings were growing was decanted prior to heat treatment to facilitate a rapid and constant temperature rise. The seedlings were then exposed to a dry atmospheric temperature of 50 C for 5 minutes or 45 C for 45 minutes. No visibly detectable heat damage of the treated plants was observed.

Measurement of seedling responses.— The number of uredosori in a specified area of abaxial leaf surface (between 2.0 to 5.5 cm from the distal leaf end) was

counted at 7 days after inoculation using a × 30 dissecting microscope. Small, brown necrotic lesions were observed in incompatible hypersensitive reactions at day 3 after inoculation, and the degree of hypersensitive necrosis was estimated after the completion of the response at day 7. The hypersensitive necrotic host reactions were graded into "degree-of-necrosis types" as follows: 'A' represents the absence of necrosis; 'B' the presence of several small necrotic spots; 'D' denotes a necrotic area covering half of the leaf; 'F' necrosis of the whole leaf; and 'C' and 'E' are respective intermediate degrees of necrosis. The percentage of leaves which expressed each degree of necrosis was calculated with approximately 200 seedlings assessed per treatment.

RESULTS.—*Induced responses resulting from double inoculation.*—Shokan 1 leaves inoculated with race 226 exhibited a classic hypersensitive necrotic response of a type-F necrosis (Table 1), while leaves inoculated with race 203 gave no hypersensitive necrosis, but produced many uredia. Leaves inoculated with mixed 226 and 203 inoculum (1:1, w/w) gave intermediate hypersensitive necrosis without decreased numbers of uredia.

Double-inoculation experiments, in which leaves were first inoculated with incompatible race 226 and then with race 203, resulted in milder hypersensitive responses, regardless of the time interval between the successive inoculations. The leaves responded with progressively greater hypersensitive necrosis as the time interval was prolonged between inoculations (Table 1). A transition of leaves from essentially compatible to essentially incompatible occurred between the 8th and 12th hours. The trends were also reflected in the uredia formation, since leaves inoculated with race 203 at 12 or more hours after the 226 inoculation produced smaller numbers of uredia than those reinoculated with 203 after 4 and 8 hours. The results in Table 1 therefore suggested that hypersensitive resistance to race 226 was irreversible by reinoculation with race 203 after 12 hours.

We found that heat treatment of Shokan 1 oat leaves before inoculation with the normally incompatible mixed inoculum of 226 and 203 (95:5, w/w) gave less hypersensitive necrosis and substantial uredia production, depending on the conditions of the treatment (T. Tani et al., *unpublished).* For example, heat treatment at 50 C for 5 minutes greatly decreased hypersensitive necrosis with a slight increase of uredia production (Table 2, Fig. 1), and heat treatment at 45 C for 45 minutes increased uredia production with a moderate decrease of

TABLE 1. Hypersensitive necrotic response and uredosorus formation of Shokan 1 oat leaves simultaneously inoculated or inoculated at different time intervals with incompatible (race 226) and compatible (race 203) races of *Puccinia coronata avenae*

First inoculation[b]	Second inoculation[b]	Time between inoculations (hours)	Leaves exhibiting various necrosis classes[a](%)						Uredia per leaf (no.)
			A	B	C	D	E	F	
226 (incompatible)	. . .	. . .	0	0	0	0	0	100	2
203 (compatible)	. . .	. . .	100	0	0	0	0	0	148
226 + 203[c]	. . .	. . .	25	25	19	19	12	0	129
226	203	0	23	15	39	15	8	0	99
226	203	4	0	73	18	9	0	0	70
226	203	8	0	51	30	19	0	0	71
226	203	12	0	15	5	5	35	40	16
226	203	16	0	0	7	9	25	59	13
226	203	20	0	0	0	0	9	91	16

[a]Approximately 200 leaves were used per treatment; at 7 days after inoculation the number of uredia per leaf were counted and the degree-of-necrosis type was determined for each leaf; A = no necrosis to F = necrosis of the entire leaf; data are expressed as the percentage of leaves in each type.

[b]Race 226 was first inoculated onto the abaxial leaf surface, then race 203 was inoculated onto the adaxial surface after the indicated period of time. Single inoculations were made onto the abaxial surface.

[c]A mixture (1:1, w/w) of uredospores was inoculated onto the abaxial surface.

TABLE 2. Hypersensitive necrotic host response on heat-predisposed Shokan 1 oat leaves elicited by normally compatible race 203 of *Puccinia coronata avenae*

First inoculation[b]	Second inoculation[b]	Time between inoculations (hours)	Leaves exhibiting various necrosis classes[a] (%)					
			A	B	C	D	E	F
226 + 203	. . .	. . .	0	0	0	0	0	100
203	. . .	. . .	100	0	0	0	0	0
Heat[c] + 226 + 203	. . .	. . .	0	33	35	17	11	4
Heat[c] + 226 + 203	203	12	0	0	14	14	19	53
Heat[c] + 226 + 203	203	20	0	0	0	0	10	90

[a]Approximately 200 leaves were used per treatment; at 7 days after inoculation the number of uredia per leaf were counted and the degree-of-necrosis type was determined for each leaf; A = no necrosis to F = necrosis of the entire leaf; data are expressed as the percentage of leaves in each type.

[b]Leaves were inoculated first with race 203 or with a mixture of uredospores of races 226 and 203 (95:5, w/w); the second inoculation was with race 203 at the indicated time intervals.

[c]Heat-treated plants were exposed to 50 C for 5 minutes.

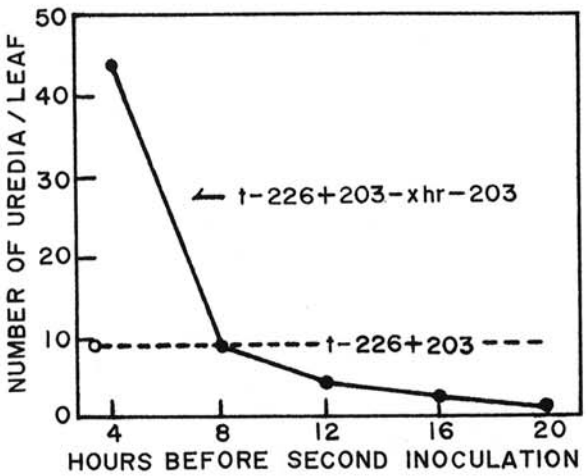

Fig. 1. Uredosorus formation on heat-treated Shokan 1 oat leaves inoculated with a mixture of races 226 and 203 and reinoculated with compatible race 203 of *Puccinia coronata avenae* at various time intervals. Leaves were exposed to 50 C for 5 minutes (t), inoculated with a mixture of uredospores of races 226 and 203 (95:5, w/w), then reinoculated with race 203 at the indicated time intervals.

TABLE 3. Uredosorus formation on heat-treated Shokan 1 oat leaves inoculated with a mixture of races 226 and 203 and reinoculated with compatible race 203 of *Puccinia coronata avenae*

First inoculation[a]	Second inoculation[a]	Uredia per leaf (no.)
226 + 203	. . .	1
226 + 203	203	16
Heat[b] + 226 + 203	. . .	91
Heat[b] + 226 + 203	203	7
Heat[b] + 226 + 203	226	0
203	. . .	198
203	226	195
Heat[b] + 203	. . .	152
Heat[b] + 203	226	178

[a]Approximately 200 leaves were used per treatment; at 7 days after inoculation the number of uredia per leaf were counted and the degree-of-necrosis type was determined for each leaf; A = no necrosis to F = necrosis of the entire leaf; data are expressed as the percentage of leaves in each type. The second inoculation was made 20 hours after the first inoculation.

[b]Heat-treated plants were exposed to 45 C for 45 minutes.

necrosis (Table 3). However, leaves that were heat treated at 50 C for 5 minutes and inoculated with mixed 226 and 203 inoculum, and then reinoculated with race 203 after 12 and 20 hours exhibited considerable hypersensitive necrosis (Table 2). On the other hand, inoculation of heat-treated leaves with race 203 only gave no necrosis. Reinoculation with race 203 at 20 hours gave greater necrosis than at 12 hours. Also, when leaves treated at 45 C for 45 minutes were inoculated with mixed 226 and 203 and were subsequently reinoculated with race 203 after 20 hours, uredia production was greatly decreased as in the case when reinoculation was made with race 226 (Table 3). Heat treatment at 45 C for 45 minutes followed by the second inoculation with race 226, gave no significant effect on the reaction of plants to race 203, high uredia production (Table 3) and no hypersensitive necrosis development was observed. Thus, the enhanced development of hypersensitive necrosis (Table 2) and the marked decrease of uredia production (Table 3) suggested that the second inoculation of compatible race 203 might possibly be recognized as an incompatible race, hence amplification of the resistant reaction by race 203 depended upon the time intervals between inoculations (Fig. 1). Leaves heat-treated and then inoculated with races 226 and 203 produced nine uredia per leaf, but when the leaves were reinoculated with race 203 at various time intervals, a large number of uredia were seen at 4 hours, but declining production of uredia at 8-16 hours. The uredial numbers at 12, 16, and 20 hours were less than on control leaves inoculated with the mixed uredospores only.

Time required for initiation of the resistant state.—To determine the exact time required for establishment of irreversible cellular conditioning toward resistance, leaves inoculated with mixed uredospores of races 226 and 203 (95:5, w/w) were subjected to heat treatment at 45 C for 45 minutes. Heat treatment under this condition gave no perceptible influence on uredia formation by a compatible race providing that the treatment was made before spore germination of within 8 to 20 hours after inoculation (T. Tani et al. *unpublished*). As shown in Fig. 2, the thermal effect was demonstrable when the heat treatment was done at 0 or 8 hours after inoculation, and a similar number of uredia were produced on leaves heated before inoculation, while it was not detected when the leaves were heat-treated at 12 or more hours after the initial inoculation. Thus, it appeared that cellular initiation of resistance was established within 8 to 12 hours after inoculation with an incompatible race.

DISCUSSION.—Our data provide additional support for the hypothesis that the early reaction of the host to the pathogen plays a key role in determining the subsequent course of infection (1, 13, 14, 18, 19) and show that once committed toward hypersensitive resistance, or alternatively toward a compatible relationship with the parasite, the host response is essentially irreversible. The resistance of Shokan 1 leaves induced by incompatible race 226 was not reversed by subsequent inoculation with race 203 after 12 hours (Table 1). Similarly, initial inoculation with race 203 prevented the appearance of subsequent hypersensitive necrosis and large numbers of uredia were produced when leaves were subsequently inoculated with the incompatible race after 20 hours (Table 3). Since these results occurred when uredospores of the two races were inoculated onto different sides of the leaves, it is not unlikely that the disease reactions occurred as a result of stomatal blockage by rust infection structures (5, 8).

We observed that inoculation of Shokan 1 leaves with a compatible race after initial inoculation with an incompatible race resulted in an enhancement of hypersensitive necrosis (Table 2) and a decrease of uredia production (Table 3 and Fig. 1). The mechanism underlying these observations is unclear, but it is intriguing to speculate that, once committed, cells responding hypersensitively recognize normally compatible races as incompatible.

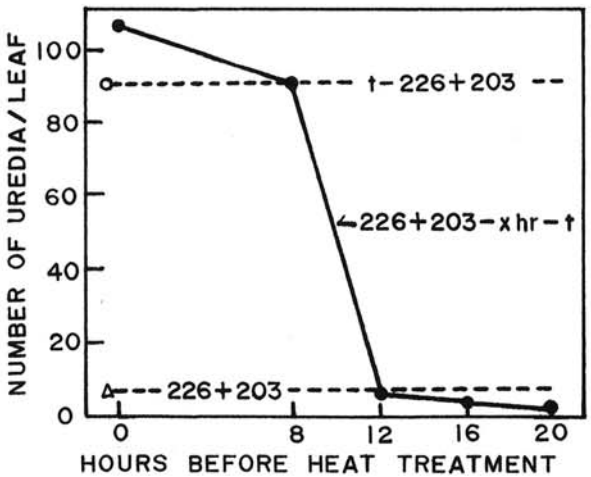

Fig. 2. Irreversible transition of resistant response of Shokan 1 oat leaves to *Puccinia coronata avenae* as assessed by the effect of heat treatment on uredosorus formation. The leaves were inoculated with a mixture of uredospores of races 226 and 203 (95:5, w/w) and were subsequently exposed to 45 C for 45 minutes after the indicated periods of time.

The data in Fig. 2 indicate that the determinative events ultimately leading to hypersensitive necrosis occur between 8 and 12 hours after initial inoculation with an incompatible race. This corresponds to the time when substomatal vesicles of the crown rust fungus are produced (11), and it is noteworthy that the initiation step precedes the formation of haustoria by the fungus. It is also of interest that the apparent time of initial pathogen recognition at 8-12 hours occurs considerably before growth cessation of the invading fungus is observed at about 40 hours after inoculation (12).

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