The Effect of Detachment on the Development of Rust Disease and the Hypersensitive Response of Wheat Leaves Infected with Puccinia graminis tritici

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ABSTRACT

Primary leaves of Khapli wheat and wheat lines with the *Sr6, Sr6b, Sr11, Sr13* and *Sr14* genes for disease reaction against race 56 of *Puccinia graminis tritici* were detached immediately after inoculation and floated on various solutions. Upon detachment, the normally incompatible reactions of attached leaves to this race were altered to greater susceptibility with the exception of wheat carrying the *Sr6* allele for resistance. Contrary to earlier reports, kinetin did not prevent the increase in susceptibility. A hypersensitive response, measured by the number and size of fluorescent areas, was evident before 48 hours after inoculation in resistant attached leaves, but not in susceptible attached leaves. The increased susceptibility of detached leaves of resistant wheat was accompanied by a hypersensitive response of at least the magnitude observed for attached leaves of the same line. Although kinetin tended to maintain the normal appearance of detached leaves, the hypersensitive reaction sites were more numerous and larger in the presence of this compound. The results support previous studies on near-isogenic lines of wheat with the *Sr6* alleles for disease reaction which indicated that the hypersensitive response of wheat to rust fungi is not a determinant in incompatibility. Rather, the response may be a general symptom of stress, and only incidental to the cellular reactions involved in host-parasite interactions.

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Evidence recently presented (14) showed that a hypersensitive response to infection by wheat leaves resistant to *Puccinia graminis tritici* (22) occurred with equal intensity under conditions where the same wheat line was completely susceptible. When grown continuously at 20 C, inoculated near-isogenic wheat carrying the *Sr6* allele for resistance had greater numbers of fluorescent areas than did a companion line carrying the recessive *sr6* allele conditioning susceptibility. Marti and Montalbini (13), as well as Howes et al. (8), have shown that fluorescent areas in rusted leaf tissue correspond with cells with the extensive browning characteristic of a hypersensitive response. Differences were found as early as 24 hours after infection and the response of the incompatible line increased progressively during infection (14). If transferred to 26 C immediately after inoculation, the *Sr6* line was susceptible yet it still showed a hypersensitive response which could not be distinguished statistically from the response at 20 C. Ethylene also induces a susceptible disease reaction with the *Sr6* allele (5), and ethylene treatment caused a hypersensitive response greater than that for untreated, resistant plants at 20 C (14).

These results with the *Sr6* allele (14) appear to indicate that the hypersensitive response in rust disease is not a determinant for incompatible reactions. Resistance controlled by the *Sr6* allele is unusual, however, because of its temperature sensitivity. Consequently, these observations might apply only to this allele (2, 4). In an attempt to substantiate them for different genes for resistance, we examined ways to induce susceptibility with other wheat lines.

Forsyth and Samborski (6) reported several methods for changing the resistance of Khapli wheat, and subsequent work indicated (16, 18) that floating excised leaves of Khapli wheat on distilled water caused a breakdown of normal resistance. The breakdown could be prevented by the addition of benzimidazole or kinetin (16, 18, 20, 26). The phenomenon seemed suitable for determining whether a hypersensitive response could be divorced from an incompatible disease reaction in cases where genes other than the *Sr6* allele control incompatibility.

MATERIALS AND METHODS.—All plants were grown at 20 C under conditions described previously and were inoculated with race 56 of *Puccinia graminis tritici* (14). After removal of the plants from the incubation chamber, 20-25 primary leaves were removed and sliced into three to four approximately equal sections. After being rinsed in distilled water, a number of sections equivalent to at least five leaves were floated on 25 ml of sterile solutions in sterile petri plates under the same temperature and light as for the intact plants. At least three replicate petri plates were used for water controls, and for each of the compounds tested.

Sufficient sections randomly were taken daily from each replicate so as to comprise a sample equivalent to three whole leaves. The sections were cut into smaller segments of approximately 0.23 cm^2, boiled in lactophenol for 1.5 minutes, and stored in water until examined for fluorescent sites (13) with the aid of Zeiss fluorescence microscope. A minimum of 30 of these segments per treatment, randomly selected, constituted the population for statistical analysis with computer programs (14). Disease development was recorded in terms of time of flecking, number of lesions, and infection type according to Stakman et al. (23). In addition, glucosamine content of samples hydrolyzed with 6N HCl (24, 25) was used as a quantitative measure of final fungus development (14). Either the absolute amount of glucosamine, or the percentage of glucosamine relative to the total ninhydrin-positive material in the hydrolyzates, was determined. In the latter instance, the ninhydrin-positive materials were calculated from a standard curve as glycine equivalents.

RESULTS AND DISCUSSION.—In preliminary experiments, a number of compounds were tested for their effects on rust development in excised leaf sections. These included kinetin, gibberellin, Ca^{++}, glucose, fructose, trehalose, mannitol, and arabitol at concentrations from 10^{-2} to 10^{-7} M. Glucose and fructose were included because of reports of their effect on rust development (18, 23), while the latter three carbohydrates are utilized rapidly during germination of rust fungi (3). In addition, various combinations such as kinetin and Ca^{++} were examined. The wheat lines employed were Khapli, Little Club, and near-isogenic (11). Chinese Spring wheat lines with the *Sr6, sr6, Sr11,* or *sr11* alleles. Monogenic lines carrying the *Sr7b, Sr13,* and *Sr14* alleles in a W2691 background were supplied by William Q. Loegering; these were tested because they may be involved in the disease reaction of Khapli.

Although not obvious from previous reports in the literature, microbial contamination frequently was a problem and was inevitable when carbohydrates and gibberellin were tested. The results described below were obtained from experiments where the solutions showed no visual microbial contamination. The monogenic lines of W2691 showed the same responses as did Khapli wheat and, in terms of the hypersensitive response, all cultivars and lines appeared to behave similarly. Therefore, only data for Khapli and the *Sr6* and *Sr11* lines on either water or kinetin at 10^{-4} or 10^{-5} M will be discussed. It should be noted that the same effects on disease development were observed at 10^{-3} M kinetin, a higher concentration than that found to be effective in maintaining resistance in other studies (16, 26). However, hypersensitivity was studied extensively only at 10^{-4} or 10^{-5} M.

As reported by others (16, 18, 26), kinetin at these concentrations prevents loss of chlorophyll in detached leaves and treated tissue remained green, while leaves floated on water began to yellow 4 or 5 days after excision. When compared with attached leaves, there was no obvious difference in the number of infected sites or the time of appearance of disease symptoms. As Person et al. reported (16), however, detached leaves on water in our study were more susceptible than attached leaves in that sporulation occurred readily with but one exception. The exception was the *Sr6* line which remained resistant when detached. Under our conditions, in contrast to previous results (16, 18, 26), sporulation occurred equally as well on kinetin solutions as with the water controls. The general results are illustrated in Fig. 1 for only the *Sr6* and *Sr11* near-isogenic wheat lines for water controls and kinetin (K) solutions.

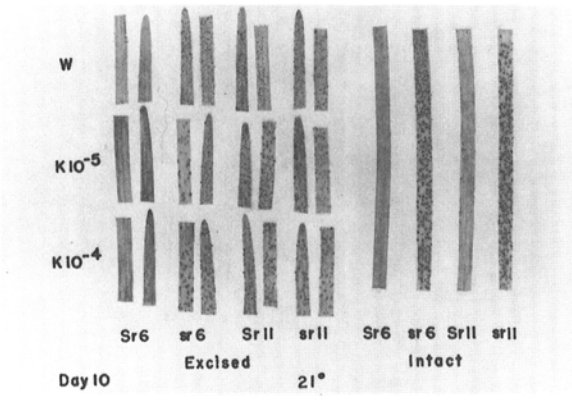

Fig. 1. Development of rust disease on intact or detached leaves of wheat lines carrying the *Sr6* and *Sr11* alleles for reaction against race 56 of *Puccinia graminis tritici*. Leaves detached on day 1 after inoculation and floated on water (W) and 10^{-4} or 10^{-5} M kinetin (K) solution at 21 C.

In a separate experiment, the extent of reversion to susceptibility that occurred with all but the *Sr6* allele was measured by glucosamine analysis (Table 1). In intact leaves on the tenth day from inoculation, total (free and bound) ninhydrin-positive materials were higher for the susceptible *sr6* and *sr11* lines than for the lines carrying the dominant resistant alleles (Table 1). However, glucosamine content is increased considerably in susceptible interactions (14), giving percentages of glucosamine in excess of 4.0 for susceptible infection tissues, but less than 1.0 percent for incompatible infections of type 0;. The infection type 1 of intact Khapli is characterized by intermediate values.

With excision, the total glycine equivalents increased (Table 1). It is known that free amino acid pools increase rapidly in excised leaves at the expense of protein. It is also reported that kinetin delays the process (18, 26). The data in Table 1, however, represent insoluble (protein), as well as free, amino nitrogen and the reason for the greater amounts of total amino nitrogen is not known. For Khapli and *Sr11* wheat leaves which showed reversion to susceptibility, the percentage of glucosamine increased to about the same percentage as for susceptible reactions. Unfortunately, an unmeasured volume of the hydrolyzate from detached *Sr11* leaves was inadvertently lost. Although chemical analysis was performed, the absolute content of glucosamine per excised leaf could not be determined directly. The percentage of glucosamine, however, is similar to all other compatible reactions. If a minimum of 18 μmoles of glycine equivalents per leaf is assumed, it can be calculated that the excised leaves of *Sr11* contained at least 0.67 μmoles of glucosamine per leaf.

Despite an increase in development of the rust fungus upon detachment, the intensity of the hypersensitive response to invasion actually was greater than with intact leaves. Table 2 compares intact leaves of the resistant *Sr11* line (infection type 0;) with susceptible *sr11* line (infection type 3-4). Even at day 2, the number of fluorescent cells in attached resistant wheat leaves was 25-fold greater than for attached susceptible leaves and statistical treatment of the data was not necessary. *Sr11* leaves floated on kinetin (Table 2) had much greater numbers of fluorescent sites earlier during infection than did attached leaves, despite the fact that kinetin treatments tend to retain the normal appearance of excised leaves. The same effect was noted for *Sr6* leaves (Table 2) although the observations were limited to only days 4 and 5 because previous work had documented similar phenomena in other ways (14). Upon excision, even the normally susceptible lines *(sr6* and *sr11)* showed a hypersensitive response in the early stages of infection.

In previous studies (14) with the *Sr6* wheat lines, the hypersensitive response was reflected by an increase in size as well as in numbers of fluorescent sites. Leaves on

TABLE 1. Glucosamine and amino acid content in 6N HCl hydrolyzates of wheat leaves infected with race 56 of *Puccinia graminis tritici* on day 10 after inoculation. Khapli wheat and Chinese Spring wheat with known alleles for rust reaction left intact or detached and floated on 10^{-5} M kinetin immediately after inoculation

		Chinese Spring			
	Khapli	*sr11*	*Sr11*	*sr6*	*Sr6*
	infection type				
Intact	1	3-4	0;	3-4	0;
Kinetin 10^{-5} M	3-4	3-4	3-4	3-4	0; (1 tr)
	μmoles glycine equivalents per leaf				
Intact	17.4	19.5	9.5	18.1	9.3
Kinetin 10^{-5} M	30.0	24.0	[a]	21.5	19.1
	μmoles glucosamine per leaf				
Intact	0.47	0.95	0.09	0.80	0.04
Kinetin 10^{-5} M	1.03	1.15	[a]	0.79	0.12
	glucosamine as percentage of glycine equivalents				
Intact	2.8	4.9	0.9	4.4	0.4
Kinetin 10^{-5} M	3.4	4.7	3.7	3.7	0.6

[a]Portion of hydrolyzate was lost, but if 18.0 μmoles of glycine equivalent is assumed, glucosamine would equal 0.67 μmoles per leaf.

kinetin solutions have fluorescent areas of larger size than intact leaves or leaves floated on distilled water (Table 3). Thus, the total leaf area affected is much greater than that indicated by the data of Table 2.

Failure to find reversion to susceptibility with the *Sr6* allele upon excision reaffirms the idea that the biochemical or physiological control of compatibility and incompatibility at this locus is distinct from control at the *Sr11* locus (4). Earlier, it was found that ethylene caused reversion in intact leaves of *Sr6* (5), but not the *Sr11* (4), line. This finding was a factor in drawing the conclusion that a specific peroxidase isozyme (19) activated in both cases of incompatibility could not be a primary gene product responsible for resistance (2, 4). The response to excision of Khapli and the monogenic lines containing the *Sr7b, Sr13,* and *Sr14,* which may be involved in the resistance of Khapli, is similar to the *Sr11* line despite the marked difference in disease reaction when normal, intact leaves are infected.

TABLE 3. Average size of fluorescent sites in 10 segments (0.23 cm^2) from leaves of Chinese Spring wheat carrying the *sr11* (compatible) or *Sr11* (incompatible) alleles for reaction against race 56 of *Puccinia graminis tritici*

Alleles and treatments	Average lesion diameter (μm) Day 3	Day 5
sr11		
Intact	< 30	< 30
Detached-H_2O	< 30	45
Detached-Kinetin	130	175
Sr11		
Intact	60	120
Detached-H_2O	100	135
Detached-Kinetin	180	180

The finding that the *Sr11* allele can be manipulated to give a hypersensitive response with a compatible disease reaction (Table 2) is significant. Previous results with the temperature-sensitive *Sr6* allele were complicated because temperatures prior to inoculation had an influence on the hypersensitive response, but not on disease reaction (14). If grown at 20 C for 6-7 days before inoculation, infected leaves then held at 26 C after inoculation showed hypersensitivity even though a compatible reaction developed. With a preinoculation temperature of 26 C, a significant hypersensitive response did not develop with the *Sr6* line at postinoculation temperatures of 26 C. Such findings reconciled our results (14) with those of Skipp and Samborski (21) who did not report a hypersensitive response at 26 C with the *Sr6* line. Light intensity, and perhaps infection density, also influences the hypersensitive response (14).

The lack of correlation between final disease development and the hypersensitive response requires a re-evaluation of the role of hypersensitivity in resistance, at least for the rust disease of wheat. In the studies of Brown and coworkers (1, 15), development of individual colonies of rust fungi was not correlated with the existence of necrotic cells in the immediate vicinity of the hyphae. They also showed that, although susceptible hosts did not exhibit extensive hypersensitivity, certain resistant hosts had hypersensitive response magnitudes which did not correlate completely with the final infection

TABLE 2. The number of fluorescent sites per 0.23 cm^2 of wheat leaves from intact plants or leaves detached immediately after inoculation and floated on water or 10^{-4} kinetin. Samples collected at the indicated days after inoculation with race 56 of *Puccinia graminis tritici*

Day	Intact		Detached H_2O		Detached Kinetin	
	sr11	*Sr11*	*sr11*	*Sr11*	*sr11*	*Sr11*
2	0.1	2.5	0.2	2.2	4.1	6.1
3	0.9	8.3	2.7	10.4	12.5	24.2
4	0.9	9.7	5.4	12.7	8.2	28.5
5	0.8	10.6	4.5	12.9	12.4	26.8
	sr6	*Sr6*	*sr6*	*Sr6*	*sr6*	*Sr6*
4	0.8	8.3	9.0	9.9	17.2	30.2
5	1.9	10.3	9.0	14.6	24.5	24.5

type. A handicap in interpreting these observations (1, 15) is the fact that the final infection type was a resistant one in instances where appreciable hypersensitivity could be detected. Our previous (14) and present observations depart significantly from this in that the response occurred in tissues where there were no visible signs of incompatibility at any stage of the infection. Development of the parasite thus appeared to be independent of the existence of the response. Much attention (7, 8, 10, 12, 13, 14, 17, 21) has centered on the hypersensitive response as a cause of resistance or at least as a symptom of incompatibility, despite cautions about the assumptions (1, 9, 15). It seems more likely that the response is a biochemical stress response (2) which occurs incidental to the cellular events involved in incompatibility.

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