Association of Hypersensitive Host Cell Death and Autofluorescence with a Gene for Resistance to *Peronospora manshurica* in Soybean

T. Érsek, M. Holliday, and N. T. Keen

First author on leave from the Institute for Plant Protection, Budapest, Hungary; second and third authors from the Department of Plant Pathology, University of California, Riverside 92521. Second author's current address is Biochemistry Department, Experimental Station, E. I. du Pont de Nemours, Wilmington, DE 19898.

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ABSTRACT

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Leaves of a soybean cultivar carrying the *Rpm* allele for resistance to *Peronospora manshurica*, but not those of a near-isogenic susceptible cultivar, microscopically reacted to infection with a typical hypersensitive response, but the leaves did not exhibit visible symptoms. Necrotic host cell death occurred around infection sites in the resistant genotype based on epifluorescence observations of sodium fluorescein uptake. Autofluorescent material, possibly the phytoalexin glyceollin or related compounds, also accumulated in hypersensitive host cells surrounding fungal penetration sites. The results therefore indicated that resistance conferred by the *Rpm* allele is associated with a classical hypersensitive reaction.

The hypersensitive response (HR) has been associated with the expression of many single genes for resistance to phytopathogens, but it has been suggested that genes resulting in rapid resistant reactions (viz no macroscopic response) may be expressed by other mechanisms not involving the HR. The *Rpm* allele in soybean against *Peronospora manshurica* (Naum.) Syd. ex Gaum. is such a gene (11), since fungus development is arrested relatively rapidly after infection. Based on the association of hypersensitivity and accumulation of the phytoalexin glyceollin with monogenic resistance to other incompatible soybean pathogens (5,6,15), we investigated whether they are also associated with resistance conditioned by the *Rpm* allele.

Holliday et al (4) found that lack of uptake of sodium fluorescein was a sensitive indicator of hypersensitive cell death in soybean leaves inoculated with nonpathogenic races of *Pseudomonas syringae* pv. *glycinea*. They also observed that hypersensitive leaf cells exhibited weak autofluorescence soon after hypersensitive death occurred, and this was attributed to accumulation of glyceollin, which fluoresces weakly in the visible range. We accordingly employed the fluorescence microscopy techniques to investigate the interactions of *P. manshurica* with soybeans containing the *Rpm* and *rpm* alleles.

MATERIALS AND METHODS

Plant and fungus culture. Seeds of the compatible soybean (*Glycine max* (L.) Merr.) cultivar Williams (*rpm*) and the near-isogenic incompatible cultivar Union (*Rpm* = SL9 [1,11]), resistant to all known North American races of *P. manshurica* were generously supplied by R. L. Bernard. Plants were grown from seed as described by Keen (6) at 22 C. Race 24 (3) of *P. manshurica* was sent by J. Dunleavy and was maintained on cultivar Williams. Induction of conidia formation, harvesting of conidia, and inoculation of primary leaves of 9-day-old plants with conidial suspensions (10^6 ml^{-1}) were carried out as described by Dunleavy (2). After inoculation, plants were incubated at 18 C under plastic bags for 12 hr and then maintained at 22 C until leaves were harvested. In some experiments, soybean leaves of both genotypes were inoculated as above with uredospores of *Uromyces phaseoli* produced on *Phaseolus vulgaris* 'Pinto' leaves.

Extraction of glyceollin and related isoflavonoid compounds. These procedures were carried out as described earlier (6) for extractions from inoculated leaves harvested at intervals after inoculation.

Microscopic observations. In order to visualize conidia and infection structures of the fungus on soybean leaves, freshly harvested *P. manshurica* conidia were labeled with the fluorescent brightener Calcofluor White M2R New (disodium salt of 4,4′-*bis*(4-anilino-6-diethylamino-*s*-triazin-2-ylamino)-2,2′-stilbene disulfonic acid, catalogue no. MR-4391, American Cyanamid Co., Wayne, NJ 07470). Calcofluor was added at 300 μg ml^{-1} to water suspensions of freshly harvested conidia of *P. manshurica* containing 0.1% Tween-20, as suggested by Tsao (14) for *Phytophthora* spp. After 2 hr, the conidia were pelleted by centrifugation at ~1,500 *g* for 1 min and washed in 0.1% Tween-20 several times until the supernatant fluids showed no fluorescence under an ultraviolet (360 nm maximum) lamp. The conidial density was then adjusted to 10^6 ml^{-1} prior to inoculation.

Host cell death was determined at intervals after inoculation by using the techniques of Holliday et al (4). Leaf segments of ~5 × 10 mm were excised from freshly harvested leaves and vacuum infiltrated with 0.01% sodium fluorescein (Fisher) in sodium citrate-phosphate buffer, pH 5.6. Leaf sections were then destained in the same buffer twice for 30 min and once for 5 min. Unstained control leaf segments were vacuum infiltrated in buffer only. All leaf segments were directly examined with a Zeiss photomicroscope equipped for epifluorescence microscopy. In some experiments, Hooker microtome sections of ~20 μm were also prepared and examined similarly. The fluorescence microscope was equipped with red suppression filter BG 38 and chromatic beam splitter FT 460. Calcofluor fluorescence was detected using excitation filters UG1 and UG5 and barrier filter LP397. The fluorescence of fluorescein and the autofluorescence of unstained soybean cells were detected using excitation filter KP490 and barrier filter LP528.

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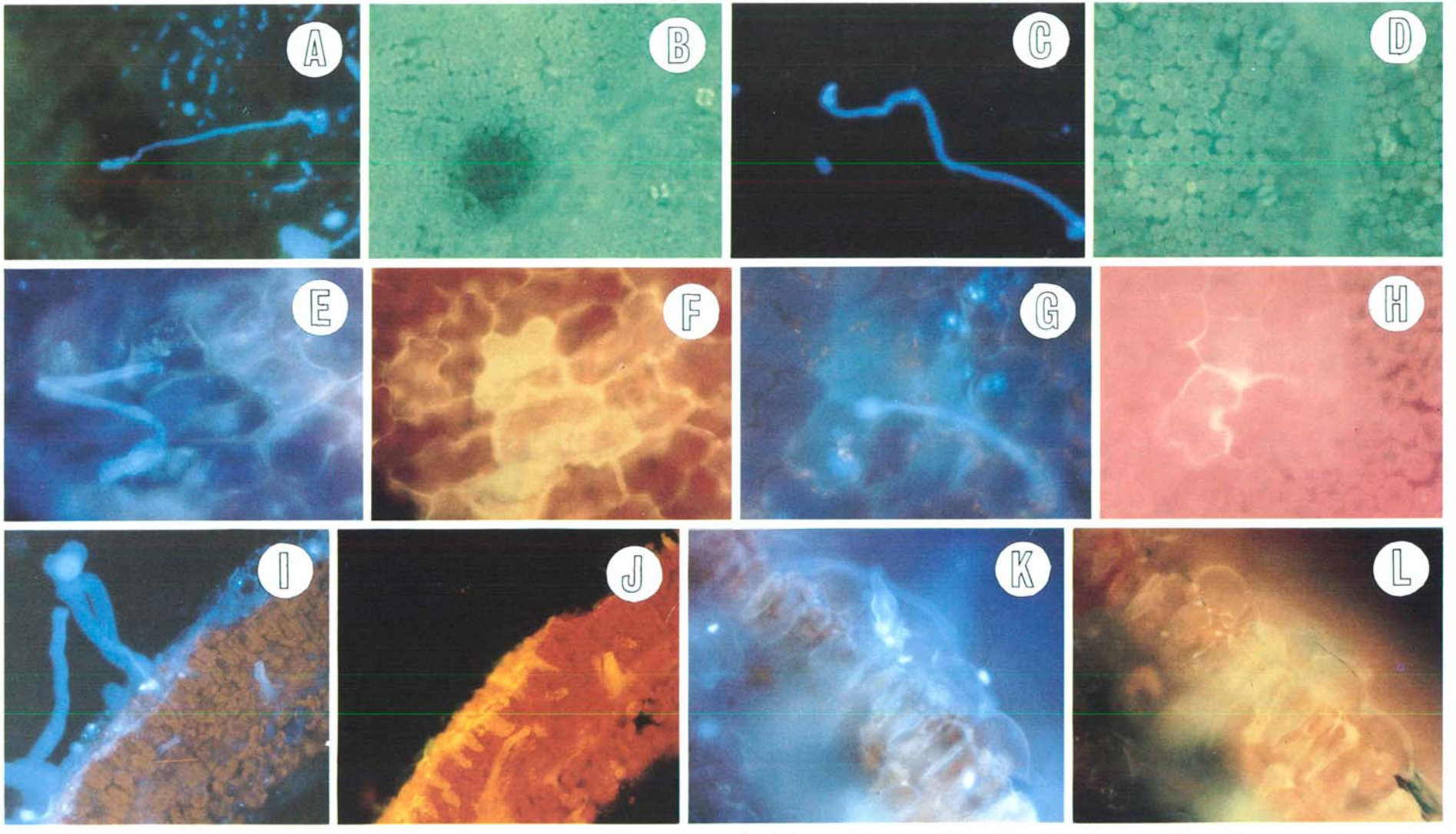

Fig. 1. Epifluorescence micrographs of soybean leaves inoculated with *Peronospora manshurica* (adjacent pairs of micrographs [eg, A and B] are of identical leaf areas, but different filters were employed): cultivar Union, 13 hr postinoculation, visualizing **A,** calcofluor and **B,** fluorescein; cultivar Williams, 24 hr postinoculation, visualizing **C,** calcofluor and **D,** fluorescein; cultivar Union, 48 hr postinoculation, visualizing **E,** calcofluor and **F,** autofluorescence; cultivar Williams, 48 hr postinoculation, visualizing **G,** calcofluor and **H,** autofluorescence; and Hooker microtome sections of cultivar Union, 24 hr postinoculation, visualizing **I and K,** calcofluor and **J and L,** autofluorescence, respectively.

RESULTS

Leaves of the compatible cultivar Williams exhibited typical chlorotic lesions on the fourth or fifth day after inoculation with *P. manshurica,* but incompatible cultivar Union plants did not exhibit any visible symptoms and would therefore classically be considered "immune." There was no difference in symptom severity on cultivar Williams plants inoculated with either Calcofluor-stained or unstained conidia, and no differences were noted in germination after 12 hr at 18 C in water containing 0.1% Tween-20. These observations indicated that Calcofluor did not affect their viability or infectivity. However, the infection efficiency on soybean leaves was low, with only ~100 successful infection sites per primary leaf of either cultivar. Infection efficiency was not increased by using higher conidial densities or by altering the incubation environment.

Calcofluor-stained conidia exhibited bright blue fluorescence under the UG1,UG5/LP397 filter system. The fluorescence was retained by germ tubes and infection structures following germination (Fig. 1), thus allowing a sensitive location of infection sites on the leaf surface. This was essential because of the poor infection efficiency. Successful penetrations occurred both through stomata and by direct appressorial penetration.

By changing from the previous filters to the KP490/LP528 filter system, fluorescein uptake by host cells could be directly observed. Small, nonstaining, necrotic areas of 10–20 host cells surrounding the penetration site appeared on leaves of cultivar Union as early as 13 hr after inoculation (Fig. 1B). These regions of necrotic host cells did not expand appreciably at later times, thus confirming the relatively rapid expression of the *Rpm* resistance gene. In contrast, inoculated leaves of the compatible cultivar Williams did not exhibit detectable host cell necrosis before the third day after inoculation (Fig. 1D), when a few necrotic cells were detected around infection sites.

Leaf cells of incompatible cultivar Union plants surrounding the fungus penetration site exhibited intense autofluorescence at 24–48 hr after inoculation (Fig. 1F), but not at 13 hr, when host cell necrosis was initially detected. The autofluorescence was most easily observed in leaf sections that had not been treated with fluorescein. The autofluorescence was observed in epidermal cell walls as well as mesophyll cells, and it became more intense from the third to fifth day after inoculation. In some cases, the cytoplasm of one or two epidermal cells penetrated by the fungus also fluoresced. The host cells exhibiting autofluorescence surrounded penetration sites and accordingly were considered dead, based on the fluorescein studies.

Inoculated compatible leaves exhibited weak autofluorescence on the second day after inoculation (Fig. 1H), and the autofluorescence became slightly stronger up to day 5, but was never as intense as that occurring at the incompatible infection sites (Fig. 1F).

Hooker microtome sections through infection sites of incompatible leaves disclosed the presence of host cell autofluorescence as early as 24 hr after inoculation (Fig. 1J and L). Autofluorescence appeared to occur in epidermal cell walls and mesophyll cells. Similar sections from inoculated compatible leaves, however, exhibited no detectable infection site autofluorescence at that time.

The phytoalexin glyceollin and related compounds were not detected by extraction of incompatible or compatible inoculated leaves at 1 and 2 days after inoculation. Traces of glyceollin were recovered after 3–5 days but could not be accurately quantitated, and no significant differences were apparent between extracts from inoculated compatible and incompatible leaves.

Soybean leaves inoculated with *U. phaseoli* exhibited host cell death patterns and autofluorescence similar to those caused by *P. manshurica* on cultivar Union. However, these lesions were macroscopically visible and glyceollin was detected by thin-layer chromatography of extracts prepared from the inoculated leaves but not from uninoculated leaves at 24–48 hr.

DISCUSSION

We were unable to devise conditions leading to a high infection efficiency by *P. manshurica* conidia on either genotype of soybean leaves. Instead, only scattered successful infections were observed, regardless of inoculum concentration or postinoculation environment. Because of this difficulty, the Calcofluor technique was essential to precisely locate fungal infection sites. Similar techniques have also proven useful with other fungal pathogens (12,14). Filter changes on the fluorescence microscope also permitted simultaneous observation of fluorescein uptake or autofluorescence in host cells at the localized infection sites (Fig. 1). Our method has the additional advantage that it eliminates the necessity of clearing and fixing procedures that extract the autofluorescent material (4).

Our observations indicated that microscopic hypersensitive death of host cells surrounding *P. manshurica* infection sites occurred in the incompatible *Rpm* genotype but not the *rpm* genotype. Hypersensitive host cells were easily detected by using the fluorescein uptake technique at 13 hr after inoculation. On the other hand, little host cell death was observed at the penetration sites of inoculated *rpm* leaves at up to 3 days after inoculation. It appears, therefore, that hypersensitive host cell death is specifically associated with the resistance reaction of *Rpm* leaves, even though visible symptoms are not macroscopically observed.

The occurrence of autofluorescence in the hypersensitive host cells of inoculated *Rpm* leaves at 24 hr was analogous to that previously observed in incompatible soybean reactions to *P. glycinea* (4) and in resistant reactions of other plants (8,9,10,13). In the former case, autofluorescence was interpreted to result from the accumulation of glyceollin and related isoflavonoid compounds in the dead host cells (4). The autofluorescence observed in *Rpm* soybean leaves inoculated with *P. manshurica* may also be due to glyceollin accumulation at fungal infection sites, but we were unable to obtain confirmation by extraction of leaves and thin-layer chromatographic quantitation. This was probably due to the low infection efficiency of *P. manshurica* conidia and the consequent low number of defensively-reacting host cells. Similarly, in previous work with the flax-flax rust system (7) and the soybean-*P. glycinea* system (4), we were unable to adequately extract and quantitate phytoalexin levels when low inoculum concentrations were used. In the present work we noted that soybean leaves of both cultivars inoculated with *U. phaseoli* exhibited small, but macroscopically visible hypersensitive lesions, and glyceollin was detected by extraction and thin-layer chromatography. The fact that host cells in the *U. phaseoli* lesion areas also contained autofluorescence, coupled with the observations of Holliday et al (4), supports the interpretation that autofluorescence in the *P. manshurica* infection sites may represent the accumulation of glyceollin.

Riggle and Dunleavy (11) concluded that expression of the *Rpm* resistance allele first occurred between 12 and 24 hr after inoculation, and fungal growth was severely inhibited by 36 hr. Since this period was precisely when host cell necrosis and autofluorescence accumulation were observed, our findings support the possible role of hypersensitive cell death and glyceollin accumulation in resistance gene expression, but proof of a cause-effect relationship remains lacking.

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