An Early Indicator of Resistance in Barley to Russian Wheat Aphid

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During early stages of infestation by Russian wheat aphids (Diuraphis noxia [Mordvilko]; RWAs), barley (Hordeum vulgare L) leaf cells collapsed and showed autofluorescence in the mesophyll and bundle sheath adjacent to the RWA stylet sheath. The response was visually similar to the hypersensitive cell death response, typical of resistance to microbial pathogens. Resistant barley produced significantly more collapsed, autofluorescent cells (CAC) than did susceptible barley. RWA stylet entry sites and sheath paths also fluoresced, making them easy to observe in whole leaf sections. The number of CAC increased with the number of RWAs and with the number of days of feeding in resistant plants. The CAC could be observed 1 d following infestation, making this the most rapid plant response toward the RWAs known to date. The response may be useful in screening for resistant plants and may provide insight into resistance mechanisms in barley.

The RWA (Diuraphis noxia [Mordvilko]) is a newly arrived insect pest in the United States (Webster et al., 1987). Since its appearance in 1986, it has caused more than $657 million damage to cereal crops (Massey and Amosson, 1991). So far only two forms of RWA have been observed in the United States: winged females and wingless females. No male forms of the RWA have been found; thus, the aphids are apparently limited to parthenogenetic reproduction. Winged females can fly only short distances, but they are capable of long distance travel on prevailing winds and air convection currents (Schotzko and Smith, 1991).

Although the advanced physical symptoms—white, yellow, or purplish streaks on leaves and stems, prostrate growth habit, and rolled leaves—of a RWA infestation are well defined (Webster et al., 1987), the early physiological responses of the plant are nearly unknown. No differences between responses of resistant (PI 366450) and susceptible (cv Morex) barley (Hordeum vulgare L) were found for relative water content, stomatal resistance, and total Chl loss in time courses of 1 to 14 DAI (Miller et al., 1994). Differences between the resistant and susceptible barley were found in induced proteins in the two-dimensional SDS-PAGE pattern at 6 DAI; at 7 DAI the susceptible barley had lower electron transfer capacity of PSII electron acceptors than did the resistant barley (Miller et al., 1994).

To date, only one study has been published describing RWA-feeding damage on a microscopic level (Fouché et al., 1984). The results showed that RWAs on susceptible wheat did not have a preferential probing site, exhibiting no preference for stomatal pores or any other particular configuration of cells. The path of the stylet into the phloem-feeding site was intercellular until the stylet pierced the phloem (Fouché et al., 1984). The stylet appears to secrete a lipoprotein sheath that surrounds the stylet and may protect it from plant wound reactions (Miles, 1990). By 5 days after infestation, feeding had caused chloroplasts and cell membranes to become disrupted or to disintegrate (Fouché et al., 1984).

In incompatible fungus-plant interactions (resistance), a hypersensitive response is frequently observed at the infection site wherein infected host cells die (Aist and Bushnell, 1991), accompanied by polymerization of phenolics in these collapsed cells (Dixon and Lamb, 1990). These cells can be easily located by a yellow autofluorescence under UV or blue light in barley (Koga et al., 1988) and in wheat (Mœrschbacher et al., 1990).

Barley germplasm resistant to the RWAs has been found (Webster et al., 1991), but the nature of the resistance mechanism(s) is unknown. Since the microscopy technique for plants resistant and susceptible to fungi identifies physiological responses that occur prior to macroscopic symptom development, it was our hope that microscopic differences would also be apparent between plants resistant and susceptible to insect infestation. We report here that CACs were formed in RWA-resistant barley mesophyll and bundle sheath within 1 d after infestation by RWAs, with considerably less development of CACs in susceptible barley.

MATERIALS AND METHODS

Plant Culture

Seeds of barley (Hordeum vulgare L.) were provided from stocks maintained by the U.S. Department of Agriculture/Agricultural Research Service Plant Science and Water Conservation Laboratory (Stillwater, OK). Barley was grown from seed in pots under greenhouse conditions (Miller et al., 1994). Four resistant barley germplasm accessions were used—Cl 1412 (Webster et al., 1991), CI ho 6925 (D.W. Mornhinweg, personal communication), PI 366444 (Webster et al., 1991).

Abbreviations: CAC, collapsed, autofluorescent cell; DAI, days after infestation; RWA, Russian wheat aphid.
PI 366450 (Webster et al., 1991)—as well as four susceptible cultivars—Morex, Excel, Klages, and Crystal (D.W. Mornhinweg, personal communication).

**Aphid Culture**

Stocks of RWAs (Diuraphis noxia [Mordvilko]) were also maintained at the Stillwater, OK, laboratory. The aphids were raised on susceptible barley (cv Wintermalt) under greenhouse conditions (Starks and Burton, 1977).

**Infestation**

Leaves were infested by placing a large number (200–300) of variously aged aphids in a 21-mm-diameter clip cage (Webster et al., 1993b) on the adaxial side of the third and fourth leaves of plants having an expanding fourth leaf (Table I). Two plants from each barley line were used. An infestation test of each barley line was made at least three times. Leaves from the last two infestation tests were used to obtain the data for Table I. At 3 DAI, leaf sections within the clip cage were excised and decolorized in boiling 70% (v/v) ethanol (Reimers and Leach, 1991). Leaves caged without aphids for 3 d were used as control (~RWA) sections. Sections were stored in 90% ethanol. Sections could be stored for at least 2 months with no loss of autofluorescence and for at least 2 years with no loss of autofluorescence but with some change in color of autofluorescence.

To produce the data for Figures 2 to 4, the procedures were similar to those for Table I except that a specified number of RWAs was put in each cage for 1, 2, or 3 d, as noted.

**Microscopy**

Transmitted white light and fluorescence studies were carried out on a Nikon Optiphot microscope. Leaf sections were mounted in water. No staining was needed for observations. Autofluorescence was observed with a ×20 fluorescence objective (CF Fluor 20×/0.75) using a 82 (blue light excitation) filtered cassette (IF 460-485, DM 510, and emission filter 520W) (Autofluorescence was observed with a X20 fluorescence objective (CF Fluor 20×/0.75) using a 82 (blue light excitation) filtered cassette (IF 460-485, DM 510, and emission filter 520W) (Reimers and Leach, 1991). Leaves caged without aphids for 3 d were used as control (~RWA) sections. Sections were stored in 90% ethanol. Sections could be stored for at least 2 months with no loss of autofluorescence and for at least 2 years with no loss of autofluorescence but with some change in color of autofluorescence.

To produce the data for Figures 2 to 4, the procedures were similar to those for Table I except that a specified number of RWAs was put in each cage for 1, 2, or 3 d, as noted.

**RESULTS**

Many entry sites and stylet sheaths were observed in RWA-resistant plants (Fig. 1, A–D). Fluorescent RWA sheaths could be tracked through several focal planes in the whole, decolorized leaf sections (Fig. 1). As had been observed for wheat (Fouché et al., 1984), the RWAs did not appear to have a preferential entry site into the barley leaf, and stylet sheaths followed an intercellular path.

Resistant barley leaves showed many CAC as well as fluorescent entry sites and sheaths (Fig. 1, A and B; Table I). In the resistant plants, nearly all fluorescent stylet sheaths extended to CACs (Fig. 1B). Sometimes CACs were also located along a sheath. Sheaths in the resistant plants often had several branches so the aphids apparently changed probing directions without completely retracting their stylets, as also noted by Fouché et al. (1984). No fluorescence of this sort was observed in the ~RWA control leaves.

After 3 d of RWA infestation, a significantly larger number of CACs were elicited in RWA-resistant barley leaves than in the susceptible barleys (Table I). Although few CACs formed in the susceptible barleys, fluorescent entry sites and sheaths could sometimes be observed (Fig. 1, E and F). In the susceptible plants the occurrence of fluorescent material varied with cultivar (Table I), from no fluorescence in many microscope fields (cv Excel) to some fluorescent entry sites and sheaths but no or few CACs (cv Crystal: Fig. 1, E and F; and cv Klages), to many fluorescent probing sheaths, some ending in CACs (cv Morex). We could also locate sheaths in white light (Fig. 1, D and H). Any collapsed cell observed under white light was fluorescent under blue light; there were no apparent nonfluorescent collapsed cells.

Increasing the number of RWAs per cage increased the number of CACs produced in resistant germplasm (Fig. 2). The two resistant barleys, CI 1412 and Clho 6925, showed much higher levels of CACs than the two susceptible barleys, Crystal and Excel, and the response showed continuous increases to 300 RWAs per cage. The increases in CACs plateaued at 100 RWAs for Excel and Crystal.

A time course of CAC production showed a large increase in the number of CACs formed in resistant barley during 3 d of feeding (Fig. 3). The susceptible barleys had no more CACs from 3 d of feeding than from 2 d. Production of CACs occurred within 1 d in both susceptible and resistant plants (Fig. 4). The total number of CACs in entire leaf sections infested with 25 RWAs was counted after 1 d. Resistant plants had 50 to 60 CACs in an entire caged area, which was severalfold more than in susceptible plants (Fig. 4).

**DISCUSSION**

The CAC of mesophyll and bundle sheath tissues in response to RWA is visually similar to the hypersensitive response of barley to incompatible fungi. In both cases whole cells collapse and become yellow fluorescent. Hypersensitive
Figure 1. (The legend appears on the opposite page.)
may, nevertheless, trigger wound reactions and/or the pro-

duction of the constituents found in papillae. In fact, feeding
monitor studies suggest that mesophyll or bundle sheath cells
are sometimes penetrated, since ingestion by RWAs occurs
from nonphloem tissue as well as from phloem (Webster et
al., 1993b).

In the hypersensitive response of resistant wheat plants
to rust fungi, the mechanism of resistance was attributed to

Table 1. Autofluorescence of CAC of barley susceptible and
resistant to RWA

<table>
<thead>
<tr>
<th>Response to RWA</th>
<th>Barley Cultivar or Germplasm</th>
<th>CAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No field</td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>Crystal</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Excel</td>
<td>0.17 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Klages</td>
<td>0.50 ± 0.22</td>
</tr>
<tr>
<td></td>
<td>Morex</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>Resistant</td>
<td>Clho 6925</td>
<td>10.5 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>PI 366444</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>CI 1412</td>
<td>14.7 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>PI 366450</td>
<td>8.8 ± 1.5</td>
</tr>
</tbody>
</table>

Leaf sections were infested for 3 d. The number of auto-
fluorescent cell groups within one optical field (0.4 mm²) were
counted. Six optical fields were sampled randomly, two fields per
leaf. Numbers are mean numbers of CAC groups in one optical
field ± se. Means of susceptible plants were significantly lower
than means of resistant plants at P = 0.05, according to Scheffe’s
S method (Ott, 1984).

death in attacked epidermal cells of barley containing the
Mia gene for resistance occurs by 24 h after inoculation with
Erysiphe graminis f. sp. hordei (barley powdery mildew fun-
gus) and is accompanied by a weak, yellow autofluorescence
that increases after death (Koga et al., 1988). A hypersensi-
tive reaction can occur in a cell in which fungal haustorium
formation is attempted, in which case collapse of the haustorium
accompanies the host cell collapse. Unattacked neigh-
boring cells may also collapse (Aist and Bushnell, 1991). With
the barley-powdery mildew interaction, the degree of hyper-
sensitive reaction corresponds with the effectiveness of in-
duced resistance, expressed as a reduction in the number of
colonies formed, in barley of three different genetic back-
grounds and four different resistance genes, indicating that
the hypersensitive reaction may be partly responsible for the
induced resistance (Martinelli et al., 1993).

In addition to the cells undergoing a hypersensitive re-
response, barley responding to E. graminis shows other kinds
of yellow autofluorescence. In both compatible and in-
compatible cultivars, epidermal autofluorescence in the host
cell wall at sites of attempted penetration (Kunoh et al., 1985;
Aist and Bushnell, 1991) is an early (within 1 h) response.
Later, fluorescent papillae (host wall appositions deposited
inside the cell wall) develop at the sites (Aist and
Bushnell, 1991). Papillae are not restricted to resistant cul-
tivars, but papillae that contain phenolics from an early acti-
vation of phenolic metabolism are apparently significant
factors in general resistance (Koga et al., 1980; Aist et al.,
1988). Papillae, formed in response to fungal attack, and
wound plugs, formed in response to mechanical puncture of
epidermal cells, both contain callose, other carbohydrates,
and protein, but only papillae contain phenols and basic-
staining material. Wound plugs, nevertheless, show some
 autolysis under blue light excitation (Russo and Bush-
nell, 1989). Although RWA stylets appear to penetrate leaf
tissue intercellularly rather than puncturing cells in their
approach to the phloem (Fig. 1; Fouché et al., 1984), they
may, nevertheless, trigger wound reactions and/or the pro-

Figure 2. The effect of increasing the number of RWAs on the
number of CACs produced by resistant and susceptible barleys.
One cage of RWAs was put on each of two leaves of barley for 3
d. The number of CACs were counted in four random microscope
fields (×10 objective) from each leaf section, for a total of eight
fields for each germplasm.

Figure 3. Time course of CAC production. Twenty-five RWAs in a
cage were allowed to feed for 1, 2, or 3 d. Three random microscope
fields (×20 objective) were observed from each of four leaf sections
from each germplasm.
addition to, lignin (Harris and Hartley, 1976). Although identification of the material would not be necessary for purposes of identifying RWA-resistant barley germplasm, knowledge of the nature of the material would facilitate investigations of the regulation of the response.

The above mechanisms of resistance attributed to the hypersensitive response would have little effect on an aphid, which can withdraw and move to another spot to feed (Fernandes, 1990). Rather, if the CACs are evidence of a resistance mechanism, that mechanism may be feeding deterrence. Studies of the feeding activity of the RWA on barley leaves have shown that RWAs probe more often and feed for less time on resistant plants than on susceptible plants (Webster et al., 1993a). RWAs take at least 6 h for each successful phloem-feeding event, including searching and probing, on susceptible barley. In a 6-h test, Webster et al. (1993a) showed that RWAs on resistant barley spend little time, less than 1 h, in each actual phloem ingestion, as opposed to more than 4 h on susceptible plants.

The short feeding period for RWAs on resistant barley, coupled with double the frequency of probing activity and one-third the successful probes, relative to RWAs on a susceptible barley (Webster et al., 1993a), may indicate production of unpalatable plant material. This unpalatable material may be related to the autofluorescent material we have observed, or precursors to that material, which is being produced in response to RWA probing and feeding. Since the autofluorescent material is probably phenolic in nature, it is likely to taste unpleasant (Mittler, 1988) to RWAs and thus result in the search for more palatable feeding sites. Among resistant plants, the number of CACs may reflect the high frequency of probing and, hence, be indicative of one type of several potential resistance mechanisms of the plant. Types of resistance mechanisms for barley include tolerance, antibiosis, and antixenosis (Webster et al., 1991). The resistant wheat lines, PI 262660 and PI 137739, have similar levels of antixenosis (Du Toit, 1987) and antibiosis (Du Toit, 1987, 1989a), but PI 262660 has higher levels of tolerance (1989a). Resistance in these two wheat lines is controlled by different genes (Du Toit, 1989b).

As in the fungal-plant interactions, resistant barleys produced more CACs than susceptible barleys (Table I). A possible exception, as evidenced by the low numbers of CACs produced, is the resistant PI 366444, which may have a separate resistance mechanism, since RWAs on PI 366444 have feeding activities similar to RWAs on susceptible plants (Webster et al., 1993a). If PI 366444 is regarded as having a separate resistance mechanism, and disregarded in this comparison of CAC production, the difference between resistant and susceptible cultivars is all the more apparent.

CAC production was positively correlated with the number of RWAs feeding on resistant barley (Fig. 2). A difference in CAC production between resistant and susceptible barleys could be seen with as few as 25 RWAs. The low numbers of CACs in susceptible barley did not appear to be the result of slower production of CACs, since CAC production did not increase after 2 d in the susceptible cultivars (Fig. 3). The resistant plants, on the other hand, had large increases in CACs measured during the 3-d period.

The higher number of CACs (Fig. 2) found in CI 1412 when compared to CIho 6925, each infested with 300 RWAs, may be due to the higher number (144) of nymphs produced on CI 1412 after 3 d relative to the number (112) produced on CIho 6925. Conversely, but still related to CAC production, with 100 RWAs in the initial infestation, more nymphs (88) were produced on CIho 6925 than on CI 1412 (74). If there is a relationship between nymph number and CAC production, it was not evident on susceptible plants. The number of nymphs produced on the susceptible plants was at least as high and generally higher than the number produced on resistant plants. Whereas CAC production in resistant plants may be correlated with the number of prohambios (Webster et al., 1991), but CIho 6925 has higher levels of tolerance (1989a). Resistance in these two wheat lines is controlled by different genes (Du Toit, 1989b).

Because of the recent occurrence of the RWA in the United States, no resistant barley cultivars are yet available to growers (Webster et al., 1991). Current methods of evaluating barley lines for resistance are time consuming and subjective (Webster et al., 1991). The production of CACs is the most rapid plant response to RWA feeding reported thus far. Infestation for 1 d was sufficient to elicit the production of severalfold more CACs in resistant than in susceptible plants (Fig. 4). For barleys in which resistance is associated with high CAC production, counting CACs to rapidly differentiate susceptible from resistant barley in screening tests could avoid the environmental effects that hamper long-term tests. Studies to determine the genetics of CAC production and its correlation with particular mechanisms of resistance await the availability of genetic material. The analysis of CAC production may also be useful in the study of the physiology and control of this resistance mechanism. The use of whole leaf sections, in addition to eliminating the process of sectioning, also allows the location of the sheaths and entry sites associated with CACs. Furthermore, many difficulties of staining and storing stained material are eliminated by using cleared material and looking for autofluorescence.

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LITERATURE CITED


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