Resistance Induction in Barley Coleoptile Cells by Intracellular pH Decline

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Cytoplasmic acidification in suspension-cultured plant cells has been characterized as a common intracellular response of some kinds of plant cells to elicitors. Expression of various defense genes in these cells has been increased by the cytoplasmic acidification itself without treatment by elicitors. It is not evident, however, whether or not cells with acidified cytoplasm actually exhibit resistance to the pathogen because of the lack of an adequate infection system between cultured plant cells and some pathogens. Using barley coleoptiles rather than suspension-cultured cells, we demonstrated both detection of cellular pH decline and increased resistance to Blumeria graminis. The cytoplasmic pH of barley coleoptile cells floated on 1 mM citrate buffer (CB), pH 4.0, became 0.5 unit lower than that of cells floated on 1 mM CB, pH 8.0, within 30 min after treatment. The penetration efficiency of B. graminis into the coleoptile was decreased in a pH-dependent manner; that is, when the coleoptiles were floated on 1 mM CB, pH 8.0, the penetration efficiency of the fungi was about 80%. In contrast, when the coleoptiles were floated on acidic buffers, the penetration efficiency decreased in parallel the decline of pH and the penetration efficiency reached 0% when coleoptiles were floated on 1 mM CB, pH 4.0. Morphogenesis of appressoria on the coleoptiles floating on CB was not influenced. The lowered penetration efficiency at lower pH was partially cancelled when the coleoptiles were floated on 1 mM CB, pH 8.0. The penetration efficiency of the fungi was about 80%. In contrast, when the coleoptiles were floated on acidic buffers, the penetration efficiency decreased in parallel the decline of pH and the penetration efficiency reached 0% when coleoptiles were floated on 1 mM CB, pH 4.0. Morphogenesis of appressoria on the coleoptiles floating on CB was not influenced. The lowered penetration efficiency at lower pH was partially cancelled when the coleoptiles were floated on 1 mM CB, pH 8.0. The penetration efficiency of the fungi was about 80%. In contrast, when the coleoptiles were floated on acidic buffers, the penetration efficiency decreased in parallel the decline of pH and the penetration efficiency reached 0% when coleoptiles were floated on 1 mM CB, pH 4.0. Morphogenesis of appressoria on the coleoptiles floating on CB was not influenced. The lowered penetration efficiency at lower pH was partially cancelled when the coleoptiles were floated on 1 mM CB, pH 8.0. The penetration efficiency of the fungi was about 80%. In contrast, when the coleoptiles were floated on acidic buffers, the penetration efficiency decreased in parallel the decline of pH and the penetration efficiency reached 0% when coleoptiles were floated on 1 mM CB, pH 4.0. Morphogenesis of appressoria on the coleoptiles floating on CB was not influenced. The lowered penetration efficiency at lower pH was partially cancelled when the coleoptiles were floated on 1 mM CB, pH 8.0. The penetration efficiency of the fungi was about 80%. In contrast, when the coleoptiles were floated on acidic buffers, the penetration efficiency decreased in parallel the decline of pH and the penetration efficiency reached 0% when coleoptiles were floated on 1 mM CB, pH 4.0. Morphogenesis of appressoria on the coleoptiles floating on CB was not influenced. The lowered penetration efficiency at lower pH was partially cancelled when the coleoptiles were floated on 1 mM CB, pH 8.0.

Key words: Blumeria graminis f. sp. hordei — Cytoplasmic acidification — Hordeum vulgare — Resistance induction.

Abbreviations: CB, citrate buffer; FDA, fluorescein diacetate; HR, hypersensitive reaction.

Introduction

One of the most important early events of plant defense systems is the hypersensitive reaction (HR). Several reports have demonstrated that electrolyte loss occurs during the early stages of the HR of the various hosts to pathogenic bacteria (Burkowicz and Goodman 1969, Cook and Stall 1968, Goodman 1968, Lyon and Wood 1976). It has been reported that some kinds of elicitor cause extracellular alkalization of plants accompanying, for example, K+ efflux and Ca2+ influx (Atkinson et al. 1996), it has also been demonstrated that some kinds of elicitor cause cytoplasmic acidification of suspension-cultured tobacco cells (Mathieu et al. 1996) and rice cells (Kuchitsu et al. 1997). On the other hand, cytoplasmic acidification itself has been reported to trigger defense gene activation in tobacco and rice cell suspensions without elicitor treatment (He et al. 1998, Lapous et al. 1998). Thus, it is likely that some cells with acidified cytoplasm could exhibit some resistance to a pathogen. Suspension-cultured plant cells, however, are not appropriate for inoculation with some pathogens because the acidified solution itself could affect the pathogen. Although there have been some reports of infection systems consisting of cultured plant cells and the pathogens (Able et al. 1998, Guo et al. 1998, Tenhaken and Rubel 1998), the disease response of such cells could not be evaluated properly because of the lack of an adequate system to observe the infection process of the pathogen.

For cytological studies of powdery mildew, Bushnell et al. (1967) provided a host cell system comprised of partially dissected barley coleoptiles, which consisted of one-cell layer of epidermis (Fig. 1). These cells are relatively uniform in morphology. When powdery mildew fungi are inoculated on coleoptiles floated on an incubation solution, the ectoparasitic fungi produce all their vegetative and reproductive organs except haustoria on the surface of these coleoptiles. Using this infection system, our primary questions were:

1. Does cytoplasmic acidification of barley coleoptile cells occur promptly when they are floated on acidified solution?
2. Is the resistance of the coleoptile cells increased by cytoplasmic acidification when powdery mildew fungi are inoculated on them?

Materials and Methods

Host plants and fungal material

Seedlings of barley (Hordeum vulgare L. cv. Kobinkatagi) were grown under fluorescent light (ca. 23.6 W) in a 14-h photoperiod in growth chambers at 70% relative humidity. The fungus Blumeria graminis DC f. sp. hordei EM. Marchal, race 1, was applied as conidia to leaves of 8- to 12-day-old seedlings, and plants were incubated for up to 14 d. Conidia were used as soon as available, typically 6 d after inoculation. Coleoptiles were excised from seedlings 9 d after sowing, and single-cell epidermal layers of partially dissected coleoptiles were prepared as described previously (Takamatsu et al. 1978).

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Coleoptiles were inoculated with 40–50 freshly harvested conidia of *B. graminis* using a brush, floated on 1 mM CaCl₂ or 1 mM citrate buffer (CB) containing 1 mM CaCl₂ at various pHs and incubated at 20°C for 24 h. The penetration efficiency (PE) of *B. graminis* was determined as follows:

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PE = \left( \frac{\text{number of haustoria}}{\text{number of appressoria with a penetration pore}} \right) \times 100 \%
\]

Using CaCl₂ instead of distilled water has been shown to result in constant high penetration efficiency of *B. graminis* to coleoptiles (70–80%). Therefore, we used 1 mM CaCl₂ for the incubation solution.

**Measurement of cytoplasmic pH under a cryomicroscope**

The changes in cytoplasmic pH in coleoptile cells were detected under a cryomicroscope using fluorescence pH-ratiometry (Yoshida 1994, Yoshida 1995). Prior to detection of pH change, coleoptile cells were loaded with 10 μM fluorescein diacetate (FDA) for 5 min at 26°C and immediately cooled on ice. The dye-loaded coleoptiles were washed, mounted on the cryomicroscope stage, and covered with a cover slip. The conditions were optimal for specific loading of fluorophore into the cytoplasm and minimising the localization of the fluorophore into vacuoles. The dye-loaded coleoptile cells were excited alternately at 495 nm and 435 nm by a 100-W mercury lamp with 10 nm band-pass interference filters (Asahi Optics, Tokyo, Japan). Fluorescence images were focused on an SIT video-camera through a zoom lens (×1.0–×2.5) and recorded with an image processor that averaged 16 video frames, as reported elsewhere (Yoshida 1994, Yoshida 1995). The ratio image (495 nm/435 nm) was calculated after subtraction of the dark signal.

**UV light irradiation**

Coleoptiles were partially dissected to expose the inner epidermis and were irradiated under a 15-W UV lamp (Toshiba Electric Co., Japan) at a distance of 50 cm for 5 min prior to inoculation of *B. graminis*.

**Western blotting**

Total protein was extracted from barley cotyledons (1 g) by grinding in a chilled mortar and pestle with 1.5 ml of ice-cold buffer (25 mM Tris-HCl, pH 7.0, 30 mM MgCl₂, 2 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride). The slurry was centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was saved. Protein concentration was determined by BioRad protein assay kit using bovine serum albumin as the standard. Total protein (50 μg) was separated by SDS-PAGE using a 10% acrylamide separating gel and electro-transferred to a Hybond C super (Amersham) nitrocellulose membrane filter according to the protocols of the manufacturer. Detection of PR1 protein (Schweizer et al. 1997) was performed using ECL system (Amersham) with antibody (1:200 dilution) raised against the major basic PR1 of tomato and horseradish-peroxidase-linked goat anti-mouse IgG.

**Results and Discussion**

Barley (*H. vulgare* L. cv. Kobinkatagi) coleoptiles, which were excised from seedlings 9 d after sowing, and single-cell epidermal layers of partially dissected coleoptiles were prepared as described previously (Fig. 1) (Takamatsu et al. 1978). When coleoptiles were floated on 1 mM CB, pH 8.0, the obtained pH-ratio images are those shown in Fig. 2C. In contrast, when coleoptiles were floated on 1 mM CB, pH 4.0, the pH-ratio images changed within 30 min (Fig. 2A). The relative cytoplasmic pH values were estimated from a calibration curve generated using pH-equilibration buffer containing 10 μM free fluorescein. The cytoplasmic pH value of coleoptiles floated on pH 4.0 solution was 0.5 units lower than that of coleoptiles floated on pH 8.0 solution (Fig. 3).

Dissected barley coleoptiles were inoculated with *B. graminis* (f. sp. *hordei* EM. Marchal, race 1), floated on 1 mM CB including 1 mM CaCl₂ at each pH, and incubated at 20°C for 24 h. As shown in Fig. 4, the penetration efficiency was about 80% at pH 8.0 and decreased gradually in a pH-decline-dependent manner. At pH 4.0, conidial germlings induced papillae below their appressoria but never formed haustoria (0% penetration efficiency) (Fig. 5C). Since *B. graminis* is an obligate parasite, the fungus cannot form a haustorium in a dead plant cell. The infection process of the fungi is completed within 24 h after inoculation. However, the coleoptile cells floating on pH 4.0 solution were still alive as determined by observation of emergence of cytoplasmic streaming even 96 h after inoculation. This means that the absence of haustorial formation of the fungi at pH 4.0 was not a result of coleoptile cell death.

Because the difference in penetration efficiency between
pH 4.0 and pH 5.0 was large, the relationship between penetration efficiency and pH was investigated in more detail in this pH range (Fig. 6). The penetration efficiency of B. graminis was increased by increasing the pH of 1 mM CB from pH 4.0 to 5.0. However, the standard deviation was quite high at pHs 4.3, 4.4, and 4.5, indicating that the response of barley coleoptile cells to B. graminis fluctuates within this pH range.

It has been reported that the resistant response was suppressed when the coleoptiles were irradiated with UV light for 5 min prior to inoculation with B. graminis (Yamaoka et al. 1990). If the low penetration efficiency of B. graminis at low pH is the result of resistance induced by cytoplasmic acidification, the induced resistance in coleoptiles floating on a low-pH solution might be cancelled by pre-treatment with UV irradiation. As shown in Figure 6, the penetration efficiency of B. graminis at pH 4.2 is about 10%. In contrast, the penetration efficiency of B. graminis on coleoptiles floating on CB at the same pH was much higher (27%) when the coleoptiles had

**Fig. 2** Fluorescence image of barley coleoptile cells 30 min after floating on 1 mM citrate buffer (CB) including 1 mM CaCl₂ at each pH.

**Fig. 7** Induced resistance in barley first leaves whose proximal ends were soaked in 100 mM CB, pH 4.0, for 24 h after inoculation with Blumeria graminis. (A) Suppression of colonization of Blumeria graminis on the first leaves soaked in 100 mM CB, pH 4.0, or water for the first 24 h and then transferred to H₂O. The photograph was taken 7 d after inoculation. (B) Western blot analysis of the specific PR1 proteins extracted from first leaves 24 h after inoculation with B. graminis. water: Uninoculated first leaves soaked in water. water+B.g: Inoculated first leaves soaked in water. CB: Uninoculated first leaves soaked in 100 mM, CB, pH 4.0. CB+B.g: Inoculated first leaves soaked in 100 mM, CB, pH 4.0.
been irradiated with UV for 5 min prior to inoculation. The cancelling of resistance by UV irradiation was also observed at pH 4.3 and 4.4. However, the induced resistance at low pH was not totally cancelled by the UV irradiation, and the penetration efficiency was much lower than that of control. This result suggests that the low pH of the incubation medium might directly affect the infection process of the fungi. However, the morphogenesis of the fungi seemed to be normal (Fig. 5B, C). It also seems likely that the fungi are hardly affected by the acidic solution because they conduct their morphogenesis on the surface of coleoptile floating on acidic solution. Moreover, the fungi that failed to form haustoria always had papillae under their appressoria (data not shown), indicating that almost all the conidia tried to penetrate the coleoptile cells. These results, together with the cancelling of resistance by UV irradiation, indicate that the decrease in penetration efficiency of *B. graminis* on the coleoptiles with acidified cytoplasm is the result of resistance of barley coleoptile cells induced by acidification, not the result of fungi affected directly by the acidified solution, although it cannot be denied that invisible parts of the infection process might be affected by the pH decline of the coleoptile cells.

In separate experiments, we investigated whether or not defense-related genes could be induced by the cytoplasmic acidification of barley coleoptile cells. Because each coleoptile contains a small amount of protein, a very large number of coleoptiles would be needed in order to prepare enough proteins for Western blot analysis. Therefore, we adopted barley first leaves instead of coleoptiles for Western blot analysis. We first investigated the induced resistance to *B. graminis* when a proximal site on barley first leaves was soaked in 1 mM CB, pH 4.0, for 7 d. However, the colonization of *B. graminis* on these first leaves soaked in 1 mM CB, pH 4.0, was not at all suppressed compared with the control (H2O) (data not shown). This result was the same when the first leaves soaked even in 10 mM CB, pH 4.0. Therefore we used 100 mM CB, pH 4.0, for the first 24 h. As shown in Fig. 7A, the colonization of *B. graminis* on these first leaves soaked in 1 mM CB, pH 4.0, was not at all suppressed compared with the control (H2O) (data not shown). This result was the same when the first leaves soaked even in 10 mM CB, pH 4.0. Therefore we used 100 mM CB, pH 4.0, for the first 24 h and then transferred to H2O, was extremely suppressed 7 d after inoculation. At the same time, the expression of the specific PR1 protein, which was extracted 24 h after inoculation, was detected more strongly in the inoculated first leaves soaked in 100 mM CB, pH 4.0 (Fig. 7B).

The mechanism of the induced resistance by cytoplasmic acidification is unclear. He et al. (1998) reported that treatment...
Resistance induced by intracellular pH decline

of suspension-cultured rice cells with propionic acid resulted in a rapid decrease in the cytoplasmic pH and concomitantly resulted in the accumulation of the mRNAs for some of the defense-related genes activated with an elicitor for suspension-cultured rice cells. Therefore, it is plausible that some resistance-related genes might be induced in barley coleoptile cells floating on the acidic buffer before *B. graminis* tried to penetrate the cells. Furthermore, He et al. (1998) reported that direct acidification of suspension-cultured rice cells by propionic acid did not induce the production of reactive oxygen species, although the elicitor, *N*-acetylchitooligosaccharides, induces production of reactive oxygen species in the cells. Thus, establishing resistance might require not only an acidic cytoplasm but also some component of the fungus. This hypothesis might be supported by the result that fungal attack was needed to increase the expression of resistance-related genes such as the PR 1 gene, as shown in Fig. 7B. The reinforced resistant state seems to need continuous cytoplasmic acidification of the plant, because the resistant state was readily cancelled by transferring the coleoptiles from 1 mM CB, pH 4.0, to 1 mM CaCl$_2$ just before the penetration of *B. graminis* was attempted (data not shown).

Some investigators have challenged suspension-cultured cells of plants with bacterial pathogens (Baker et al. 1993, Guo et al. 1998, Tenhaken and Rubel 1998) or fungus (Able et al. 1998). Such suspension-cultured cells have been used for investigating extracellular alkalinization and oxidative burst (Baker et al. 1993, Tenhaken and Rubel 1998) or phytoalexin accumulation (Guo et al. 1998) by inoculating the cells with an avirulent strain or an incompatible race of bacteria. Tobacco cell cultures have also been challenged with avirulent zoospores of the pathogenic fungus, *Phytophthora parasitica*, for investigating the production of superoxide radicals (Able et al. 1998). However, inoculation systems using suspension-cultured plant cells are not adequate for precisely evaluating resistance to the challenging pathogens because of the lack of disease symptoms and because it is impossible to investigate suppression of elongating hyphae of pathogenic fungi or suppression of the multiplication of the pathogenic bacteria. Furthermore, exogenous treatment with such a reagent as propionic acid for cytoplasmic acidification could directly affect the infection process of the pathogens. Therefore, there have been no reports that directly relate acidified cytoplasm to induced resistance to a pathogen, except for the expression of defense-related genes using suspension-cultured cells. Our results are the first to indicate directly that cells with cytoplasmic acidification show resistance to a challenging plant pathogen.

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Resistance induced by intracellular pH decline

References


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