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Full Length Research Paper

Ca^{2+} and reactive oxygen species are involved in the defense responses of rice callus culture to rice blast disease

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The role of Ca^{2+} and reactive oxygen species in the defense responses of callus cultures of rice (*Oryza sativa* L., cv. Zenith) to infection with avirulent strain of rice blast fungus (*Magnaporthe grisea*, strain Ina168) was investigated. It was observed that rice calli, especially after mild blast infection, exude substances (diffusate) that inhibit spore germination of the avirulent blast fungus. This fungitoxic calli diffusate led to superoxide dismutase-sensitive reduction of Nitro-blue-tetrazolium. Treatment of rice calli with crude elicitor from the blast fungus also led to hypersensitive necrotic response. Addition of antioxidant reagents diminishes the necrotic response of calli to the elictor treatment, implicating the involvement of reactive oxygen species in the hypersensitive necrotic response. When ethyleneglycoltetraacetic acid (Ca^{2+} chelator) or $LaCl_3$ (Ca^{2+} channel blocker) was added, the necrotic response of calli to elicitor treatment was also significantly weakened, implying the involvement of Ca^{2+} in the defense response.

Key words: *Oryza sativa*, *Magnaporthe grisea*, hypersensitive response, reactive oxygen species, calcium.

INTRODUCTION

Plant defense responses against pathogens involve the recognition of invading pathogens and activation of signal transduction pathways leading to hypersensitive reaction (Mehdy et al., 1994; Hammond-Kosack and Jones, 1996; Somssich and Kahlbrock, 1998; Richter and Roland, 2000). Hypersensitive reaction (HR) in plants is generally characterized by a rapid, localized cell death around the infection site and the accumulation of antimicrobial agents (Hammond-Kosack and Jones, 1996; Richter and Roland, 2000). In the rice-blast disease system, it has been demonstrated (Aver'yanov et al., 1988) that leaves of resistant, but not susceptible rice varieties, respond to blast attack by excreting substances that are toxic to the invading blast fungus. There is now a growing interest in elucidating the mechanisms by which plant cells invoke these defense responses against pathogens.

Several literature reports suggest that calcium ions (Ca^{2+}) participate in the induction of defense response in plants (Chen et al., 1993; Lamb et al., 1994; Lock et al., 1994; Mehdy, 1994; Ward et al., 1995; Dmitriev et al., 1996; Levine et al., 1996; Ishihara et al., 1996; Zimmermann et al., 1997; Allen et al., 2000; Blume et al., 2000; Romeis et al., 2001). Recently, Lecourieux and colleagues (2002) observed rapid increase in cytosolic free calcium levels in cells of *Nicotiana plumbaginifolia* in response to treatment with elicitors. Such stress-induced elevation of cytosolic calcium levels has been linked to the opening of calcium channels and influx of calcium into the cytosol (Engstrom et al., 2002). The interesting aspect

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Abbreviations: EGTA, ethyleneglycoltetraacetic acid; SHAM, salicylhydroxamic acid; DPI, diphenyleneiodonium; ROS, reactive oxygen species; SOD, superoxide dismutase; NBT, nitroblue tetrazolium.

of the influx of extracellular calcium in response to pathogen attack is that such an influx could lead to oxidative burst in plant cells (Blume et al., 2000).

Oxidative burst is part of the downstream defense responses in plant cells, which involves the large and rapid generation of reactive oxygen species (superoxide, hydrogen peroxide, hydroxyl, peroxyl and alkoxyl radicals) that can cause cell damage. The literature is rich with information on the role of oxidative burst in plant defense responses (Doke, 1983; Doke and Chai, 1985; Lamb et al., 1989; Levine et al., 1994; Goodman, 1994; Low and Merida, 1996; Tzeng and DeVay, 1996; Wojtaszek, 1997; Vanacker et al., 2000; Park et al., 2000). The fungitoxic excretions in leaves of blast-resistant rice varieties have also been associated with reactive oxygen species (Aver'yanov et al., 1987). The key component of the oxidative burst is hydrogen peroxide, which in the presence of a metal reductant, can form the highly reactive oxygen radical, hydroxyl radical (Mehdy, 1994). The role of hydrogen peroxide in defense response of plants to external stress factors has recently been demonstrated in cells of *Betula pendula* (Pellinen et al., 2002).

Plant cell cultures have been extensively used to investigate defense signal transduction in several plant species, but not much work has been carried out with rice callus cultures in this regard. The present study reports the role of calcium ions and reactive oxygen species in the defense responses of callus culture of rice to blast disease. Studies on the involvement of oxidative enzymes in blast disease resistance response are also included.

MATERIALS AND METHODS

Preparation of plant material

Callus cultures of cv. Zenith, a rice cultivar resistant to blast strain Ina168, was used. For callus induction, surface-sterilized mature Zenith caryopses were placed on MS medium (Murashige and Scoog, 1962), supplemented with 4 mg L^{-1} 2,4-D. They were then incubated in the dark at 27°C for 2 - 3 weeks. The induced calli were maintained on MS containing less auxin - 2 mg L^{-1} 2,4-D, with frequent sub-culturings after every 3-4 weeks. Calli were used 7 days after sub-culturing on freshly prepared MS medium.

Fungal culture

The *M. grisea* strain, Ina168, which is avirulent to cv. Zenith was used. Cultures of Ina168 were maintained on semi-solid carrot medium, consisting of 50 g L^{-1} dry carrot + 20 g L^{-1} agar. Crude elicitor from *M.grisea* was obtained by autoclaving homogenized mycelia of the fungus. Elicitors from blast fungus have been shown to induce defense responses in rice (Schaffrath et al., 1995).

Callus inoculation and collection of exometabolites

Callus fragments (4-5 mg) were placed into a well of a 96-well tissue culture plate (”Linbro”, Flow Laboratories) containing 50 µl of distilled water. Then another 50 µl of water or blast spore suspension (200 thousand spores/ml) was added to the callus fragments. The plate was then incubated in the dark for 18 h at 23°C. Then, the liquid was collected with simultaneous removal of inoculum spores (Lapikova et al., 1994) and is further referred to as “exometabolites” in this report.

Estimation of fungitoxicity of callus exometabolites

Estimation of fungitoxicity of exometabolites were conducted according to the method described by Lapikova et al. (1998). 80 µl of callus exometabolite was poured into wells of 96- well plate and 10 µl of freshly prepared spore suspension (3.5×10^4 /ml) was added. 10 µl of water was also added to the mixture and incubated for 5 h at 23°C. Then under inverted microscope, the number of spores that germinated was counted in 5 replicates of 100 spores. The measure of fungitoxicity of exometabolites was their capacity to inhibit fungal spore germination. The inhibition of germination was determined against spores incubated with 80 µl of water in place of a diffusate. All values are represented as means ± standard deviations (n = 5).

Necrotic response

Necrotic reactions of rice cells were evaluated visually 48 h after treatment with elicitor or after blast infection. The number of calli that turned dark brown was counted and presented as percentage of total calli treated.

Role of calcium ions in fungitoxic response and necrosis

To test the participation of calcium in the induction of the fungitoxic response, calli, during inoculation, were submerged in 50 µl of 0.1 mM EGTA (Serva) or $LaCl_3$ (0.05 to 10 mM) instead of water (see above). The first agent is the Ca^{2+} chelator, and the second is the blocker of calcium channels in plasma membrane. Then 50 µl water spore suspension (or water in mock inoculation) was added following incubation and estimation of the diffusate toxicity as described above. The role of calcium in elicitor-induced necrosis was evaluated by adding EGTA or $LaCl_3$ during treatment with elicitor.

Assessment of involvement of ROS in elicitor-depended hypersensitive response

Solutions of different antioxidant reagents sequestering particular ROS were added, instead of 10 µl water (see above), to rice cells, treated with fungal elicitor. To destroy superoxide radical, Tiron (1 mM, sodium salt of 4,5-dihidroxy-1,3-benzene disulfonic acid, Serva Heidelberg) was used. To decompose hydrogen peroxide dimethylthiourea (30 mM, Sigma) (Toth et al., 1989) was employed similarly. To test the role of hydroxyl radical, its scavengers sodium formate (1 mM, Merck Darmstadt), mannitol (10 mM, Serva) or thiourea (0.5 mM, Sigma) were used.

Generation of superoxide anion

Generation of superoxide radical in the exometabolites of rice cells was assessed by the method of Nitro-Blue Tetrazolium (NBT) reduction (Doke, 1983) and measured with digital spectrophotometer “Shimadzu” UV-260 (Japan) in the absorption spectrum of 560 nm.

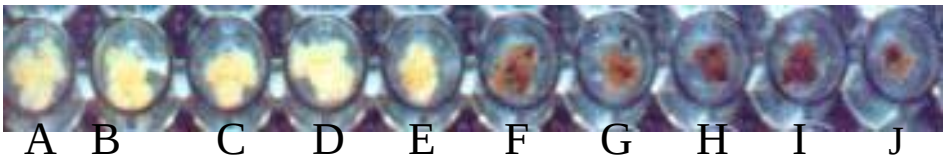

Figure 1. Necrotic reaction of callus culture to elicitor treatment. A, B, C, D and E are control calli that were not treated with elicitor, while F, G, H, I,, and J are calli, treated with elicitor, showing intense browning (necrosis).

Role of oxidative enzymes

Diphenelene iodonium (DPI, inhibitor of NAD(P)H-oxidase) (Cross and Jones, 1986) and Salicylhydroxamic acid (SHAM, inhibitor of peroxidase and lipoxygenase) were used in a pharmacological experiment to determine the role of oxidative enzymes in elicitor-dependent necrotic reactions of cells. Cells were treated with elicitor together with either of the above-mentioned inhibitors or none after which necrosis is assessed.

RESULTS

Defense responses of rice calli to blast infection

Inoculation of calli of resistant rice cultivar Zenith with spores of avirulent *M. grisea* strain Ina168 resulted in the production of exometabolites that inhibited the germination of fungal spores by 70%. On the other hand, the exometabolites from non-inoculated Zenith calli did not inhibit, but rather stimulated spore germination by as little as 2%. Distilled water, as expected, was not toxic, as the fungus had about 68% absolute spore germination in it.

Apart from the blast-induced secretions of antimicrobial substances, intense browning (necrosis) of callus tisssues was also observed after treatment of calli with crude elicitor from blast fungus (Figure 1). Therefore, callus cultures of cv. Zenith respond to blast attack by accumulating antimicrobial substances and necrosis. It is interesting to find out the role of calcium ions in the induction of these blast-induced defense responses in Zenith calli.

Involvement of Ca^{2+} in the defense reactions of rice calli to blast infection

To investigate the involvement of calcium ions in blast-induced defense responses of callus cultures, we used the Ca^{2+} chelator, EGTA, or the Ca^{2+} channel blocker, $LaCl_3$. The addition of any of these compounds to infected rice calli inhibited the production of fungitoxic diffusate by the calli (Table 1). Also, when Ca^{2+} is removed from medium by adding EGTA, necrotic reaction of cells to elicitor treatment was significantly weakened (Table 2) . The addition of 1 mM $CaCl_2$ after EGTA treatment reactivates the necrotic reaction of cells to elicitor treatment. However, other factors, like reactive oxygen species might also be involve in such defense responses.

Table 1. Effect of EGTA or $LaCl_3$ on the fungitoxicity of diffusates of rice calli (var. Zenith), infected with blast fungus (strain Ina 168).

Treatment	Inhibition of spore germination, %
Diffusate (without EGTA or $LaCl_3$)	70 ± 9.0
Diffusate + EGTA	8±0.6
Diffusate + $LaCl_3$	8±0.8

Table 2. Effect of EGTA on necrotic reaction of rice calli to elicitor treatment.

Treatment	% necrotic calli
No treatment	0±0.0
Elicitor treatment	66 ± 3.6
EGTA, then elicitor	13 ± 1.0
EGTA, then $CaCl_2$, then elicitor	51.3 ± 1.5

Generation of superoxide radical

Superoxide radical is known to specifically reduce NBT. We observed that exometabolites collected from infected rice calli also reduce NBT, signifying the presence of superoxide radical in the exometabolites (Table 3). Addition of SOD (an enzyme that specifically destroy superoxide radical) to the exometabolites diminishes its potential to reduce NBT. However, other reactive oxygen species (ROS) may also participate in the defense response of blast-infected calli.

Table 3. Reduction of nitroblue tetrazolium (NBT).

Treatments	A_{560} *x* 10^3
Diffusate	112 ± 16
Diffusate + SOD	30±8
± SOD	75

Role of ROS in elicitor-dependent necrotic reactions of cultured cells of rice

In order to demonstrate the role of ROS ($^{\bullet}OH$, H_2O_2, $O_2^{\bullet}$, $^1O_2$) in necrotic response of calli to blast elicitor treatment, we added the following antioxidative reagents to the cells

together with the elicitor: mannitol, formate or thiourea (these are known to specifically scavenge $^{\bullet}OH$), dimethythiourea (scavenger of H_2O_2), tiron (scavenger of $O_2^{\bullet}$), or histidine ($^1O_2$ quencher). All these reagents, especially dimethylthiourea and mannitol, weakened necrotic reaction (Table 4), indicating the participation of different ROS, particularly hydroxyl radical and hydrogen peroxide in elicitor-induced calli necrosis.

Table 4. Effect of different antioxidative reagents on elicitor-dependent necrotic reactions of cultured rice cells (cv. Zenith).

Treatment	% necrotic calli
Elicitor treatment	97.7 ± 2.5
+ 2.3 mM histidine	56.0 ± 5.3
+ 30 mM dimethylthiourea	0.0 ± 0.0
+ 1 mM tiron	38.7 ± 3.2
+ 1 mM formate	40 ± 1.0
+ 10 mM mannitol	19.0 ± 3.6
+ 0.5 mM thiourea	39 ± 1.0

Role of oxidative enzymes in elicitor-induced callus necrosis

The different ROS implicated in the blast-induced calli necrosis might be generated by oxidative enzymes, like peroxidase and NADPH-oxidase. We investigated the role of these enzymes in blast-induced calli necrosis with the help of specific enzyme inhibitors: SHAM for inhibition of peroxidase and DPI for inhibition NADPH-oxidase. As shown in Table 5, addition of these enzyme inhibitors reduces elicitor-dependent necrotic reactions of callus cultures, implying the participation of peroxidase and NADPH-oxidase in this reaction.

DISCUSSION

It has been shown that callus cultures of blast-resistant rice cultivar, Zenith, exudes fungitoxic exometabolites in response to inoculation with avirulent blast strain, Ina168. The fact that the exometabolites of non-inoculated calli were not fungitoxic may mean that the defense response in callus culture is not constitutive, but induced by the blast fungus. These results confirmed earlier observations that blast infection induces excretion of antimicrobial substances in leaves of blast resistant rice cultivars (Lapikova et al., 1994; Pasechnik et al., 1998).

However, the exometabolites of blast-inoculated plant cell may be toxic not only to the blast fungus, but also to the host cell itself. This may explain the intense tissue browning (necrosis) we observed in callus cultures in response to treatment with elicitor from *M. grisea*.

Probably, cells of resistant rice cultivar, not only when in tissues of leaves, but also while being isolated, recognize elicitor from avirulent blast fungus and induce a cascade of defense responses, leading, in parts, to exudation of fungitoxic exometabolites and plant tissue necrosis.

Calcium ions (Ca^{2+}) have been shown to participate in the induction of defense responses in several plant species (Levine et al., 1996; Sze et al., 2000; Harmon et al., 2000; Romeis et al., 2001). Recently, Engstrom and colleagues (2002) tested some pharmaceuticals that modulate the activity of calcium channels to demonstrate the role of calcium influx into cytozol in defense signalling. Using pharmaceuticals such as calcium chelator (EGTA) and calcium channel blocker ($LaCl_3$), we have also demonstrated that the influx of extracellular calcium into cytozol is essential for the blast-induced excretion of fungitoxic exometabolites and cell necrosis in rice callus cultures. Our results correspond with earlier findings by Dmitriev et al. (1996) and the recent conclusions of Lecourieux et al. (2002), who also used EGTA and $LaCl_3$ to confirm the role of Ca^{2+} in plant defense responses.

It was earlier suggested that Ca^{2+} influx is necessary as second messenger for the elicitation of oxidative burst in plant cells (Lock and Price, 1994; Blume et al., 2000; Lecourieux et al., 2002), and that the oxidative burst, in turn, induces several subsequent defense responses of plants (Doke et al., 1996; Low and Merida, 1996; Orozco-Cardenas et al., 2001; Pellinen et al., 2002). We have also demonstrated the role of reactive oxygen species (ROS) in elicitor-dependent necrosis in rice calli. When we infected rice calli with blast fungus, we observed that

Table 5. Role of NADPH-oxidase and peroxidase (by the inhibitory effects of DPI and SHAM) in elicitor-dependent necrosis of rice calli (cv. Zenith).

Treatment	% necrotic cells	
	No elicitor	With elicitor
No additions of inhibitors	0.0±0	99.0 ± 1.0
Addition of 2 µM DPI	16.3 ± 0.6	44.0 ± 3.6
Addition of 100 ∝M SHAM	17.0 ± 1.0	33.0 ± 1.0

the exometabolites of the calli lead to SOD-sensitive reduction of NBT, implying the generation of superoxide radical in the exometabolites.

The oxidative enzymes, NADPH oxidase and peroxidase, have also been implicated in plant defense response to stress factors (Wojtaszek, 1997; Orozco-Cardinas et al., 2001; Pellinen et al., 2002; Shivakumar et al., 2003). Though the cellular targets of most pharmaceuticals are yet to be established in plants, pharmaceuticals are still widely used as important tools for identifying candidate components of signal transduction pathways (Engstrom et al., 2002). DPI, for example, is a pharmaceutical that specifically inhibits NADPH oxidase (O'Donnell et al., 1993) and has been successfully employed to investigate the role of NADPH oxidase in defense responses (Park et al., 2000; Orozco-Cardenas et al., 2001). We also used similar approach to confirm the involvement of NADPH oxidase and peroxidase in the elicitor-dependent necrosis of rice callus cultures.

It appears, therefore, that during incompatible rice-blast interactions, the blast fungus elicits the rapid increase of Ca^{2+} in rice cytosol which activates the generation of ROS through oxidative enzymes, like NADPH-oxidase and peroxidase. Thereafter, the generated ROS could be toxic to the invading blast fungus, and could also induce necrosis in rice cells. These findings may be critical in understanding the mechanisms of blast disease resistance in rice.

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