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PLANT CELL WALL WITH FUNGAL SIGNALS MAY DETERMINE HOST-PARASITE SPECIFICITY

SHIRAISHI T., A. KIBA, A. INATA, M. SUGIMOTO, K. TOYODA,
Y. ICHINOSE, T. YAMADA
Laboratory of Plant Pathology & Genetic Engineering, College of Agriculture,
Okayama University, Okayama, 700-8530, Japan

For improvement of plants in disease resistance, it is most important to elucidate the mechanism to perceive and respond to the signal molecules of invaders. A model system with pea and its pathogen, *Mycosphaerella pinodes*, showed that the fungal elicitor induced defense responses in all plant species tested but that the suppressor of the fungus blocked or delayed the expression of defense responses and induced accessibility only in the host plant. In the world, many researchers believe that the pathogens' signals are recognized only on the receptors in the plasma membranes. Though we found that the ATPase and polyphosphoinositide metabolism in isolated plasma membranes responded to these fungal signals, we failed to detect specific actions of the suppressor *in vitro* on these plasma membrane functions. Recently, we found that ATPase (NTPases) and superoxide generating system in isolated cell wall were regulated by these fungal signals even *in vitro*, especially, by the suppressor in a strictly species-specific manner and also
that the cell wall alone prepared an original defense system. The effects of both fungal signals on the isolated cell wall functions *in vitro* coincide perfectly with those on defense responses *in vivo*. In this treatise, we discuss the key role of the cell wall, which is plant-specific and the most exterior organelle, in determining host-parasite specificity and molecular target for improvement of plants.

INTRODUCTION

Plant-parasite interactions, resulting in rejection or acceptance of pathogens, is strictly specific. As a model system, we have been using a glycopeptide elicitor (mol. wt.>70 kDa) and glycopeptide suppressors (mol. wt.<5 kDa) of pea defense responses in spore germination fluid of a pea pathogenic fungus, *Mycosphaerella pinodes* (Berk. et Blox.) Vestergren (29-31,40,42). The elicitor induces defense responses such as the production of both phytoalexin (37,45) and an as yet-unidentified infection-inhibitor (46), and the activation of pathogenesis-related proteins, endochitinase and endo-β-1,3-glucanase, in pea tissues (48). Since this elicitor also induces the defense responses of other plant species (3,48), the action of the elicitor is not species-specific. On the other hand, the suppressor inhibited these defense responses and induced local susceptibility (accessibility) only in host plant of *M. pinodes*, whereas the suppressor alone acts as an elicitor on non-host plants of *M. pinodes* (3,31,39,40,48). From these findings, the suppressor is thought as a determinant of specificity (31).

It was found that elicitor activated polyphosphoinositide (PI) metabolism in pea tissues and in isolated plasma membranes within

5 sec but the simultaneous presence of the suppressor inhibited the activation (43,44). The suppressor inhibited ATPase activity in both pea cells and isolated plasma membranes (20,36,38,40). A P-type ATPase inhibitor, orthovanadate, suppressed PI metabolism and defense responses, suggesting that the plasma membrane ATPase was tightly associated with the regulation of defense system (40,48,49) and that "cross-talk" between the ATPase and PI metabolism may exist (40; Toyoda and Shiraishi, unpublished).

We had expected the specific action of the suppressor on the plasma membrane functions but the suppressor nonspecifically inhibited these functions in plasma membranes prepared from both of the host and nonhosts of *M. pinodes* (36). This finding is contradictory to *in vivo* effects of the suppressor on nonhost plants. A cytochemical observation, however, showed that treatment of uninjured leaves with the suppressor severely inhibited ATPase activity only in pea cells out of five plant species tested (36). Thus, *in vivo* action of the suppressor on defense response and ATPase is strictly species-specific. These findings led us to a hypothesis that the primary target(s) of the suppressor may exist in cell walls and that the target(s) may regulate the downstream involving ATPase and PI metabolism in plasma membranes.

Recognition Of Elicitor On Plant Cell Surface

When the *M. pinodes*-elicitor was placed on the surfaces of uninjured tissues, it did not induce phytoalexin biosynthesis. However, an as yet-unidentified infection-inhibitor was produced, resulting in rejection reaction against pathogens within 2 h (46). Observations on the distribution of Fe-labeled elicitor by STEM and EDX showed that the elicitor localized only to the surface of pea cells (Shiraishi et al., unpublished). Thus, the elicitor did not migrate from the cell surface into the plasma membrane or cytoplasm but it was able to induce resistance. In this connection, Hoyos et al., (17) suggested that the cell wall is crucial for the induction of the hypersensitive reaction in tobacco suspension-cultured cells by harpinpss, a peptide elicitor from *Pseudomonas syringae* pv. syringae. They found that exogenous harpinpss, which was localized to the outer part of cultured cells but was undetectable on the surface of protoplasts, induced alkalinization of extracellular medium of cultured cells but not that of protoplasts (17). Together with these data, it is probable that plants are able to recognize and respond to the exogenous elicitors. Simultaneous treatment with the M. pinodes-suppressor, however, negates induction of defense response by the elicitor and conditioned pea cells to be susceptible even to avirulent pathogens (46; Shiraishi et al., unpublished).

Cell Wall-Bound ATPase

It was previously reported that phosphatases including ATPase existed in the cell wall of corn coleoptile (25). However, a role of these phosphatases has been unknown for a long time. We also found the activities of NTPase, p-nitrophenol-phosphatase and pyrophosphatase in cell wall prepared from several leguminous plants (22,24). The cell wall-bound ATPase was different from that of the plasma membranes in several properties such as molecular weight, sensitivity to neomycin, and dependence upon both divalent cations and pH (22,41). Surprisingly, the elicitor from *M. pinodes* enhanced nonspecifically cell wall-bound ATPases and the *M. pinodes*-suppressor inhibited that only in pea cell wall. The suppressor rather enhanced those of nonhosts of the fungus. Thus, the action of the suppressor on cell wall-bound ATPase was strictly species-specific even *in vitro*.

As described above, the suppressor, which was placed on the surface of leaves, inhibited the

ATPase activities in all membrane systems only in pea cells out of five plant species tested, whereas the suppressor inhibited *in vitro* the ATPase activities of plasma membranes isolated from both of the host and non-hosts of *M. pinodes*. These results suggested that the cell wall controls the functions of membrane systems of other organella. Moreover, inhibition of the cell wall function seems to result in suppression of defense responses and in acceptance of pathogens. This hypothesis is supported by the fact that a tight connection between cell walls and the cytoskeleton via plasma membranes was reported to exist in plant cells (1,2). It was also reported that "Hechtian strands" in epidermal cells might be drawn out from the cytoplasmic face of a transmembrane protein (complex) bound to a wall-to-membrane linker (32). The result, that cell wall-bound ATPases responded to the elicitor and the suppressor, also suggests that the receptor for these fungal signals might be tightly associated with the cell wall-bound ATPases or that the cell wall-bound ATPases might act as receptors for these fungal signals.

O_2 Generation On Leaf Surface

In plants, the oxidative burst was first reported in potato tuber tissues inoculated with *Phytophthora infestans*, the cause of potato blight (12,13). At present, there are many reports that generation of active oxygen species (AOS), in particular, hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), lipid peroxides (LOO•) and superoxide anion (O_2^-), is induced in cultured plant cells, protoplasts, and tissues by treatment with elicitors or by inoculation with pathogens (6). Hydrogen peroxide and O_2^- are well documented for a possible role in plant-pathogen interactions. Hydrogen peroxide is thought to play an important role in lignification (28), oxidative cross-linking of hydroxyproline- and/or proline-rich glycoproteins in cell walls (9,10), and induction of systemic resistance (11). The superoxide anion is thought to play an important role in plant defenses not only as a toxicant to pathogens but also as a signal molecule for the hypersensitive response. In this section, we present the role of the elicitor and suppressor from *M. pinodes* on O_2 generation on the surfaces of leaves of pea and cowpea seedlings.

Kiba et al., (23) showed generation of O_2 at the infection site on uninjured leaves of pea and cowpea. As compared to the water control, formation of blue formazan from nitroblue tetrazolium was rapidly and significantly enhanced on the surface of pea leaves within 10 min after inoculation with an avirulent pathogen of pea, *Colletotrichum lagenarium* and with a hypovirulent strain (OMP-av) of *M. pinodes*. In case of inoculation with the virulent strain (OMP-1) of *M. pinodes*, however, the amount of blue formazan was as the same to or lower level than the water control. On the other hand, blue formazan rapidly and significantly increased on cowpea leaves by inoculation with all these fungi avirulent to cowpea. In addition, the reaction mixture did not affect fungal morphogenesis and these pathogens themselves produced negligible amount of O_2. These results showed that O_2 generation occurred more intensively and rapidly in incompatible combinations even on uninjured plant tissues, suggesting that plants are able to recognize and respond immediately to avirulent pathogens which contact with the leaf surfaces.

Nextly, the effects of the elicitor and suppressor from *M. pinodes* on blue formazan-formation were examined. The elicitor from *M. pinodes* enhanced the formation on leaves of pea and cowpea within 5 min as compared to the water control. On the other hand, the suppressor markedly decreased the elicitor-enhanced formation on pea leaves to the level of the water control. On cowpea leaves, however, the suppressor did not inhibit the elicitor-enhanced formation and the suppressor

alone rather enhanced the formation of formazan. Thus, the suppressor from *M. pinodes* acts as an elicitor on a non-host plant of *M. pinodes*. Blue formazan-formation on leaves of both plants decreased in the presence of superoxide dismutase (SOD) in a dose-dependent manner. For example, the formation was suppressed by 300 units of SOD to the level of the water control. These findings indicate that O_2^- generation is one of the extremely rapid responses even of uninjured plant tissues to fungal signals.

Such elicitor-activated blue formazan-formation on surfaces of pea and cowpea leaves was reduced to the level of the water control by the presence of an inhibitor of peroxidase, salicylhydroxamic acid (SHAM) (4). On the other hand, an inhibitor of mammalian neutrophil NADPH oxidase, imidazole (18), slightly inhibited the blue formazan-formation. Another inhibitor of NADPH oxidase, quinacrine (4), did not affect the formation at all. These findings suggest that O_2^- generation on the surfaces of pea and cowpea leaves may be mediated mainly by peroxidase(s). Orthovanadate and neomycin inhibited phytoalexin production of pea wounded tissues induced by the elicitor (43,44,47). One mM orthovanadate remarkably inhibited elicitor-induced blue formazan-formation. One mM neomycin, however, showed little effect on the formation. Together with a report that orthovanadate acts as an inhibitor of peroxidase (34), there are two possibilities that the suppression of O_2^- generation by orthovanadate results from direct inhibition of peroxidase or from indirect action via inhibition of ATPase. The results also indicate that the phospholipase C/PtdInsP2-regulated system seems to participate slightly in O_2^- generation on the surface of leaf tissue as well as reported by Legendre et al.,(26) with cultured cells of soybean.

O_2^- Generation In Cell Wall Preparations

It was reported that a plasma membrane redox enzyme, NADPH oxidase, was involved in O_2^- and H_2O_2 synthesis in rose cultured cells (4). Doke and Miura (14) reported that a NADPH-dependent O_2^- generation system was contained in plasma membranes, which were isolated from potato tuber tissues inoculated with an incompatible race of P. infestans and that the system in plasma membrane from healthy potato tuber was activated by hyphal wall component of P. infestans. It was also shown that peroxidase in cauliflower plasma membranes was able to oxidize NADH, catalyzing the formation of O_2^- (5). On the other hand, another hypothesis was presented by several groups that cell wall peroxidase(s) catalyzed O_2^- production by a complex pathway involving NADH, NAD· and NAD+ (15,16,19,27). Bolwell's group (7,8) reported that the wall-bound peroxidase participated in generation of H_2O_2 in bean suspension-cultured cells in response to elicitor.

We found an O_2^- generating system in the cell wall fractions from pea and cowpea (21). The activity of blue formazan-formation in both fractions was markedly reduced in the absence of NADH (NADPH), manganese ion or p-coumaric acid. The activity was also reduced by the addition of a scavenger of H_2O_2, catalase, in a dose-dependent manner. A requirement for these co-factors and inhibition by catalase strongly indicate that such an O_2^- generating system is dependent upon certain cell wall-bound peroxidase(s) as described by Halliwell (16). Rubinstein and Luster (33) doubted the physiological significance of an O_2^- generating system dependent upon peroxidase(s) reasoning that the NAD(P)H concentrations required for this reaction were not likely in the apoplast. It was, however, reported that NAD was contained in the cell wall free space (35) and that apoplastic fluids from the elicited kidney bean contained an as yet-unidentified reductant (7).

Such a concept that cell wall-bound

peroxidase(s) is responsible for O_2^- generation is also supported by the results from experiments with several inhibitors. Inhibitors of NADPH-oxidase, quinacrine, imidazole, pyridine and diphenyleneiodonium scarcely affected O_2^- generation in the fractions solubilized from pea and cowpea cell wall. On the other hand, SHAM remarkably inhibited O_2^- generation in these fractions. Superoxide generation in both cell wall fractions was not affected by neomycin but was markedly inhibited by orthovanadate. The effect of orthovanadate or neomycin on the formazan-formation seems to coincide with those on the activity of cell wall-bound ATPases. As described above, it is unknown whether the inhibitory effect of orthovanadate on O_2^- generation results from the inhibition of a cell wall-bound peroxidase or ATPase.

The elicitor from M. pinodes also enhanced blue formazan-formation in the fractions solubilized from cell walls of pea and cowpea in a non-specific and dose-dependent manner (21). On the other hand, even the concomitant presence of the suppressor with the elicitor decreased blue formazan in pea fraction to the level of the water control. However, the elicitor-induced formation in the cowpea fraction was not inhibited by the suppressor. Inversely, the suppressor alone enhanced the formazan-formation in cowpea fractions in a dose-dependent manner. These results showed that the activity of O_2^- generation in the solubilized cell-wall fraction is clearly regulated by these fungal signals and that the suppressor acts on that in a strictly species-specific manner. These results suggest that O_2^- generation in cell wall fractions of pea and cowpea might be regulated together with cell wall-bound ATPases. In fact, both enzymes were co-purified by affinity chromatography with an ATP-conjugated resin and by immuno-precipitation (Kiba and Shiraishi; unpublished data). Taken together these *in vivo* and *in vitro* findings indicate that the system responsible for O_2^- generation on leaf surfaces may exist mainly in plant cell walls and is composed of peroxidase and ATPase.

Formation Of Infection-Inhibitor In Cell Wall

Glycopeptide elicitors, which were prepared from germination fluids of *M. pinodes*, *M. ligulicola* and *M. melonis*, induced the accumulation of a phytoalexin, pisatin, in wounded pea tissues but not in uninjured tissues. However, treatment of uninjured pea leaves with these elicitors induced within 2 h local resistance to the infection by *M. pinodes* that may be correlated with the production of an as yet unidentified infection-inhibitor on the surface of uninjured pea leaves (46). Based on these findings, we examined whether isolated cell walls produced an infection-inhibitor in response to the elicitor. After incubation of cell-wall fraction with the elicitor even for 15 min, an ethylacetate-extractable infection-inhibitor was detected (above 30% decrease of penetration frequency as compared to the water-treatment). The production of this substance reached a maximum 30 min after treatment with the elicitor (above 60% decrease of penetration frequency). The extract did not affect the germination of conidiospores of several pathogens even at a concentration of 500 μg/ml, but, severely inhibited the penetration through heat-killed pea epidermis and the infection on pea leaves. A similar substance was also produced in cowpea cell walls incubated with the *M. pinodes*-elicitor. The *M. pinodes*-suppressor, however, markedly inhibited the production of infection-inhibitor in pea cell walls but not in cowpea cell walls. In the latter case, the suppressor alone acted as an elicitor. Thus, the species-specific action of the suppressor also appeared on *in vitro* production of infection-inhibitor in cell walls. This result clearly show that plant cell wall itself is able to

respond to fungal signals and to evoke active defense accompanied with the infection-inhibitor.

Interestingly, the production of an infection-inhibitor induced by the elicitor was suppressed by tiron, SOD, catalase or mannitol (Inata and Shiraishi, unpublished). These results suggest that the production of infection-inhibitor is tightly associated with AOS generation system in the cell wall and that the infection-inhibitor may be generated through Harber-Weiss or Fenton reactions such as $O_2^- + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$ In addition, these scavengers are able to negate the decrease of the infection frequency of *M. pinodes* on pea leaves treated with the elicitor. Structural determination of the infection-inhibitor of pea plants is under way. In this connection, (+)-catechin in strawberry and 3, 5-dicaffeoylquinic acid and caffeoylarbutin in pear leaves were determined as infection-inhibitors induced by an elicitor in spore germination fluids of *A. alternata* (Yamamoto 1986 Ph. D. Thesis, Nagoya Univ.; Kodama 1989, Proceedings of Molecular Mechanism in Signal Transduction Leading to Defense Reactions, Phytopathol. Soc. Jpn.).

CONCLUDING REMARKS

For a long time, it has been believed that the fungal signals are perceived by the plasma membranes of plants. Binding proteins for the elicitor were actually isolated from the plasma membrane fractions as described above. We also found that the ATPase and PI metabolism in isolated plasma membranes were able to respond to the elicitor and suppressor from *M. pinodes*. The response of isolated plasma membranes to the suppressor, however, was not species-specific *in vitro* (36; Toyoda and Shiraishi unpublished) and therefore was contradictory to the phenomenon *in vivo*. This fact led us to the concept that the apparatus regulating the signal transduction in plasma membranes exists in cell walls.

Our findings presented in this treatise clearly show that: 1) Plants are able to recognize and respond rapidly to the elicitor even on the surface of uninjured tissues; 2) Plant cell wall prepares the ATPase (NTPase) and the system(s) generating AOS and an infection-inhibitor that are able to respond *in vitro* to the elicitor and the suppressor; 3) The suppressor from *M. pinodes* affects these systems both *in vivo* and *in vitro* in a strictly species-specific manner (the suppressor alone acts as an elicitor of these functions in cell walls of non hosts of *M. pinodes*); 4) The systems for NADH-dependent O_2^- generation and for the production of an infection-inhibitor in cell walls are tightly correlated with the cell wall-bound ATPase; 5) *In vitro* response of cell walls to fungal signals coincides perfectly with *in vivo* response of tissues to these signals. Further investigations are needed to elucidate not only the receptors for these fungal signals in cell walls but also the apparatus regulating the downstream leading to defense responses. Nevertheless, these results strongly support the concept that the primary apparatus switching defense responses on or off exists in the cell wall. In other words, the cell wall, the most exterior and plant-specific organelle, along with the suppressor may play a crucial role in determining plant host-parasite specificity at least in species-species combinations. Here we propose "Cell Wall Concept".

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