Superoxide Generation in Extracts from Isolated Plant Cell Walls Is Regulated by Fungal Signal Molecules

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ABSTRACT

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Fractions solubilized with NaCl from cell walls of pea and cowpea plants catalyzed the formation of blue formazan from nitroblue tetrazolium. Because superoxide dismutase decreased formazan production by over 90%, superoxide anion (O_2^-) may participate in the formation of formazan in the solubilized cell wall fractions. The formazan formation in the fractions solubilized from pea and cowpea cell walls was markedly reduced by exclusion of NAD(P)H, manganese ion, or *p*-coumaric acid from the reaction mixture. The formazan formation was severely inhibited by salicylhydroxamic acid and catalase, but not by imidazole, pyridine, quinacrine, and diphenyleneiodonium. An elicitor preparation from

the pea pathogen *Mycosphaerella pinodes* enhanced the activities of formazan formation nonspecifically in both pea and cowpea fractions. The suppressor preparation from *M. pinodes* inhibited the activity in the pea fraction in the presence or absence of the elicitor. In the cowpea fraction, however, the suppressor did not inhibit the elicitor-enhanced activity, and the suppressor alone stimulated formazan formation. These results indicated that O_2^- generation in the fractions solubilized from pea and cowpea cell walls seems to be catalyzed by cell wall-bound peroxidase(s) and that the plant cell walls alone are able to respond to the elicitor nonspecifically and to the suppressor in a species-specific manner, suggesting the plant cell walls may play an important role in determination of plant-fungal pathogen specificity.

Additional keywords: *Pisum sativum*, species specificity, *Vigna sinensis*.

A strict specificity is observed in plant-parasite interactions that results in rejection or acceptance of invading pathogens. A pea pathogen, *Mycosphaerella pinodes* (Berk. & Bloxam) Vesterg., secretes in its pycnospor germination fluid a glycopeptide elicitor (29) and glycopeptide suppressor of pea defense responses (31,37–40). In pea tissues, the elicitor induces defense responses such as the production of phytoalexin (36,44) and an, as yet, unidentified infection-inhibitor (45), and the activation of the pathogenesis-related proteins endochitinase and endo- β -1,3-glucanase (47). Since the elicitor also induces the defense responses in other plant species (45,47), the action of the elicitor is thought not to be species specific. On the other hand, the action of the suppressor preparation was species specific, since it inhibited these defense responses and induced susceptibility only in host plants of *M. pinodes* (32,38,39,47). Thus, the suppressor is thought to be a determinant of host-parasite specificity (32).

The involvement of superoxide anion (O_2^-) generation in plant defense was first shown in the interaction between potato and *Phytophthora infestans*, the cause of potato blight (12,13). Since this initial work, there have been many reports that the production of active oxygen species, particularly hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and O_2^- , were triggered in cultured plant cells, protoplasts, and tissues by treatment with elicitors or by inoculation with potent pathogens (3). Among these active oxygen species, the possible roles of H_2O_2 and O_2^- have been well demonstrated in plant-pathogen interactions. H_2O_2 is thought to be required for lignification of the cell wall (27,28) and for oxidative cross-

linking of hydroxyproline-rich glycoproteins in the cell wall (7,8). It was also suggested that H_2O_2 acted as a signal molecule in salicylic acid-mediated, systemic acquired resistance in tobacco plants (10).

O_2^- was thought to play an important role in plant defenses not only as a toxicant to pathogens, but also as a signal molecule for active defense. Treatment with hyphal cell wall components of *P. infestans* and inoculation with an incompatible race of *P. infestans* induced O_2^- generation in potato tubers (14). O_2^- has been thought to act as a signal molecule to induce the defense response, since superoxide dismutase (SOD), the scavenger of O_2^- , blocked hypersensitive cell death in potato tubers (12). Recently, it was found that the elicitor preparation from *M. pinodes* also nonspecifically induced O_2^- generation on the leaf surfaces of pea and cowpea, but the suppressor showed a species-specific effect on the O_2^- generation (23).

In mammalian cells, a neutrophil NADPH oxidase is responsible for the generation of O_2^- on the extracellular surface of the plasma membrane via a one-electron reduction of dioxygen (11). In plants, it has also been thought that an O_2^- -generating NADPH oxidase exists in the plasma membrane (1,15). On the other hand, peroxidase in plasma membranes has also been reported to be responsible for the synthesis of O_2^- (2). Another study has pointed out the oxidation of NADH by cell wall-bound peroxidases, resulting in the generation of O_2^-/H_2O_2 through the complex pathway involving the apoplastic NADH, $NAD\cdot$, and NAD^+ cycles (19,20).

Our previous report suggested that plant cell walls might play an important role in the recognition of fungal signals and in the determination of host-parasite specificity. We found that a cell wall-bound ATPase responded to the elicitor and suppressor preparations from *M. pinodes*. In particular, the response to the suppressor preparation was in a species-specific manner (24,25). Though there are several reports that active oxygen species were produced in plant cell walls, as described above, it has been unclear whether the production of active oxygen species in cell wall fractions is

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directly affected by fungal signal molecules. In this report, therefore, we examined whether O_2^- is generated in fractions solubilized from pea and cowpea cell walls, and whether the elicitor and suppressor preparations from *M. pinodes* are able to regulate such O_2^- generation.

MATERIALS AND METHODS

Chemicals. Tris, diphenyliodonium (DPI), NADH, NADPH, quinacrine, paracoumaric acid (*p*-CA), salicylhydroxamic acid (SHAM), catalase (EC 1.11.1.6), and SOD (EC 1.15.1.1) were purchased from Sigma Chemical Co., St. Louis. Other chemicals were from Wako Pure Chemical Inc., Osaka, Japan.

Plant materials. Seeds of pea (*Pisum sativum* L. cv. Midoriusui) and cowpea (*Vigna sinensis* (L.) Savi ex Hassk. cv. Sanjakusage) were sown in vermiculite in plastic containers. Seedlings were grown at 20 to 24°C for 8 days in the dark for isolation of the cell wall fractions.

Preparation of cell wall fractions and solubilization of cell wall-bound proteins. Preparation of the cell wall fractions and solubilization of proteins from cell wall fractions with 0.5 M NaCl were carried out by the method described previously (25). Purity of cell wall fractions from pea and cowpea seedling was assessed by examining the activity of 11 marker enzymes (25). The activities of 5'-nucleotidase and glucose-6-phosphatase, marker enzymes of the nuclear membrane, were not detectable in fractions solubilized from pea and cowpea cell walls. No activities of fumarase and cytochrome c oxidase, marker enzymes of mitochondria, were detected in these fractions. The activities of ribulose biphosphate carboxylase, NAD(P)H cytochrome c reductase, and glucan synthase I, marker enzymes of chloroplasts; endoplasmic reticulum; and Golgi bodies were also not detectable in these fractions. From the results, it was shown that the fractions solubilized from both plant species were relatively free of contamination by nuclei, chloroplast, Golgi bodies, mitochondria, and endoplasmic reticulum. On the other hand, these fractions included the activities of acid phosphatase, but not alkaline phosphatase and nitrate-sensitive ATPase, indicating that tonoplast had also been excluded from the fractions from pea and cowpea cell walls. The activity of glucan synthase II was not detectable in the cowpea fraction, but the specific activity of this enzyme was 0.4 $\mu\text{mol}/\text{mg}$ of protein/h in the pea fraction. However, this activity was markedly lower than that in plasma membrane fractions, because the specific activities in plasma membrane fractions from pea and cowpea seedling were 15.16 and 18.95 $\mu\text{mol}/\text{mg}$ of protein/h, respectively. Thus, contamination by plasma membrane of the pea cell wall fraction also seemed to be below 3% with respect to the activity of glucan synthase II. Concentrations of protein in solubilized cell wall fractions were determined by the method of Bradford (6), with bovine serum albumin (BSA) as the standard. The fractions, which were solubilized from pea and cowpea cell walls, were stored at -20°C until used.

Preparation of the elicitor and suppressor from *M. pinodes*. The elicitor and the suppressor preparations were prepared from pycnospor germination fluid of *M. pinodes* strain OMP-1 (IFO-30342, ATCC-42741) by the method of Yoshioka et al. (48). Pycnosporos formed on Czapek agar medium were suspended in sterilized distilled water. The suspension was shaken in a shaking flask at 20 to 24°C for 20 h, and then it was filtered through a Whatman No. 1 filter paper. The filtrate was separated into low- and high-molecular-weight fractions by passage through a ultrafilter (type PTGC 142-05; Millipore Corp., Bedford, MS). The high-molecular-weight fraction (molecular weight > 10,000) was dialyzed against sterilized water at 0 to 4°C for 12 h, and the inner solution was used as the elicitor preparation. This preparation contained a glycopeptide (molecular weight > 70 kDa) of which a sugar chain is glucose-mannose-mannose-*O*-serine (29). The lyophilized low-molecular-weight fraction was suspended in a small amount of distilled

water, and fractionated by gel-filtration chromatography with a Toyopearl HW 40F column (Tosoh Co., Tokyo). Fractions suppressing the accumulation of pisatin were used as the suppressor preparation. In this report, we used the partially purified suppressor preparation including mucin-type glycopeptides, suppressors A and B (37), for measuring the overall effect of suppressor activity on O_2^- generation in solubilized cell wall fractions of pea and cowpea. Concentrations of the elicitor and suppressor preparations were determined by the methods of Dubois et al.

Fig. 1. Blue formazan formation in the fractions solubilized from cell walls of pea and cowpea. The assay was carried out in Tris-morpholineethanesulfonic acid (pH 6.5) containing 20 mM MnCl_2 , 2.5 $\mu\text{g}/\text{ml}$ of nitroblue tetrazolium, 0.5 mM NADH, and 0.5 mM *p*-coumaric acid in the absence (○) or presence (●) of 100 units of superoxide dismutase (SOD). The blue formazan was determined by the method of Nathan et al. (30). Arrow heads indicated the addition of 100 units of SOD 30 s after the start of reaction. Each value represents the mean with standard deviation from triplicate experiments.

(16) and Lowry et al. (26), with glucose and BSA as the standards, respectively.

Determination of O_2^- generation. The O_2^- generation via NAD(P)H oxidation in the solubilized cell wall fractions was measured by the method of Ishida et al. (22), with a slight modification. The assay mixture was composed of 30 mM Tris-2-(*N*-morpholino)ethanesulfonic acid (pH 6.5), 0.5 mM NADH, 20 mM $MnCl_2$, 0.5 mM *p*-CA, 2.5 μ g/ml of nitroblue tetrazolium, and 1.0 μ g of solubilized cell wall fractions of pea and cowpea in the absence or presence of several inhibitors, catalase, and SOD, at 100 μ g/ml of the elicitor or suppressor preparation of *M. pinodes*, alone or in combination. The reaction was started by the addition of NAD(P)H, and the formation of blue formazan was monitored at 560 nm every 10 s for 2 min with a spectrophotometer (DU series 600; Beckman Instruments, Inc., Fullerton, CA). The amount of blue formazan was determined by the method of Nathan et al. (30).

RESULTS AND DISCUSSION

It is reported that the systems for generation of H_2O_2 and O_2^- in the cell walls of horseradish and tobacco are involved in lignin synthesis (19,20,27,28). However, the role of such a generation system in plant-microbe interactions is obscure. We also demonstrated that O_2^- was generated in fractions solubilized from cell walls of pea and cowpea, since blue formazan formation, which was detectable in the fractions of both plants, was decreased by 90% in the presence of 100 units of SOD (Fig. 1). Moreover, formazan formation was also inhibited by the addition of SOD 30 s after the start of the reaction (Fig. 1).

As shown in Table 1, formazan formation in the solubilized cell wall fractions of pea and cowpea was markedly reduced in the absence of NADH. Because the activity in the absence of NADH was only restored to about 50% by the addition of the same molarity of NADPH, NADH was shown to be preferable to NADPH as the electron donor. The activity of NADH-dependent O_2^- generation in the fractions solubilized from pea and cowpea cell walls was also markedly decreased in the absence of manganese ion or *p*-CA (Table 1). The formazan formation was also reduced in the presence of catalase, a scavenger of H_2O_2 , in a dose-dependent manner (Fig. 2). These results showed that manganese ion, *p*-CA, and H_2O_2 were required for O_2^- generation. Halliwell (20) showed that peroxidase-catalyzed H_2O_2/O_2^- generation depended on manganese ion and phenolic compounds, and was also accelerated by H_2O_2 during NAD(P)H oxidation. Thus, the requirement of these cofactors for O_2^- generation and inhibition by catalase indicate that the O_2^- generation in the fractions solubilized from pea and cowpea cell walls is dependent upon the activity of certain cell wall-bound peroxidase(s). The involvement of cell wall-bound peroxidase(s)

TABLE 1. Effect of manganese ion (Mn), *p*-coumaric acid (*p*-CA), and NAD(P)H on blue formazan formation in the fractions solubilized from cell walls of pea and cowpea

	Pea		Cowpea	
	Blue formazan ^a	% ^b	Blue formazan ^a	% ^b
Complete ^c	14.31 $\pm$ 2.70	100	20.90 $\pm$ 1.89	100
-Mn	5.46 $\pm$ 0.34	38	8.93 $\pm$ 1.56	42
- <i>p</i> -CA	4.58 $\pm$ 0.21	32	4.66 $\pm$ 1.11	22
-NADH	2.02 $\pm$ 0.21	14	2.61 $\pm$ 0.13	12
+NADPH ^d	5.82 $\pm$ 0.88	41	11.66 $\pm$ 0.36	56

^a Blue formazan (micromoles per milligram of protein per minute) was determined at 25°C for 1 min by the method of Nathan et al. (30), and each value represents the mean with standard deviation from triplicate experiments.

^b The percentage of the mean value is shown instead of that of the complete reaction mixture.

^c The complete reaction mixture was as described in Figure 1.

^d NADPH was used as an electron donor, instead of NADH, in the complete reaction mixture.

in O_2^- generation is also supported by additional results. Inhibitors of NADPH oxidase, quinacrine and DPI, that bind to flavoproteins (11) did not inhibit O_2^- generation in the solubilized cell wall fractions of pea and cowpea. Other inhibitors of NADPH oxidase, imidazole and pyridine, that bind to *b*-type cytochromes (21) also scarcely affected O_2^- generation in the solubilized cell wall fractions of both plant species (Table 2). On the other hand, SHAM, an inhibitor of peroxidase (2), markedly inhibited the O_2^- generation in fractions of both plants (Table 2). Taken together with the

Fig. 2. Effect of catalase on blue formazan formation in the fractions solubilized from cell wall fractions of pea and cowpea. The assay was carried out in the reaction mixture as described in Figure 1 in the absence (○; 0 unit) or presence of 50 units (●) or 100 units (▲) of catalase. Each value represents the mean with standard deviation from triplicate experiments.

data in Table 1, these data support the hypothesis that certain cell wall-bound peroxidase(s) participate in the NADH-dependent O_2^- generation in these cell wall fractions.

Several reports showed that cell wall-bound peroxidase participated in generation of H_2O_2 and O_2^- (19,20,27,28). However, it has not been clarified whether electron donors such as NAD(P)H exist in extracellular spaces or not. Recently, it was reported that NAD was contained in intercellular fluid (34), and cell wall-bound malate dehydrogenase has been reported to exist (17,18). The fractions solubilized from pea and cowpea cell walls also contained the activity of malate dehydrogenase (A. Kiba, K. Toyoda, Y. Ichinose, T. Yamada, and T. Shiraishi, *unpublished data*). Taken together with these results, cell wall-bound malate dehydrogenase might act as a supplier for electron donors. Another study reported that apoplastic fluid aspirated from cell walls of elicited-French bean cells contained an, as yet, unidentified reductant (4). This result suggested that electron donors might be either secreted into cell walls or produced in response to elicitors.

Our previous reports showed that ATPase activity and poly-phosphoinositide metabolism are involved in recognition and signal transduction leading to induction of defense responses in pea plants (41,42,48). Orthovanadate, an inhibitor of P-type ATPase, prevented the accumulation of pisatin (47) and mRNAs encoding phenylalanine ammonia-lyase and chalcone synthase (46), key enzymes in the pisatin biosynthetic pathway, in a manner similar to the *M. pinodes*-suppressor (44). An inhibitor of phospholipase C, neomycin, also blocked the defense responses of pea (42). Moreover, neomycin induced susceptibility of pea cells even to avirulent pathogens (41). Based on these findings, we investigated the effects of inhibitors of ATPase and phospholipase C on the blue formazan formation in fractions solubilized from pea and cowpea cell walls. Though neomycin did not affect O_2^- generation in either fraction, orthovanadate markedly inhibited the activity of the fractions from both plants (Table 2). The results are similar to those with cell wall-bound ATPases. That is, the cell wall-bound ATPase was inhibited by orthovanadate, but not by neomycin (25), whereas the plasma membrane ATPases were inhibited by both inhibitors (48). Thus, it is possible that the O_2^- generation in solubilized cell wall fractions from pea and cowpea might be associated with cell wall-bound ATPases. However, since it has been shown that vanadate acts as an inhibitor of peroxidase (33), it is currently unknown whether the inhibition of O_2^- generation by orthovanadate resulted from the inhibition of cell wall-bound peroxidase or ATPase.

The elicitor preparation from *M. pinodes* enhanced nonspecifically the blue formazan formation in the fractions from both pea

TABLE 2. Effects of several inhibitors on blue formazan formation in the fractions solubilized from cell walls of pea and cowpea

	Pea		Cowpea	
	Blue formazan ^a	% ^b	Blue formazan ^a	% ^b
Water control	19.07 ± 0.74	100	19.39 ± 0.65	100
+Na ₃ VO ₄	8.68 ± 0.75	46	2.90 ± 0.47	15
+Neomycin	19.18 ± 1.16	101	19.38 ± 1.73	100
+Imidazole	17.39 ± 0.81	91	21.08 ± 1.32	108
+Pyridine	21.18 ± 0.55	111	18.96 ± 0.66	98
+Diphenyleneiodonium	17.52 ± 0.69	92	18.29 ± 0.76	94
+Quinacrine	20.44 ± 0.68	107	22.73 ± 1.15	117
+Salicylhydroxamic acid	6.78 ± 0.36	36	8.43 ± 0.35	43

^a The assay was carried out at 25°C for 1 min in the reaction mixture, as described in Figure 1, in the absence or presence of 1 mM Na₃VO₄, 1 mM neomycin, 10 mM imidazole, 20 mM pyridine, 25 μM diphenyleneiodonium, 0.5 mM quinacrine, or 5 mM salicylhydroxamic acid. The amount of formed formazan (micromoles per milligram of protein per minute) was determined by the method of Nathan et al. (30).

^b The percentage of the mean value is shown instead of that of the water control.

Fig. 3. Time course study and effects of the elicitor and suppressor from *Mycosphaerella pinodes* on blue formazan formation in the fractions solubilized from cell walls of pea and cowpea. The assay was carried out at 25°C for 2 min in Tris-morpholineethanesulfonic acid (pH 6.5) containing 20 mM MnCl₂, 2.5 μg/ml of nitroblue tetrazolium, 0.5 mM NADH, and 0.5 mM *p*-coumaric acid in the absence (x; water control) or presence of 100 units of superoxide dismutase (SOD) (◇), 100 μg/ml of elicitor alone (●), 100 μg/ml of suppressor alone (○), elicitor plus suppressor (◐), elicitor plus SOD (◆), and suppressor plus SOD (◻). The blue formazan was determined by the method of Nathan et al. (30). Each value represents the mean with standard deviation from triplicate experiments.

and cowpea in a dose-dependent manner (Figs. 3 and 4). On the other hand, the suppressor preparation from *M. pinodes* inhibited blue formazan formation in the pea fraction in a dose-dependent manner (Figs. 3 and 4), and even the concomitant presence of the suppressor with the elicitor decreased the blue formazan formation in the pea fraction to the level of the water control (Fig. 3). On the other hand, the suppressor preparation did not inhibit elicitor-enhanced blue formazan formation in the cowpea fraction (Fig. 3). Moreover, the blue formazan formation in the cowpea fraction was not inhibited, but was rather enhanced by the suppressor alone in a dose-dependent manner (Fig. 4). The elicitor and suppressor preparations from *M. pinodes* did not contain the activity to form blue formazan by themselves (data not shown).

Fig. 4. Effects of the elicitor and suppressor preparations from *Mycosphaerella pinodes* on blue formazan formation in the fractions solubilized from cell walls of pea and cowpea. The assay was carried out as described in Figure 1 in the absence (0 μg/ml) or presence of several concentrations of elicitor (○) or suppressor (■). Each value represents the mean with standard deviation from triplicate experiments.

These results showed that O_2^- generation in the solubilized cell wall fractions is regulated by these fungal signal molecule preparations, and, especially, that the suppressor acts on the activity in a species-specific manner.

Though O_2^- generation in the solubilized cell wall fractions is regulated by these fungal signal molecule preparations, as described above, it has been obscured how O_2^- generated in the cell wall participates in plant-pathogen interactions. Bolwell (4) and Bolwell et al. (5) reported that cell wall-bound peroxidase participated in production of H_2O_2 in suspension-cultured cells of French bean in response to the elicitor. On the other hand, we recently found that the elicitor preparation from *M. pinodes* induced O_2^- generation on the surfaces of pea and cowpea leaves, and that the O_2^- generation was affected by the suppressor preparation from its fungus in a species-specific manner (23). The O_2^- generation on the leaf surfaces of both plants induced by the elicitor preparation was markedly inhibited by an inhibitor of peroxidase, SHAM, not by inhibitors of NADPH oxidase, imidazole and quinacrine (23). Since these properties of O_2^- generation on the leaf surfaces of pea and cowpea strictly coincide with those in the fractions solubilized from pea and cowpea cell walls, cell wall-bound peroxidase from pea and cowpea might act as the supplier for O_2^- generation on the surfaces of pea and cowpea leaves.

The suppressor from *M. pinodes* was proposed to be a determinant of host-parasite specificity, since it inhibited defense responses and induced susceptibility in only host plants of *M. pinodes* (32, 38,46). One of the major effects of the suppressor was thought to be inhibition of ATPase activity of the host plant. Cytochemical observation showed that the suppressor specifically inhibited the ATPase activity of pea, but not those of the nonhost plant species barley, cowpea, kidney bean, and soybean, whereas ATPase activities in plasma membranes isolated from these five plant species were nonspecifically inhibited by the suppressor (35). On the other hand, the cell wall-bound ATPases were regulated even in vitro by the suppressor in a species-specific manner (24,25). Thus, the cell wall-bound ATPase was thought to be tightly correlated to defense responses and determination of host-parasite specificity. In our current results, NADH-dependent O_2^- generation, which may be mediated by cell wall bound-peroxidase(s), was also regulated by the suppressor in a species-specific manner (Figs. 3 and 4). These results suggest the possibility that the activity of cell wall-bound ATPase is tightly correlated with NADH-dependent O_2^- generation by peroxidase(s). In fact, we recently found that the cell wall-bound ATPases of pea and cowpea were copurified by affinity chromatography with an ATP-conjugate agarose column with peroxidase(s) activity (A. Kiba, K. Toyoda, Y. Ichinose, T. Yamada, and T. Shiraishi, unpublished data).

It has been thought that plant cell walls play an important role in plant defense responses, such as acting as a physical barrier against invasion by potent pathogens. Insolubilization of pre-existent protein (7,8) or lignification (43) rapidly induced by inoculation with pathogens or by treatment with elicitors seems to strengthen cell walls. Another report showed that polygalacturonase-inhibiting proteins in cell walls may contribute to the formation of elicitor-active oligogalacturonide fragments (9). Thus, plant cell walls act as not only physical barriers, but also as a site for generating second messengers for defense responses. Together with our previous reports (24,25), our current data show that plant cell walls alone are able to recognize and respond to fungal signals, since cell wall-bound ATPase(s) and the O_2^- generation system are nonspecifically stimulated by the *M. pinodes*-elicitor, but are affected by the *M. pinodes*-suppressor in a species-specific manner. We, therefore, present the hypothesis that cell walls might play a crucial role in recognition of fungal signal molecules, regulation of defense responses, and determination of plants-pathogens specificity.

Further experiments are needed to elucidate not only the relationship between the receptors for these fungal signals and the

regulatory systems for O_2^- generation in cell walls, but also the role of O_2^- in signal transduction leading to the defense responses of plants.

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