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Generation of Active Oxygen Species (AOS) and Induction of β-Glucanase Activity by Fungal Elicitor Xylanase in the Suspension Cultured Cells of Tobacco

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It was investigated that active oxygen species (AOS) involved in the plant defense responses induced by fungal elicitor xylanase. When xylanase from the fungus *Trichoderma viridae* was treated to tobacco suspension cultured cells as an elicitor, β-glucanase activity was increased markedly. Lignin biosynthesis was also increased and peaked at 72 h after the treatment with xylanase. The treatment of H_2O_2 also dramatically increased β-glucanase activity at 24 h, which was much earlier than that of xylanase did. Using lucigenin- and luminol-dependent chemiluminescence, the effects of xylanase on oxidative burst were examined. Superoxide anion (O_2^-) production was peaked at 40 h and 52 h after xylanase treatment and hydrogen peroxide (H_2O_2) release was peaked at 44 h and 56 h, suggesting H_2O_2 burst was followed by O_2^- generation. The scavengers of AOS, n-propyl gallate (PG) and mannitol, inhibited xylanase-induced β-glucanase activity by 85% and 50%, respectively. The activity of superoxide dismutase (SOD), which catalyzes the dismutation of O_2^- to H_2O_2, began to increase from 24 h and reached to maximum at 48 h after xylanase treatment. Pretreatment of N,N,-diethyldithiocarbamate (DDC), known as a SOD inhibitor, caused the inhibition of H_2O_2 generation by 80% and reduced the β-glucanase activity by 60%. Treatment of 2,5-norbonadiene (NBD), a specific ethylene-action inhibitor, did not have any significant effect on xylanase-induced β-glucanase activity. This result suggested that ethylene did not involve in xylanase-induced response. Our results strongly suggest that the AOS generation is an essential component in plant defense response, in which cell wall degrading enzyme, glucanase, contributes to remove the necrotic tissue induced by pathogens.

Keywords: xylanase, AOS, plant defense response, β-glucanase, signal transduction

INTRODUCTION

Plant disease resistance to pathogens such as fungi, bacteria, and viruses often depends on whether the plant is able to recognize the pathogen early in the infection process (Mehdy, 1994). The recognition event leads to a rapid tissue necrosis at the site of infection, which is called the hypersensitive (HR). The HR deprives the pathogen of nutrients and/or release toxic molecules, thereby confining pathogen growth to a small region of the plant. This response provides resistance to the great majority of potential pathogens.

Several rapid processes characteristic of the HR appear to involve primarily activation of preexisting components rather than changes in gene expression. One of these rapid processes is the striking release of active oxygen species (AOS), which is termed the oxidative burst. The AOS are toxic intermediates that result from successive one electron steps in the reduction of molecular O_2. The predominant AOS detected in plant-pathogen interactions are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). The oxidative burst is correlated with the HR and therefore may be an important element contributing to disease resistance.

Recent findings suggest that AOS may be involved to perform multiple physiological functions (Legrand *et al.*, 1993; Degousee *et al.*, 1994; Green and Fluhr, 1995; Robertson *et al.*, 1995). For instances, i) they act directly as toxic defense agents

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against pathogens (Adam *et al.*, 1989), ii) they serve as inducers of various defense reactions associated with systemic acquired resistance (SAR) in addition to HR (Lamb *et al.*, 1989), iii) they promote the peroxidase-catalyzed cross linking of cell wall polymers such as lignin, proteins or pectins to protect against pathogen attack (Bradly *et al.*, 1992).

The filamentous fungus *Trichoderma viridae* formed xylan-inducible xylanase, a 24 kD protein, to degrade the xylan backbone of hemicellulose into monomeric pentosan units that are used by bacterial and fungal populations as primary carbon source (Prade, 1996). It has been known that the xylanase purified from *Trichoderma viridae* stimulates production of PR proteins in tobacco. An ethylene biosynthesis-inducing xylanase (EIX) produced by the fungus *Trichoderma viridae* (Dean and Anderson, 1991) elicits ethylene biosynthesis and plant defense responses. When EIX is applied to cut petiol on an intact plant, it moves through the vascular to leaves above and below the point of application, particularly into the mesophyll tissue, where ethylene production is enhanced and the tissue becomes necrotic (Avni *et al.*, 1994). But the signal transduction mechanism of xylanase is still obscure. Moreover, it was known that xylanase signaling is independent of the change of intracellular calcium level and protein phosphorylation state (Raz and Flur, 1992, 1993).

Plant developmental processes involving modifications to cell wall structure, such as cell expansion, organ abscission and fruit ripening, are accompanied by increased enzyme activity of glucanase (Brummell *et al.*, 1997). Specially, glucanase activity significantly increased within plant organs undergoing cell separation to act cooperatively in mediating cell wall disassembly and degrading structural polysaccarides.

In this paper, we investigated the involvement of AOS in the plant defense response by measuring the glucanase activity after the treatment with the fungal elicitor xylanase in tobacco suspension cultured cells.

MATERIALS AND METHODS

Plant Materials

Callus cells induced from tobacco (*Nicotiana tabacum* cv. Wisconsin 38) leaf were cultured in Murashige and Skoog (MS) liquid medium with 0.3 μg/mL NAA and 1 μg/mL kinetin. The suspension cells were subcultured at every two weeks and maintained at 28°C with shaking at 140 rpm.

Treatment of Chemicals

Xylanase from *Trichoderma viridae* which was used as an elicitor was purchased from Sigma (USA). The 7 day-cultured cells were harvested by the vacuum filtration. Cells were washed with assay buffer (0.25 M sorbitol, 10 mM MES, pH 6.0) and xylanase was treated at 50 ng/mL. n-Propyl gallate and mannitol was pretreated for 6 h at 0.5 μM and 1 mM, respectively.

Assay of β-Glucanase Activity

β-glucanase activity was determined by measuring the amount of reduced sugars from β-glucan (Abeles, 1970). Cells were harvested and homogenized with 1 mL of 50 mM potassium acetate buffer (pH 5.0) and centrifuged at 18,000 rpm. The resulting supernatant was used as an enzyme source. To 0.5 mL of enzyme source, 0.5 mL of 2% (w/v) β-glucan was added and then incubated at 50°C for 2 h. The reaction was terminated by adding 3 mL of dinitrosalicylic reagent (prepared by adding 300 mL of 4.5% NaOH to 880 mL of a solution containing 8.8 g of dinitrosalicylic acid and 255 g of potassium sodium tartrate) and heated for 5 min at 100°C. After cooled to 25°C, the optical density was measured at 500 nm. The results are given as absorbance units.

Measurement of Lignin Content

Lignin content was measured by derivatization from alcohol-insoluble residues (AIR) of suspension cultured cell materials with adding thioglycolic acid (Whitmore, 1978). Cells were harvest and homogenized in methanol, vacuum-filtered through Whatman GF/A filter paper and dried at 60°C. The resulting AIR was used for lignin determination. To 50 mg of AIR, 5 mL of 2 N HCl and 0.5 mL of thioglycolic acid were added and incubated at 100°C for 4 h. Following centrifugation at 30,000×g for 10 min at RT, the resulting pellet was resuspended in 5 mL of 0.5 N NaOH and incubated at 25°C for 18 h with gentle agitation to extract the lignin thioglycolate (LGTA). After centrifugation (30,000×g, 10 min), the supernatant was neutralized by adding 1 mL of HCl and allowed to precipitate at 4°C for 4 h. Following centrifugation, the orange-brown colored pellet was dissolved in 10 mL of 0.5 N NaOH, and

the optical density at 280 nm was measured. The results are given as absorbance units.

Measurement of Superoxide Anion and Hydrogen Peroxide Contents

Contents of superoxide anion were measured by lucigenin-dependent chemiluminescence (LCDC) (Corbisier, 1987). To 0.2 g of cells, 0.2 mL of lucigenin solution (1 mM lucigenin, 1 mM EDTA, 1 mM DDC, 1 mM sodium salicylate, 0.1 M glycine buffer, pH 9.0) was added and measured the bioluminescence using scintillation counter. Contents of hydrogen peroxide were also measured by luminol-dependent chemiluminescence (LMDC) (Murphy, 1990). In this assay, luminol is oxidized in presence of H_2O_2 by peroxidase present in the reaction mixture and an unstable derivative emits light upon return to its ground state. To 0.2 g of cells, 0.2 mL of luminol solution (1 mM luminol, 1 mM potassium ferricyanide, 20 mM potassium phosphate buffer, pH 7.4) was added and measured the bioluminescence using scintillation counter.

Measurement of Ethylene Production

Cells were enclosed in a gas-tight glass vial and incubated at 28°C in dark. One mL sample of gas was withdrawn and analysed with a Gas Chromatography (GC-3BF, Shimadzu, Japan) equipped with an activated alumina column and a flame ionization detector.

Assay of Superoxide Dismutase Activity

Activity of SOD was measured by the method of McCord and Fridovich (1969). The standard assay was performed in 1 mL of 0.05 M potassium phosphate buffer at pH 7.8 containing 0.1 mM EDTA in a 1.0 cm cuvette thermostated at 25°C. The reaction mixture contained 10 μM ferricytochrome c, 50 μM xanthine, and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome c at 550 nm of 0.025 absorbance unit per min. The concentration of xanthine oxidase in the cuvette was usually 6 nM but may vary with different preparations of the enzyme source. Under these defined conditions, the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% (i. e. to a rate of 0.0125 absorbance unit per min) is defined as 1 unit of activity.

RESULTS

Effects of Xylanase on β-Glucanase Activity in Tobacco Suspension Cultured Cells

When xylanase as an elicitor was treated to tobacco suspension cultured cells, β-glucanase activity was increased at 24 h and peaked at 72 h after xylanase treatment (Fig. 1A). β-glucanase is one of the well known pathogenesis-related (PR) protein, and has an anti-fungal activity. Antifungal activity may attribute to the digestion of the β-glucan polymer, which is a cell wall constituent in certain classes of phytopathogenic fungi (Kauffmann *et al.*, 1987; Wray, 1992).

It is widely known that signal transduction pathway of various pathogen infection is mediated by plant hormone, ethylene (Ohashi and Matsuoka, 1987). Many plants respond to an attack by pathogens with an enhanced ethylene production (Yang and Hoffman, 1984). Exogenously applied ethylene has been found to activate or enhance biochemical

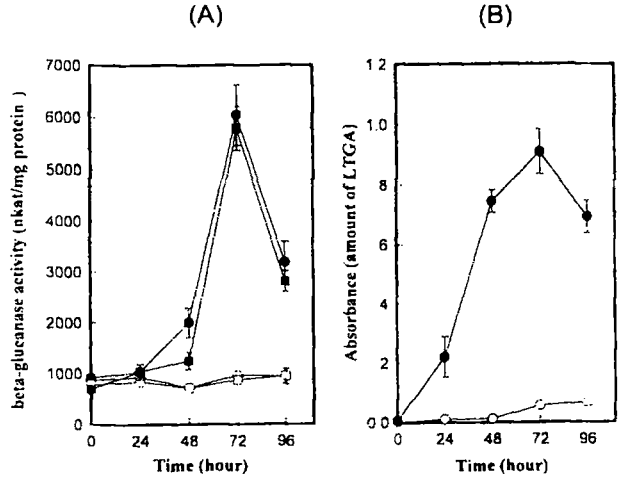

Fig. 1. Effects of xylanase on β-glucanase activity (A) and lignin biosynthesis (B). (A) Cells were treated with 50 ng of xylanase and then the β-glucanase activity was measured. 2,5-norbonadiene (NBD) was pretreated for 6 h. The enzyme activity was expressed as the nkat/mg protein. 1 nkat as defined as the amount of enzyme catalyzing the formation of 1 ng of reducing sugar per min. (B) The lignin contents were measured from 50 mg of alcohol insoluble residue (AIR). Results were expressed as the absorbance of lignin thioglycolic acid (LGTA) at 280 nm. Each value is the mean ±S.E. from three or four independent experiments. Control: ○ - ○, 50 ng of xylanase treated: ● ●, 1000 ppm of NBD treated: □ — □, 1000 ppm of NBD and 50 ng of xylanase treated: ■ — ■.

defenses against potential pathogens in a number of cases (Yang and Hoffman, 1984; Boller, 1990). Therefore, it has been hypothesized that the endogenously produced ethylene may function as a signal for the plant to defense against pathogens (Latt and Vanloon, 1982; Ecker and Davis, 1987). But the ethylene-independent signal transduction system also exist. In this pathway, ethylene is not essential for the induction of pathogenesis-related (PR) protein (Paradies *et al.*, 1980; Mauch *et al.*, 1984).

The treatment of 2,5-norbonadiene (NBD), a specific ethylene-action inhibitor, has no significant effect on xylanase-induced β-glucanase activity (Fig. 1A). This means that the xylanase induced signaling is independent of ethylene action in the suspension cultured cells of tobacco (*Nicotiana tabacum* cv. Wisconsin 38). Xylanase from *Trichoderma viride* elicits ethylene biosynthesis in leaf tissue of *Nicotiana tabacum* cv. Xanthi but not in cv. Hicks (Avni *et al.*, 1994). Ethylene priming enhances ethylene production in response to cell-wall-digesting enzymes. However, not all cultivars of tobacco respond to EIX treatment, suggesting a requirement for EIX-specific recognition factors in the plant. The EIX-incentive cultivars, Hicks, however, produce ethylene in response to other cell-wall-digesting enzymes (Sharon *et al.*, 1993). The cultivar-specific response of EIX may be compared to the hypersensitive response in plant-pathogen interactions, where single dominant genes predominate as the controlling factors (Avni *et al.*, 1994).

Lignin is known as a kind of barrier of plant to pathogen attack (Whetten and Sderoff, 1995). Therefore, we studied the effect of xylanase on lignin biosynthesis (Fig. 1B). As a result, lignin biosynthesis was increased immediately after treatment of xylanase and then reached to maximum at 72 h. Since active oxygen species (AOS) has been known to drive the biosynthesis of lignin (Freudenberg, 1965), the possibility that AOS were involved in the xylanase-induced plant defense responses as a signal transducer was investigated.

Effects of H_2O_2 on β-Glucanase Activity and Measurements of the Oxidative Burst After the Treatment with Xylanase

To investigate the involvement of AOS in xylanase signaling forward induction of β-glucanase, the effect of H_2O_2 on β-glucanase activity were studied. The treatment of H_2O_2 prominently induced β-glucanase activity and rapidly increased until 48 h,

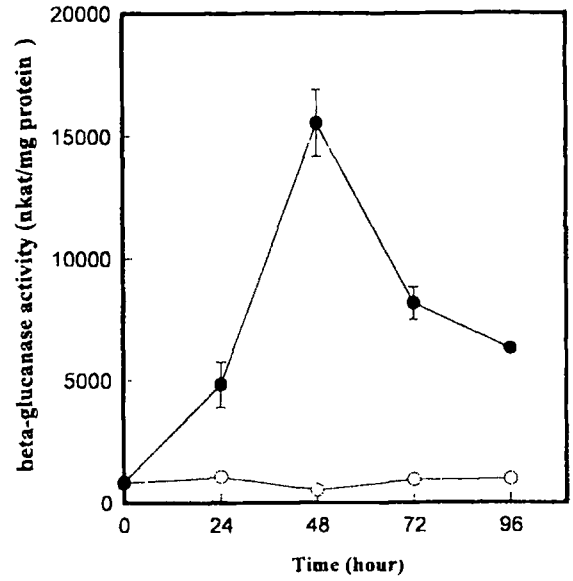

Fig. 2. Effects of hydrogen peroxide on β-glucanase activity. Cells were treated with 10 mM of hydrogen peroxide and then the β-glucanase activities were measured. Each value is the mean ± S.E. from three or four independent experiments. Control: ○ ○, 10 mM of hydrogen peroxide treated: ● ●.

which is earlier than the treatment of xylanase did (Fig. 2). Therefore, β-glucanase was more rapidly induced by H_2O_2 than by xylanase.

Using lucigenin- and luminol-dependent chemiluminescence, we examined the effect of xylanase on AOS production. O_2^- production increased after treatment of xylanase, which was peaked at 40 h and 50 h (Fig. 3A), and also H_2O_2 release at 44 h and 56 h (Fig. 3B). These patterns of AOS production showed a transient increase and a characteristic double peak. Rapid generation of AOS that made a first peak is known as a distinct response of plant cells to microbial elicitors or challenge to an avirulent pathogen. When plant cells were infected by pathogens, second oxidative burst was occurred by virulent pathogens, but not by avirulent pathogens (Levine *et al.*, 1994). These facts indicate that first and second oxidative burst are distinguished from their functions. The first burst is concerned to simple perception of pathogens and the second, which has a rather long duration time, is related to induction of plant defense responses (Levine *et al.*, 1994).

Effects of Reducing Agents and Antioxidant Enzymes on the Xylanase-induced β-Glucanase Activity

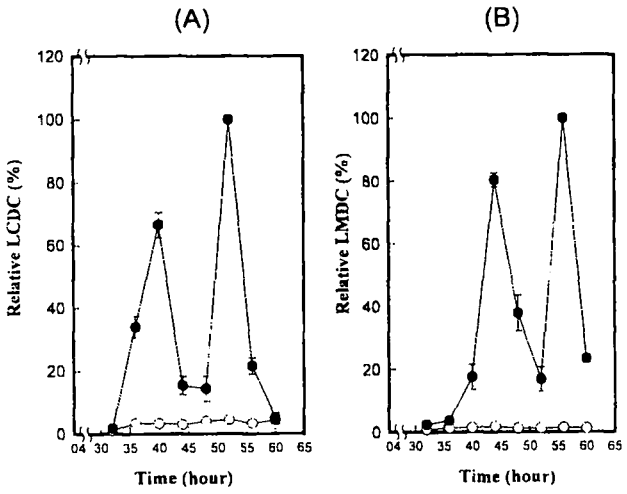

Fig. 3. Effects of xylanase on the accumulation pattern of superoxide anion (A) and hydrogen peroxide (B). Accumulation of superoxide and hydrogen peroxide were measured by the chemiluminescence of lucigenin and luminol, respectively. The ordinate shows the relative lucigenin-dependent chemiluminescence (LCDC) (A), and relative luminol-dependent chemiluminescence (LMDC) (B). Each value is the mean ±S.E. from three or four independent experiments. Control: ○ -○, 50 ng of xylanase treated: ● -●.

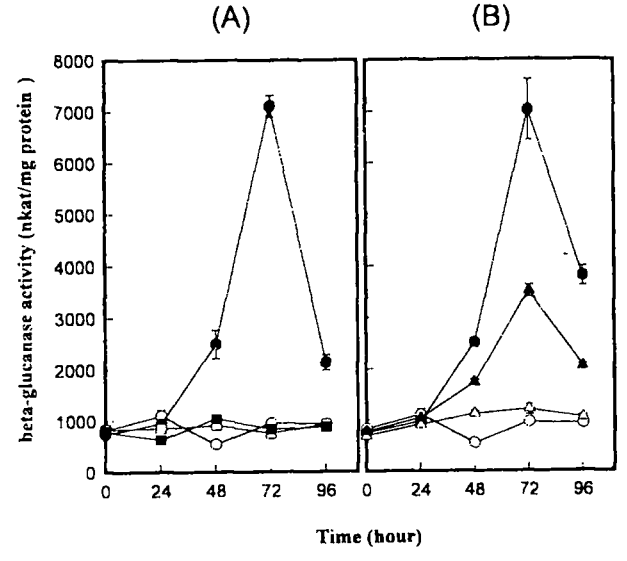

Fig. 4. Effects of n-propyl gallate (PG) (A) and mannitol (B) on xylanase-induced β-glucanase activity. Cells were preincubated for 6h with PG or mannitol, and then β-glucanase activities were measured with the treatment of xylanase at indicated time. Each value is the mean ±S.E. from three or four independent experiments. Control: ○ -○, 50 ng of xylanase treated: ● -●, 0.5 μM of PG treated: □ -□, 0.5 μM of PG and 50 ng of xylanase treated: ■ -■, 1 mM of mannitol treated: △ -△, 1 mM of mannitol and 50 ng of xylanase treated: ▲-▲.

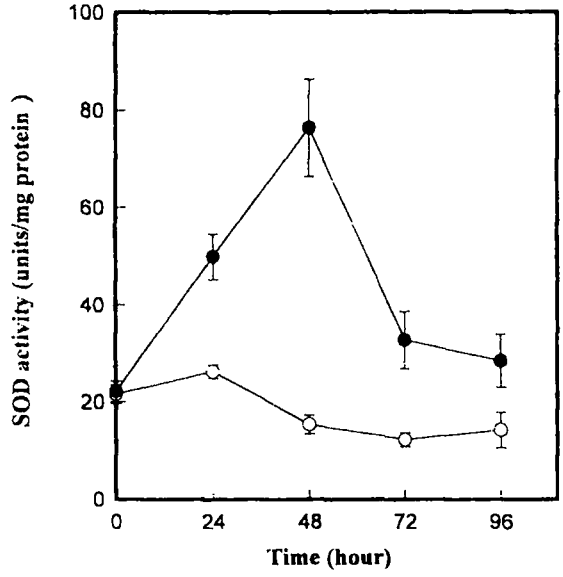

Fig. 5. Effects of xylanase on superoxide dismutase (SOD) activity. Cells were treated with 50 ng of xylanase and then the SOD activity was measured at the indicated time. The activity of the enzyme was expressed as the unit/mg protein. 1 unit was defined as the amount of enzyme required to cause 50% inhibition of cytochrome c reduction measured at 550 nm. Each value is the mean ± S.E. from three or four independent experiments. Control: ○ -○, 50 ng of xylanase treated: ● -●.

It has been known that n-propyl gallate (PG) is a phenolic compound that functions as a free radical scavenger (Kacperska and Zebalska, 1989). When tobacco suspension cultured cells were pretreated with PG, xylanase-induced β-glucanase activity was completely blocked into control level at concentration of 0.5 μM (Fig. 4A). For further confirmation, we used another antioxidant, mannitol, which is also known as a free radical scavenger, for this inhibitory effect (Estrella *et al.*, 1992). Pretreatment with mannitol inhibited xylanase-induced β-glucanase activity by 50% (Fig. 4B). These results enforce that the AOS may play an important role in the xylanase-induced β-glucanase activity.

When we examined the production of H_2O_2 and O_2^- in the time course experiments after treatment of xylanase, it was found that the H_2O_2 burst was followed by O_2^- burst. This result confirms to the idea that O_2^- is converted to H_2O_2 by superoxide dismutase. In fact, the activity of SOD began to increase after xylanase treatment and peaked at 48 h (Fig. 5). Furthermore, pretreatment of N,N-diethyldithiocarbamate (DDC), an inhibitor of SOD, caused the inhibition of H_2O_2 production and reduced β-glu-

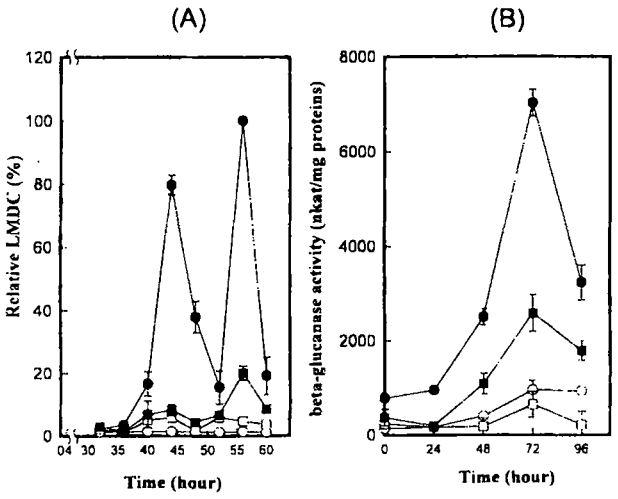

Fig. 6. Effects of DDC on the accumulation of hydrogen peroxide (A) and β-glucanase activity (B). Cells were preincubated with N,N-diethyldithiocarbamate (DDC) for 24 h before the treatment of xylanase. The amount of hydrogen peroxide was measured by luminol dependent chemiluminescence (LMDC). Each value is the mean ± S. E. from three or four independent experiments. Control: ○ ○, 50 ng of xylanase treated: ● ●, 1 mM of DDC treated: □ □, 1 mM of DDC and 50 ng of xylanase treated: ■ ■.

canase activity by 80% and 60%, respectively (Fig. 6A, B). These findings suggest that xylanase-induced SOD activity is responsible for transition of O_2^- to H_2O_2, and these oxidative bursts are related to the induction of PR protein, such as β-glucanase.

DISCUSSION

Several plant receptors that bind plant or fungal cell wall-derived elicitors have been localized to the plasma membrane. In soybean, plant cell wall-derived polygalacturonic acid and fungal cell wall-derived carbohydrate/(glyco)protein preparations are known to stimulate the oxidative burst and their receptors were found on the plasma membrane (Mehdy, 1994). Elicitor binding probably leads to production of signals that induce other effects such as pH changes and ion fluxes, which may be related to membrane acylation of sterol glycosidase, protein phosphorylation, or inositol phospholipid kinase activity (Avni *et al.*, 1994). One reasonable pathway consistent with the data is that elicitor receptor coupled with a G protein leads to Ca^{2+} influx, which then activates a Ca^{2+}-dependent protein kinase and ultimately the O_2^--generating oxidase (Mehdy, 1994).

Numerous mechanisms are available to limit the duration of the oxidative burst and its toxic consequences in plant cells (Mehdy, 1994). It was reported AOS directly reduce pathogen viability. Also, the oxidative burst appears to play a novel role in strengthening the plant cell wall to pathogen attack. The possibility that AOS serve as signal intermediates that induce phytoalexin biosynthesis has received considerable attention. In recent research, it was directly detected that the secretion of cellulases including β-glucanase and that of xylanases occurred later than the start of infection, suggesting a role of these cell-wall degrading enzymes in the digesting of dead host tissues (Toedemann, 1997). Also, the scavengers of AOS, catalase and mannitol, significantly reduced the severity of infection. Therefore, the AOS may function extracellularly as agents for pathogen killing, both at the level of cell wall for oxidative cross-linking of proteins and intracellularly as signal intermediates triggering changes in gene expression and possibly as toxic molecules contributing to host cell death by inducing cell wall degrading enzymes. Our results based on β-glucanase activity which was increased by the treatment of xylanase suggest that the fact cell wall degrading enzyme, β-glucanase, mediated with AOS burst leads to a rapid tissue necrosis at the site of infection, which was due to cell separation, and then deprive the pathogen of nutrients and/or release toxic molecules, thereby confining pathogen growth to a small region of the plant.

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