Induced Resistance to Powdery Mildew by 2,6-Dichloroisonicotinic Acid is Associated with Activation of Active Oxygen Species-mediated Enzymes in Cucumber Plants

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Powdery mildew infection in cucumber plants was significantly reduced by foliar application of 2,6-dichloroisonicotinic acid (INA). Although the overall isozyme banding patterns of superoxide dismutase (SOD) and peroxidase (POD) were very similar, there were differences in the densities of major bands between the leaves of control and INA-applied plants. When plants were subjected to foliar applications of INA, the densities of SOD and POD isoforms was increased more abruptly 3 days after foliar application compared with the control plants; the high density was maintained thereafter. The specific activities of SOD and POD were increased rapidly by foliar application of INA. Although enzyme activities were increased with the development of colonies 9 days after the inoculation of pathogen in control plants, it did not reach the level of activity in the leaves of INA-applied plants. The expression of β-1,3-glucanase was only detected in the leaves of INA-applied plants. The specific activity of β-1,3-glucanase was also significantly increased in the leaves of INA-applied plants. Activities of SOD and POD by foliar application of INA were significantly reduced by diphenylene iodinium (DPI), an inhibitor of oxidative burst. The expression of β-1,3-glucanase was also restricted by foliar application of INA with DPI. However, the β-1,3-glucanase isozyme band was detected ambiguously with increasing of SOD and POD 6 days after foliar application. Although gel and specific activity of SOD and POD were significantly increased in the leaves of H2O2-applied plants, no β-1,3-glucanase isoforms were detected. These results suggest that activation of AOS-mediated enzymes by foliar application of INA seem to take part in defense reaction to induce the resistance to powdery mildew in cucumber plants.

Key Words: antioxidant enzymes, cucumber, β-1,3-glucanase, INA, powdery mildew.

Introduction

The inducible resistance system, known as systemic acquired resistance (SAR), is an effective control program against plant pathogens including, viruses, bacteria, and fungi (Ryals et al., 1996). The SAR is associated with various plant defense mechanisms such as accumulation of active oxygen species (Ryals et al., 1996), synthesis of pathogenesis-related (PR) proteins (Delaney, 1997), phytoalexins (Hwang, 1995), and reinforcement of the cell wall (Brisson et al., 1994). The oxidative burst, the rapid production of active oxygen species (AOS), is one of the early and initial responses of plants to pathogen attack. It leads to a hypersensitive response (Levine et al., 1994) including the expression of PR proteins encoded by SAR genes (Orozco-Cardenas et al., 2001). The generation and accumulation of AOS such as superoxide radicals and H2O2 were more fully reported mechanisms for SAR (Álvarez et al., 1998; Lamb and Dixon, 1997). It was suggested that AOS perform a crucial role in early defense responses against pathogens. The defense responses by AOS include direct antimicrobial action (Lu and Higgins, 1999), reinforcement of the cell wall (Brisson et al., 1994), and the expression of PR proteins by triggering the SAR gene (Lamb and Dixon, 1997).

Plants respond to various exogenously applied chemical stresses by the synthesis of PR proteins such as β-1,3-glucanase and chitinase. It has been reported that several plant defense activators including acibenzolar-S-methyl (ASM), DL-β-amino-n-butyric acid (BABA), and 2,6-dichloroisonicotinic acid (INA) not only induced resistance in plants against pathogens, but also elicited the accumulation of PR proteins (Gorlach et al., 1996; Kauss et al., 1992; Silue et al.,...
The expression of PR proteins in SAR systems is correlated to the levels of accumulation of SA by plant defense activators. The accumulations of SA are mediated through the generation of AOS (Kawano and Muto, 2000). Elevated levels of AOS have beneficial effects on the defense mechanisms as signal mediators or direct activators against pathogens, but they also have harmful effects by way of disrupting metabolic functions and damaging cellular components in the plants. Regardless of the type of effect, an obvious response to elevated levels of AOS is the activation of antioxidant enzyme systems. AOS generated via oxidative burst are under the control of antioxidant enzymes, such as superoxide dismutase (SOD) and peroxidase (POD). SODs are metal-containing enzymes that convert superoxide radicals to oxygen and H2O2 by dismutation (Fridovich, 1986). The toxic H2O2 produced by dismutation of SOD is reduced to H2O by POD, which is the key enzyme involved in H2O2 scavenging (Asada and Takahashi, 1987). There have been several reports on the role of SOD during plant infection by pathogens. SOD activity increased in the interaction of host plants and pathogens (Babitha et al., 2002; Yun et al., 1995). It was suggested that the enhanced activity of SOD resulted from the generation of AOS in the interaction of host plants and pathogens, seemingly playing a crucial role in reducing oxidative stress. PODs activated by overproduction of AOS are oxireductive enzymes that participate in the reinforcement of the cell walls through suberization (Espelie et al., 1986), lignification (Whetten et al., 1998), cross-linking of cell walls (Brisson et al., 1994), and activation of defense genes as a defense reaction against pathogens (Borden and Higgins, 2002).

A lot of studies on the response of plants to these defense activators have focused on the induction of PR proteins associated with the accumulation of salicylic acid, and can be viable alternative methods to traditional fungicides for controlling pathogen invasion (Bokshi et al., 2003; Cohen et al., 1994; Hwang et al., 1997). Although several studies reported that induction of antioxidant enzymes such as SOD, POD, and lipoxygenase are related to the restriction of fungal diseases in resistance plants (Babitha et al., 2002; Nagarathna et al., 1992; Sreedhara et al., 1995), there has been no report on the activation of AOS-mediated enzymes by plant defense activators.

In the present study, cucumber plants exposed to foliar applications of INA were analyzed in terms of induction of resistance to powdery mildew and activation of AOS-mediated enzymes. We also examined whether exogenously applied-H2O2 could be related to the activation of AOS-mediated enzymes and induction of resistance to powdery mildew in cucumber plants.

Materials and Methods

Plant materials and growing conditions

Cucumber (Cucumis sativus L. Heukjinju) seeds were sown in a plastic tray (45 cm × 35 cm × 8 cm) containing commercial soil (Shinan Grow, Chjinju, Korea), and transplanted into plastic pots (11 cm × 11 cm) containing the same commercial soil at the one-leaf stage. Cucumber seedlings were grown in a growth chamber set at 20°C, 12 h photoperiod, and a light intensity of 200 μmol·s−1·m−2. When the cucumber plants have three expanded leaves, the plants were transferred in the glasshouse at 25 ± 5°C, and grown with irrigation once every day to field capacity.

Foliar application

First experiments were carried out to determine effects of 2,6-dichloroisonicotinic acid (INA) on the induction of resistance to powdery mildew and activation of AOS-mediated enzymes. Cucumber plants at three fully expanded leaves were uniformly applied with 0.01 mM INA containing 0.01% Tween-20 as surfactant. The control plants were applied with water containing 0.01% Tween-20. The foliar applications were conducted three times at 2-days intervals.

Second experiments were carried out to determine effects of H2O2 and AOS inhibitor on the induction of resistance to powdery mildew and activation of AOS-mediated enzymes. Cucumber plants at three fully expanded leaves were uniformly applied by foliar application of 30 mM H2O2 and 0.01 mM INA with 3.0 μM diphenylene iodinium (DPI, specific inhibitor of AOS) containing 0.01% Tween-20 as surfactant. The control plants were applied with water or 0.01 mM INA containing 0.01% Tween-20 as surfactant.

Fungal inoculation

Inoculation of fungal disease was done after final foliar application. The conidia of powdery mildew were collected 2h before inoculation by shaking mature colonies on naturally infected glasshouse-grown cucumber leaves into a triangle flask of distilled water containing 0.01% Tween-20. The number of conidia in suspension was determined using a haemocytometer, and then sprayed onto the upper surfaces of the leaves at 500–1000 conidia per leaf. The number of colonies of powdery mildew was counted 15 days after inoculation.

Total soluble protein extraction

Samples were collected from second leaves, and homogenized in 0.5 M sodium acetate buffer (pH 5.2) containing a 15 mM 2-mercaptoethanol with polytron (PT-3100, Kinematica, Switzerland). The homogenate was centrifuged to remove insoluble materials at 20,000 g at 5°C for 60 min, and the supernatant was collected. The supernatant was precipitated from crude extracts by incubating in four volumes of cold acetone at −20°C overnight. The precipitate was collected by centrifugation at 15,000 g for 15 min, washed at least twice with cold 80% acetone, and dried in a speed vac. The protein pellets were dissolved in 30 mM sodium
H₂O₂ in darkness at 30 °C (pH 10.2), 1.3 reaction mixtures contained 50 mM Na-carbonate method following Beyer and Fridovich (1987). The Enzyme activity analysis for antioxidant enzymes were measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

Native PAGE and isoenzyme analysis for antioxidant enzymes
The isoenzymes were separated electrophoretically in 10% polyacrylamide slab gels under native conditions. For each sample, 20 μg of total proteins was loaded on the gel using a micropipette. Electrophoresis was run using Tris-glycine (pH 8.3) electrode buffer at 4 °C for 5 h with a constant current of 30 mA. After electrophoresis, the gels were stained for enzyme activity analysis.

Gels were stained for superoxide dismutase (SOD, EC.1.15.1.1) isoforms by equilibrating with 50 mM potassium phosphate (pH 7.8) containing 2.5 mM nitro blue tetrazolium (NBT) in darkness at 30 °C for 25 min, followed by soaking in 50 mM potassium phosphate (pH 7.8) containing 30 mM riboflavin and 2.5 mM NBT in darkness for 30 min. Gels were then exposed to light until achromatic bands appeared. Staining of peroxidase (POD, EC. 1.11.1.7) isoforms was achieved by incubating the gels in sodium citrate buffer (pH 5.0) containing 9.0 mM ρ-phenylenediamine and 4.0 mM H₂O₂ in darkness at 30 °C for 15 min.

Enzyme activity analysis for antioxidant enzymes
SOD activity was determined by the NBT reduction method following Beyer and Fridovich (1987). The reaction mixtures contained 50 mM Na-carbonate (pH 10.2), 1.3 μM riboflavin, 13 mM methionine, 65 μM NBT, and enzyme extracts. The reaction was initiated by light illumination in a chamber at 25 °C for 10 min. The development of purple color was then determined by the measurement of absorbance at 560 nm. Unilluminated samples served as control. One unit of SOD activity is defined as the amount required to inhibit the photoreduction of NBT by 50%. One unit was determined by (V-v)–1, where V and v were the slopes of changes in absorbance in the absence and presence of the enzyme extract, respectively.

POD activity was determined spectrophotometrically by monitoring the formation of tetraguaiacol from guaiacol (extinction coefficient at 460 nm = 6.39 mM⁻¹·cm⁻¹) in the presence of H₂O₂ (Putter, 1974). The reaction mixtures consisted of 10 mM potassium phosphate buffer (pH 7.0), 0.3 mM guaiacol, 0.1 mM H₂O₂, and enzyme extracts. The reaction was initiated by adding H₂O₂, and absorbance was recorded for 1 min at 25 °C. Activities were defined as μmol guaiacol oxidized per min per mg protein.

Native PAGE and detection of β-1,3-glucanase isoform
Polyacrylamide gel electrophoresis (PAGE) of protein under native conditions was carried out on 10% polyacrylamide separating gels. The gels were prepared by mixing 16.0 mL distilled water, 15.0 mL of 30% acrylamide, 10.0 mL of 1.5 M Tris-HCl (pH 8.8), 1.0 mL of 1.0% ammonium persulfate, and 50 μL TEMED. One hundred micrograms of total proteins was applied with a micropipette. Electrophoresis was run using Tris-glycine (pH 8.3) electrode buffer at 4 °C for 5 h with a constant current of 30 mA. After native PAGE, the resolved gels were equilibrated in 0.05 M sodium acetate (pH 5.2) for 10 min, and then incubated at 40 °C for 30 min in a mixture containing 75 mL of 0.05 M sodium acetate buffer (pH 5.0) and 1 g of laminarin dissolved in 75 mL of water by heating in a boiling water bath. The gels were incubated in a mixture of methanol, water, and acetic acid (5:5:2, v/v/v) for 5 min, and then washed three times with distilled water. The gels were stained with 0.3 g of 2,3,5-triphenyltetrazolium chloride in 200 mL of 1.0 M NaOH solution in a boiling water bath until red bands appeared. The gels were stained in the staining solution (glacial acetic acid, methanol and distilled water, 1:5:5, v/v/v) containing 0.25% Coomassie Brilliant Blue R-250 for 3 h at room temperature. Then, gels were destained in the destaining solution (glacial acetic acid, methanol and water, 1:3:9, v/v/v).

Enzyme activity analysis for β-1,3-glucanase
The activity of β-1,3-glucanase was measured on the basis of reducing sugars formed from laminarin by β-1,3-glucanase by a colorimetric assay using laminarin as a substrate. The substrate buffer was 0.1 M sodium acetate buffer (pH 5.2) containing 0.1% laminarin. The reaction mixture contained 0.9 mL substrate buffer and 0.1 mL enzyme extracts. It was incubated at 37 °C for 1 h. The reducing sugars were measured by the method of Nelson (1944) using glucose as a standard. A katal was defined as the enzyme activity catalyzing the formation of one mole of glucose equivalents per second.

Results
Restriction of powdery mildew in cucumber plants
Foliar application of INA was able to induce resistance against powdery mildew on the leaves of cucumber plants (Fig. 1). Powdery mildew disease was strikingly developed on the leaf of water-applied (control) plants after fungal inoculation. The progress of the disease on the leaves of control plants increased with time after inoculation, and most of the leaves developed severe fungal infection 15 days after inoculation. The colonies began to develop on the surface of cucumber leaves 3 days after inoculation; it was about 243 per leaf 15 days after inoculation. However, colonies of powdery mildew
slowly developed on the leaves of INA-applied plants before inoculation. The number of colonies on the leaves of INA-applied plants was significantly reduced to 18 colonies per leaf 15 days after inoculation.

Changes in gel and specific activities of antioxidant enzymes

Isozyme bands of SOD detected the presence of six isoforms in cucumber leaves (Fig. 2). The cucumber leaf has a total of four major bands (SOD-1, -2, -3, and -6) and two minor bands (SOD-4 and -5) in the resolving gel. Although the overall banding patterns of SOD were very similar, different densities of isozyme band in three major bands (SOD-1, -2, and -3) were found between the leaves of control and INA-applied plants. In the leaves of INA-applied plants, the isozymes of three major bands dramatically increased their activities after foliar application. However, increasing gel activity in the leaves of control plants was detected with the development of powdery mildew colonies 9 days after fungal inoculation. When gels were stained for POD activities, four isoforms were detected in cucumber leaves (Fig. 2). The gel activities of POD isozymes by density of band abruptly increased after foliar application with INA compared with the leaves of control plants. The gel activities of POD in the leaves of control plants were slightly increased 9 days after inoculation.

The time course of SOD and POD specific activities in leaves of control and INA-applied plants were shown Figure 3. The specific activities of SOD and POD

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**Figure 1.** Effects of foliar application of INA on the development of powdery mildew colonies in the leaves of cucumber plants 15 days after inoculation. Plants were applied with water as a control and 0.01 mM INA containing 0.01% Tween-20 as surfactant. After final foliar application of water and INA, fungal inoculation was done by sprayed on to the upper surfaces of the leaves at 500–1000 conidia per leaf obtained from naturally infected glasshouse-grown cucumber plants. The number of colonies was counted from the second leaf by adding the number of plants (three replicates of 10 plants per treatment). Bars represent standard error of the means. Pictures are symptoms of development of powdery mildew colonies in the leaf of water-applied (control) and INA-applied cucumber plants 10 days after inoculation.

**Figure 2.** Changes in gel activities of superoxide dismutase (SOD) and peroxidase (POD) in the leaves of cucumber plants. Proteins were extracted from leaves applied with water as a control and 0.01 mM INA containing 0.01% Tween 20 as surfactant. Electrophoresis was conducted using a 10% polyacrylamide slab gel without SDS.

**Figure 3.** Time courses of SOD and POD specific activities in the leaves of control and INA-applied cucumber plants. Proteins were extracted from leaves applied with water as a control (●) and 0.01 mM INA (○) containing 0.01% Tween-20 as surfactant. SOD activities are defined as unit per mg protein. One unit of SOD activity is defined as the amount required to inhibit the photoreduction of NBT by 50%. POD activities are defined as μmole guaiacol oxidized per min per mg protein. Data are means of nine replicates, and bars indicate standard error of the means.
increased rapidly in the leaves of INA-applied plants 3 days after foliar application, and were maintained at high level for 15 days. The specific activity of SOD and POD 3 days after foliar application of INA was higher as 145% and 329% than those in leaves of control plants, respectively. However, specific activity of SOD and POD in the leaves of control plants was increased gradually during 6 days following fungal inoculation. The levels of specific activity in the leaves of control plants increased significantly at 9 days with the development of colonies of powdery mildew after fungal inoculation. However, it did not reach the level of specific activity in the leaves of INA-applied plants during the whole time course.

Activation of gel and specific activities of β-1,3-glucanase

Isozyme banding patterns of β-1,3-glucanase were detected in the leaves of control and INA-applied plants by using native PAGE gels (Fig. 4). Only one β-1,3-glucanase isoform was detected in the leaves of INA-applied plants. At the beginning of incubation in boiling water for staining, two or three minor red bands appeared in the leaves of control and INA-applied plants. However, we could not take a photograph because it disappeared with time lapsed for staining of the major band. The time course of β-1,3-glucanase specific activities was monitored over a 15 days period after foliar application (Fig. 5). Induction of β-1,3-glucanase activities in the leaves of INA-applied plants was significantly different from that in the leaves of control plants. In the leaves of control plants, β-1,3-glucanase levels remained constant for 6 days, and then increased slightly over 9 days when powdery mildew developed. However, β-1,3-glucanase levels were abruptly increased 2.2 times, to 5.82 μkat per mg protein 3 days after foliar application of INA. Six days after foliar application, specific activity in the leaves of INA-applied plants was increased to 8.43 μkat per mg protein, but no significant differences were observed in the following days.

Effects of exogenous H2O2 and DPI on the restriction of powdery mildew and activation of SOD, POD, and β-1,3-glucanase

The number of colonies on the leaves of control plants was rapidly increased to 201 colonies per leaf 15 d after inoculation. However, the colonies of powdery mildew spread slowly on the leaves of plants that were foliar applied with exogenous H2O2 or INA before inoculation (Fig. 6). The number of colonies on the leaves of exogenous H2O2-applied and INA-applied cucumber plants was reduced by 42% and 92% as 117 and 16 colonies per leaf compared with that on the leaves of control plants, respectively. The effectiveness of restriction on the development of powdery mildew colonies by INA were reduced by DPI, a specific inhibitor of oxidative burst. When the plants were applied with INA supplemented with DPI, the number of colonies was increased to 3.6 times, as 58 colonies per leaf, compared with that on the leaf of INA-applied plants.

Figure 7 shows the changes in gel activities of SOD, POD, and β-1,3-glucanase in the leaves of exogenous H2O2-applied and INA-applied plants. The gel activity of SOD and POD significantly increased in the leaves of exogenous H2O2-applied plants during the 4 days after foliar application. However, gel activity of SOD and POD decreased gradually throughout the time after foliar application. The β-1,3-glucanase isoform was not

Fig. 4. The β-1,3-glucanase isozyme-banding patterns (red arrow) in the leaves of control and INA-applied cucumber plants. Proteins were extracted from leaves applied with water (control) and 0.01 mM INA containing Tween-20 as surfactant. Electrophoresis was conducted using a 10% polyacrylamide slab gel without SDS.

Fig. 5. Time course of β-1,3-glucanase specific activities in the leaves of control and INA-applied cucumber plants. Proteins were extracted from leaves applied with water as a control (●) and 0.01 mM INA (○) containing 0.01% Tween-20 as surfactant. Activities are defined as μkat per mg proteins. A katal was defined as the enzyme activity catalyzing the formation of one mole of glucose equivalents per second. Data are means of nine replicates, and bars indicate standard error of the means.
detected in the leaves of exogenous H$_2$O$_2$-applied plants. The effects of DPI, a specific inhibitor of oxidative burst, on the activation of AOS-mediated enzymes in responses to foliar applied INA were investigated. The activation of SOD and POD gel activity were reduced significantly by DPI (Fig. 8). However, gel activity of SOD and POD increased slowly throughout the time after foliar application. The β-1,3-glucanase isoform was not detected in the leaves until 6 days after foliar application of INA with DPI but was detected ambiguously 6 days after foliar application.

Figure 9 shows the time course of POD specific activity in the leaves of exogenous H$_2$O$_2$-applied and
INA-applied (with or without DPI) cucumber plants. The specific activity in the leaves of INA-applied plants was abruptly increased 2 d after foliar application. The activation of specific activities by INA was significantly reduced by DPI. Although specific activities in the leaves of DPI-applied plants were increased gradually throughout the time after foliar application, but it did not reach the level of activity in the leaves of INA-applied plants during whole time. The specific activities in leaves of H$_2$O$_2$-applied plants increased abruptly during the first 2 days after foliar application; it then declined slowly.

**Discussion**

This study confirms that INA induces resistance against infection with powdery mildew in cucumber plants. Disease symptoms appeared 3 days after fungal inoculation, and colony development was strikingly increased in the leaves of control plants. However, the development of powdery mildew colonies was significantly reduced in the leaves of INA-applied plants before fungal inoculation (Fig. 1). Various plant defense activators, such as ASM, BABA and INA have been reported to act in defense-activating systems and parts of biological activation of resistance (Cools and Ishii, 2002; Narusaka et al., 1999; Soylu et al., 2003). It was suggested that these defense activators may be alternative to agricultural chemicals in controlling plant diseases. The INA was the first synthetic compound that was shown to activate SAR with the development of a broad spectrum of resistance in plants against pathogens (Metrax et al., 1991). Earlier work has been studied on the induction patterns of resistance against powdery mildew by synthetic INA in cucumber plants (Hijwegen and Verhaar, 1994). This study shows that effectiveness of INA on the induction of resistance to powdery mildew was higher on partially resistant cultivars than on a susceptible cultivar. Plant defense activators may have a phytotoxic effect on the host plants because of excessive generation of oxidative burst (Conrath et al., 2000). Foliar application of BABA at higher concentrations induced necrosis in tomato (Cohen and Gisi, 1994). Kauss and Jeblick (1995) reported that pre-treatment with INA or SA in parsley augmented the induction of the elicitor of early oxidative burst. This phytotoxicity was accompanied by the formation of AOS, lipid peroxidation, and accumulation of SA in the leaves (Siegrist et al., 2000). In our study, the foliar application of 0.1 mM INA showed a greater restriction of powdery mildew than the foliar application of 0.01 mM INA (data not shown). However, foliar application concentrations higher than 0.01 mM INA induced damages such as tip burn and necrosis on cucumber leaves. The water-soluble protein and chlorophyll contents in the leaves applied with 0.1 mM INA were lower (23.1% and 31.0%, respectively) than that of foliar application of 0.01 mM INA (data not shown).

In this study, we observed a significant increase in gel and specific activities of SOD and POD in the leaves of INA-applied plants compared with the leaves of control plants (Figs. 2 and 3). Earlier works have been carried out on the induction of SOD activity during plant infection by several pathogens in horticultural crops (Edreva et al., 1991; Daza et al., 1993). These studies showed that SOD activity was significantly increased in a resistant genotype upon inoculation with pathogens. Yun et al. (1995) also reported that SOD activity was higher in downy mildew-resistant genotypes of cucumber than in susceptible ones. SODs are metalloenzymes that are found in almost all organisms, catalyzing the dismutation of superoxide radicals to H$_2$O$_2$ and molecular oxygen (Bowler et al., 1992). Tenhaken et al. (1995) reported that the enhanced SOD activity might increase oxidative stress due to increased production of H$_2$O$_2$. Hydrogen peroxide has a part not only as a local signal for triggering hypersensitive reaction but also as a diffusible signal for the induction of defense genes (Alvarez et al., 1998). PODs are oxidoreductive enzymes that participate in various physiological processes such as auxin catabolism (Lagrimini et al., 1997), senescence (Abeles et al., 1988), suberization (Espelie et al., 1986), lignification (Whetten et al., 1998), linking of cell wall structural proteins (Fry, 1986), and defense gene activation against pathogens (Chittoor et al., 1999). The specific pathogen-inducible POD has been reportedly induced by infection with pathogens and was categorized into a family of PR proteins (Harrison et al., 1995; Hiraga et al., 2000; Rasmussen et al., 1995; Vera et al., 1993). Many studies have reported that POD was involved in defense mechanisms and that it played important roles against pathogen infection. The crucial roles of POD in defense mechanisms included reinforcement of cell wall barriers and enhanced production of AOS as antimicrobial compounds or signal mediators (Wojtaszek, 1997).

Plants can induce SAR to become more resistant to diseases through various biotic or abiotic agents. The development of SAR is accompanied by the synthesis of PR proteins such as β-1,3-glucanase and chitinase (Seung and Hwang, 1996). There have been reports on several plant defense activators, such as ASM, BABA and INA, not only inducing resistance in plants against pathogens but also eliciting the expression of PR proteins (Cohen et al., 1994; Ziadi et al., 2001). An expression of β-1,3-glucanase was observed in the leaves of INA-applied plants, but, not in the leaves of control plants (Fig. 4). There are also significant increases in specific activity of β-1,3-glucanase in the leaves of INA-applied plants compared with leaves of control plants (Fig. 5). Similarly, it has been reported that induced resistance by application of INA in cucumber plants was associated with increased activity of β-1,3-glucanase and chitinase (Siegrist et al., 1994). It has likewise been suggested that SA was required for the expression of PR proteins
in SAR systems by using plant defense activators (Ryals et al., 1996). Kawano and Muto (2000) reported that the accumulation of SA induced the generation of superoxide anion, and that the action of SA in SAR is mediated through the production of AOS. One of the early responses to the activation of defense systems against pathogens is a burst of oxidative mechanisms that leads to the rapid generation of AOS, such as superoxide and H$_2$O$_2$ (Lamb and Dixon, 1997). To understand the role of AOS-mediated enzymes in restriction of powdery mildew infection, effects of exogenous H$_2$O$_2$ and DPI, a specific inhibitor of oxidative burst, on the activation of AOS-mediated enzymes was investigated. The number of colonies on the leaf of exogenous H$_2$O$_2$-applied plants was significantly reduced to 47% compared with that on leaf of control plants (Fig. 6). The gel and specific activities of SOD and POD were also increased by exogenously applied H$_2$O$_2$ (Figs. 7 and 9). A differential response in the leaves of H$_2$O$_2$-applied and INA-applied cucumber plants was observed in the expression of β-1,3-glucanase isozyme. An activation of POD and SOD by foliar application of INA was correlated to express the β-1,3-glucanase. Although the foliar application of exogenous H$_2$O$_2$ increased the specific activity of SOD and POD, it did not express β-1,3-glucanase. Similarly, it was reported that direct application of H$_2$O$_2$ failed to induce SAR, but plant cells rapidly metabolized exogenous H$_2$O$_2$ by activation of POD (Neuenschwander et al., 1995). An activation of SOD and POD in the leaves of INA-applied plants suggests that oxidative burst is generated endogenously, and then acts as a signal mediator to trigger the expression of defense genes. The activation of SOD and POD enzymes was significantly suppressed as a result of DPI supplement (Figs. 8 and 9). This result implies the possibility that blocking of oxidative burst, as a result of DPI supplement, may suppress the activity of antioxidant enzymes, and then reduced effects on the restriction of powdery mildew colonies. The DPI is known to be a specific inhibitor of oxidative burst in the NADPH oxidase catalyzed AOS generation system (Kawano, 2003). We also detected that the expression of β-1,3-glucanase by foliar application of INA was restricted by DPI supplement (Fig. 8). However, it was expressed past the time, 6 days after foliar application of INA with DPI. This result suggested that AOS acts as a signal factor for the expression of β-1,3-glucanase in cucumber leaves. Lee and Hwang (2005) reported that the co-infiltration of DPI with a virulent pathogen markedly inhibited POD activity as well as H$_2$O$_2$ accumulation. They concluded that the reduction of the H$_2$O$_2$ levels, as a result of DPI application, suppressed the transcription of defense-related genes. It has been well documented that the development of SAR systems in plants was associated with an oxidative burst, such as the generation of superoxide and subsequent accumulation of H$_2$O$_2$ (Alvarez et al., 1998). It is not yet known whether H$_2$O$_2$ is the primary signal mediator or whether its interactions with other intermediates such as SA and jasmonic acid induce the SAR genes (Lee and Hwang, 2005). Orazco-Cardenas et al. (2001) have demonstrated that exogenously applied H$_2$O$_2$ might have a role as a second messenger mediating the expression of various SAR genes in tomato plants.

Although foliar application of exogenous H$_2$O$_2$ does not play to induce β-1,3-glucanase, it was affects on the activation of AOS-mediated enzymes and restriction of powdery mildew infection. The reduction of effectiveness of restriction of powdery mildew by inhibitor of oxidative burst may reflect the critical role of AOS in contributing to the antifungal activity by activation of AOS-mediated enzymes.

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