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Expression Analysis of *Oryza sativa* Ascorbate Peroxidase 1 (*OsAPx1*) in Response to Different Phytohormones and Pathogens

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We have isolated and characterized an ascorbate peroxidase (*APx*) gene, *OsAPx1* from rice. Northern and Western blot analyses indicated that at young seedling stage, *OsAPx1* mRNA was expressed highly in root, shoot apical meristem (SAM) and leaf sheath than leaf. In mature plant, *OsAPx1* gene expressed highly in root, stem and flower but weakly in leaf. *OsAPx1* gene and protein expression level was induced in leaves inoculated with *Magnaporthe oryzae (M. oryzae)* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Phytohormones treatment showed that *OsAPx1* was up-regulated by jasmonic acid (JA), but was down regulated by ABA and SA co-treatments with JA, resulting that they have antagonistic effect on pathogen responsive *OsAPx1* expression. Phylogenetic analysis illustrated that Arabidopsis AtAPx1 has a close relationship with OsAPx1. In *AtAPx1* knock out lines, the accumulation of O_2^- and H_2O_2 are all highly detected than wild type, revealing that the high concentration of exogenous H_2O_2 cause the intercellular superoxide anion and hydrogen peroxide accumulation in *AtAPx1* knockout plant. These results suggested that *OsAPx1* gene may be associated with the pathogen defense cascades as the mediator for balancing redox state by acting ROS scavenger and is associated with response to the pathogen defense via Jasmonic acid signaling pathway.

Key words : Ascorbate peroxidase, phytohormone, ROS, shoot apical meristem

Introduction

Reactive oxygen species (ROS), which are formed from excitation or incomplete reduction of molecular oxygen, are unwelcome harmful by-products of normal cellular metabolism in aerobic organisms [13]. These partially reduced or activated derivatives of oxygen, such as singlet oxygen, superoxide anions, hydrogen peroxide and hydroxyl radical, are highly reactive and toxic not only to mammals, but also to the other life forms including plants, and can lead to the oxidative destruction of cells [2]. H_2O_2 is a major ROS which is generated in cells by the direct transfer of two electrons to the superoxide anion [8]. During the perception process of abiotic and biotic stresses, the rate of cellular H_2O_2 production is enhanced dramatically as a consequence of superoxide dismutase activation, which catalyze the conversion of O_2^- to H_2O_2, and also activates the NADPH-dependent oxidase system [5, 15].

In plant cells, ROS overproduction is induced by biotic and abiotic stresses, such as salt, drought, high light, pathogen, etc. To avoid the toxicity of ROS, aerobic cells are provided with a flexible set of enzymes and metabolites involved in ROS catabolism, which often acts at the site of ROS production [12]. These enzymes and metabolites maintain the homeostasis of ROS in plant cells. Major ROS-scavenging enzymes in plants include superoxide dismutase (SOD), ascorbate peroxidase (APx), catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (PrxR). Together with the antixoidants ascorbic acid and glutathione, these enzymes provide cells with highly efficient machinery for detoxifying O_2^- and H_2O_2. The ROS network of these various scavenging enzymes can be found in almost every subcellular compartment. A particular ROS signal can be scavenged by more than one enzymatic scavenger in each of the different compartments [16].

Ascorbate peroxidase (APx) exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher

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plants. APx utilizes ascorbate (ASC) as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodyehydroascorbate (MDHAR), a univalent oxidant of ASC. In ascorbate-glutathione cycle, APx functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms [3]. Recent studies showed that APx isoenzymes subjected to several environmental stresses such as ozone, high light, and extremes of temperature, salt, and paraquat. In *Arabidopsis* and Tobacco, APx was reported plays a key role in elevating H_2O_2 and results in enhanced cell death in response to pathogen attack. The regulation of APx may play an essential part of regulation of terminal and beneficial roles of H_2O_2 in plant cell. Nitric oxygen is also a dose-dependent inhibitor of APx during the process of programmed cell death (PCD) which also can influence the H_2O_2 cellular levels [6].

In rice genome, eight different ascorbate peroxidase isoforms were already characterized by southern blotting analyses (*OsAPx1* - *OsAPx8*) [23]. Two of them are cytosolic, two are putative peroxisomal, while four are putative chloroplastic. *OsAPx1* and *OsAPx2* are involved in pathogen response and growth, and plant hormones which trigger the defense/stress phenomenon [1]. Previously, based on the 2-DE-MS results, we identified 21 different oxygen detoxifying proteins in the roots of rice [18]. We demonstrated that *OsAPx1* is involved in H_2O_2 scavenging system. In this study, the infection of *Magnaporthe oryzae* (*M. oryzae*) fungal elicitor, *M. oryzae*, and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and phytohormone illuminated that *OsAPx1* is associated with response to the pathogen defense via Jasmonic acid signaling pathway.

Materials and Methods

Plants material and growth conditions

Mature rice seeds (*Oryza sativa L.*) were obtained from the National Gyeongnam Agricultural Experimentation Station. The dehulled seeds were sterilized in 70% ethanol for 10 min and then in 3% sodium hypochlorite for 20 min. To induce the callus formation, sterilized seeds were placed in R2 medium and allowed to culture in the dark at 25°C. For raising the rice seedlings, the dehulled seeds were put onto the Kimwipe paper with water in a plant culture dish, and kept in the light chambers for 3 days for germination. Germinated rice seedlings were then shifted to the greenhouse for further growth. Fourth- and fifth-leaf stage rice seedlings grown under natural light conditions in a greenhouse (20-30°C) were inoculated with the rice blast fungus, *M. oryzae*.

Arabidopsis thaliana plants were grown in growth chambers under controlled conditions: 21-22℃, with 18 hr or constant light cycle, 100 μmol m^{-2} sec^{-1}, and a relative humidity of 70%. Knockout *Arabidopsis* plants containing a T-DNA insert in *Apx1* was obtained from the *Arabidopsis* knockout facility at the Arabidopsis Biological Resource Center (ABRC) and confirmed according to the knockout facility recommended protocols (http://www. arabidopsis.org/) using the following DNA Primers: LP 5′-ATTATAAGCCAGAGCC-ATCCG-3′ and RP 5′-AACTCTTGAGCGGAGAGAAGG-3′. The plants were selected to obtain a pure homozygote line.

Chemical treatment

The calli were treated with 50 μg/ml fungal elicitor, 250 μM Jasmonic acid (JA), 5 mM Salicylic acid (SA), or 200 μM abscisic acid (ABA), and harvested at 24 and 48 hr after treatment [20]. RNA was extracted from whole plant after 3 and 6 days of treatments.

Infection with rice blast fungus and rice blight bacteria

Two races of rice blast fungus, *M.* oryzae races KJ401, were used for infecting the rice plants. KJ401 is incompatible with the Jinheung cultivar. Conidia were spread on rice polish agar medium (25 g of rice polish, 20 g of agar/1 l H_2O) and grown in the dark at 28°C for 3 days. For conidia production, the plates were incubated at 28°C for 3－4 more days under fluorescent light, after removing aerial mycelia with a sterile loop. The conidia were collected by agitating the cultures with distilled H_2O containing 0.02% Tween 20, filtered through two layers of Kimwipe to remove aerial mycelia and cell debris and washed twice [20]. The rice calli (ca. 500 mg fresh weight) were suspended in 5 ml of R2 medium in 6-well plates, and inoculated with suspensions of conidia of each race to a final concentration of $1{\times}10^5$ conidia/ml. To prepare the fungal elicitor, mycelia of rice blast fungus (race KJ401), which is avirulent to the rice cultivar Jinheung, were used. The mycelia were chopped, homogenized in 50 mM sodium acetate buffer (pH 4.5), sonicated, and centrifuged. The pellet was homogenized again in 0.1 M borate buffer, pH 8.8, and autoclaved, and the supernatant was extensively dialyzed against distilled water at 4°C and freeze-dried. Reducing sugar was determined by

the dinitrosalicylic acid method using glucose as standard [9, 10].

Northern blot analysis

Total RNA was isolated from rice leaves or suspension cells. RNA was separated on formaldehyde -denaturing agarose gel, and blotted onto nylon membranes. Each 20 μg RNA sample blotted onto a nylon membrane was hybridized with [α-^{32}P]dCTP labeled *OsAPx1*gene probe using the Prime-a-Gene labeling system (Promega, Madison, USA). Hybridization and washing conditions were as described for Southern blot analysis [21]. Equal loading of RNA were verified by intensity of rRNA staining by Ethidium bromide stain.

Semi-quantitative RT-PCR

RNA isolation and first-strand cDNA synthesis was carried out as mentioned above. A mixture comprising 500 ng mRNA, 500 ng Oligo dT18, and 500 μM dNTP was prepared at 65°C and chilled on ice. To the mixture, the following reagents were added to the respective final concentrations: 1× First-strand buffer, 5 mM Dithiothreitol, 2 units RNaseOUT, and 10 units SuperScript II RT enzyme. The mixture was incubated at 50°C for 60 min and inactivated at 70°C for 15 min. PCR was performed on a PTC-0220 PCR machine (MJ Research, Waltham, MA, USA), using synthesized cDNA as template. Gene-specific primers were designed from the coding sequence of the desired gene (Forward primer, 5′-CTTGAGTGATCAGGACATTG-3′, and Reverse primer, 5′-AGCAGTAGTAGACTAGAAACCTCT-3′). An equal amount of cDNA was used for each PCR reaction by normalization to the actin gene (Forward primer, 5′-AGGAATGGAAGCTGCGGGTAT-3′ and Reverse primer, 5′-GCAGGAGGACGGCGATAACA-3′).

Protein extraction and Western blot

Control and treated samples were harvested and pulverized in liquid nitrogen. Samples were then homogenized with protein extraction buffer (0.5 M Tris-Cl (pH 8.3), 2% (v/v) NP-40, 20 mM $MgCl_2$, 1 mM phenyl methyl sulfonyl fluoride, 2% (v/v) β-mercaptoethanol) and proteins were extracted using phenol-methanolic ammonium acetate precipitation as described previously [20].

For Western blotting, proteins (20 μg) were resolved on SDS-PAGE, and transferred to a Polyvinylidene fluoride (PVDF) membrane using a semidry electrophoretic apparatus (Hoefer, Holliston, MA). Protein detection was carried out using anti-Apx1, anti-CuZnSOD (1:1,000) and anti-Rubisco large subunit (1:5,000) as primary antibodies [20] and anti-rabbit (1:10,000) conjugated with horseradish peroxidase as secondary antibodies.

Detection of H_2O_2 and O_2^- with DAB and NBT stain

Leaves from both wild type and APx1 knockout mutant *Arabidopsis* were put into 10 mM H_2O_2 for 4 hr. Leaves were put into pH 3.8 ddH_2O with 0.05% tween-20, and infiltrated for 5 min under vacuum. For 3,3′-Diaminobenzidine (DAB) staining, leaves were infiltrated with 1mg/ml DAB solution at 28°C for 4 hr, and cleared by 10% lactic acid for 4 hr. To remove the chlorophylls, the stained samples were transferred to 70% ethanol, and incubated at 70°C for 10 min. For nitro blue tetrazolium (NBT) staining, leaves were infiltrated with 0.1% NBT solution at room temperature for 2 hr, and washed with 70% ethanol for 4hr.

Results and Discussion

Expression pattern in rice tissues

For analyzing the tissue specific expression of *OsAPx1* in rice, total RNA was extracted from different tissues of either rice seedlings or mature plants, including root (R), leaf (L), stem (S), flower (F), short apical meristem (SAM) and leaf sheath (SH). At young seedling stage, *OsAPx1* mRNA was highly expressed in root, SAM and leaf sheath, but expressed low in leaf (Fig. 1A). In mature plants, a higher level expression of *OsAPx1* was observed in root, stem and flower but weakly in rice leaf (Fig. 1B). Western blot analysis revealed that accumulation pattern of *OsAPx1* was consistent with that of RNA level (Fig. 1C). Apx is an important ROS scavenger which maintains a particular level of ROS. ROS are continuously produced during different stages of plant growth and development. Here, we also observed the transcriptional and translational accumulation of *OsAPx1* protein in different developmental organs of rice which may help in maintaining the ROS levels in the cells.

Interestingly, a previous report categorized many ROS-related proteins as belonging to the defense/stress group. It was also reported that ROS level was highly accumulated in the rice root and the growth was dramatically inhibited by ROS quenching chemicals which inhibited ROS generation in the rice root [18]. Thus, functions of APxs are to detoxify cytotoxic substrates and protect cells against oxida-

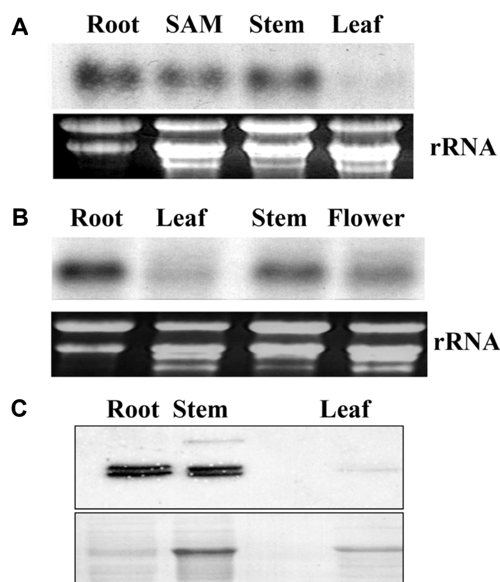

Fig. 1. Northern blot analysis of *OsAPx1* expression in different rice tissues. Total RNA were extracted form root, shoot apical meristem and leaf sheath at two leaf stage. (B) Total RNA were extracted form root, leaf, stem and flower at mature stage. (Each RNA sample (20 µg) was blotted onto nylon membranes, hybridized with ^{32}P-labeled probes, and washed at high stringency. Equal loading was verified by staining rRNA with ethidium bromide. Blots were exposed to X-ray film at -70℃ for 1 day. (C) Western blot analysis of *OsAPx1* in the leaf, stem and root tissues of rice.

tive damage in the rice root system.

Semi quantitative RT-PCR and Western blot based validation of *OsAPx1* expression

Here we characterized several proteins involved in redox regulation including *OsAPx1* and cupper/zinc- superoxide dismutase (*Cu/Zn-SOD*) were characterized in response to *Xoo* and *M. oryzae* infection. Transcription of *OsApx*1 and *Cu/Zn-SOD* were detected at 12 hpi, and increased further at 48 and 72 hpi (Fig. 2A and 2C), indicated that *OsApx*1 and *Cu/Zn-SOD* was induced in response to *Xoo* and *M. oryzae* infections. These results were further validated by Western blot analysis. The secretion of APx1 and Cu/Zn-SOD proteins were detected at 24 hpi, and were further increased at 48 hpi and 72 hpi (Fig. 2B and 2D). The intracellular protein RuBisCO was used for loading control.

In plant cells, ROS formation is induced by various factors including different biotic and abiotic stress [4, 17]. It was also reported that the ROS related-proteins including 7 glutathione S-transferases, 6 ascorbate peroxidases, 4 catalase isozymes, 3 superoxide dismutase, glutathione (GSH)-dependent dehydroascorbate reductase 1, and glutathione reductase comprised the most abundant group in root proteome [18]. These data suggest that ROS-scavenging activities may differentially regulate protein expression levels and patterns because GSH, ascorbate and diphenyleneiodonium (DPI), which were ROS quenching chemicals, have other cellular functions in addition to ROS scavenging. Thus, during plant-microbe interaction, OsAPx plays a critical role for homeostasis of ROS levels in leaves inoculated with pathogens.

Induction of *OsAPx1* gene by JA

In plant cells, biotic stress and different stress signals such as JA can induce ROS overproduction. JA is a phytohormone which is produced in response to pathogen attack. Here, we observed that the *OsAPx1* mRNA was strongly upregulated in response to JA treatment. When JA treatment was given along with either SA or ABA, a reduction in its m-RNA expression was observed. Moreover, when SA and ABA were applied together, *OsAPx1* transcription was completely inhibited (Fig. 3).

SA and JA, produced during pathogen attack, act as signaling molecules which lead to the transcriptional induction of several PR genes [14, 24]. Detailed analysis of the SA and JA induced signaling has shown a crosstalk between these two signaling pathways each other and fungal elicitors in previous studies [7, 11]. Our results showed plant hormones also have great effect on *OsAPX1* expression. Its mRNA was strongly upregulated by the presence of JA, but significantly reduced by SA and ABA. Both SA and ABA reduced the transcription level of *OsAPx1*, and also restricted the JA induced *OsAPx1* gene expression. It has been reported that increased concentrations of H_2O_2 have beneficial effects on plant defense. Several reports have suggested that pathogen attack leads to the accumulation of H_2O_2 which results in cell death (hypersensitive response, HR). Therefore, SA and ABA based inhibition of *OsAPx1* may be beneficial during pathogen attack as it would lead to the increased concentration of H_2O_2 by reducing the expression of redox detoxifying enzymes including APx1. However, which pathways and cross-talk regulate the *OsAPx1* expression is still unknown.

Phylogenetic relationship analysis and characterization of AtAPx1 knockout plant

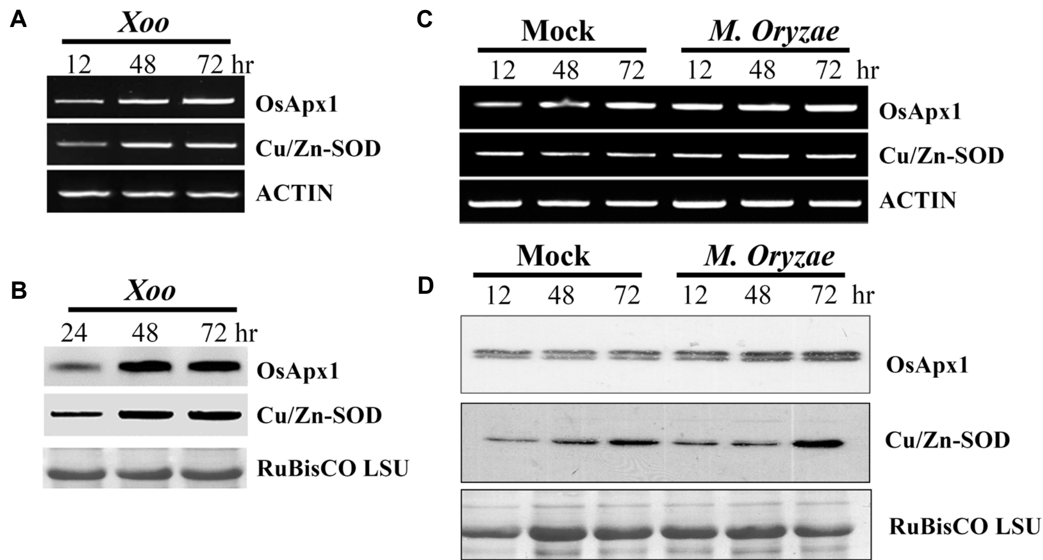

Fig. 2. Valiation of expression patterns of *OsAPX1* in leaves inoculated with *Xoo* and with *M. oryzae*. (A) Semiquantitative analysis of Apx1 and Cu/Zn-SOD transcriptional regulation in response to *X. oryzae* infection. (B) Western blot analysis of secreted Apx1 and Cu/Zn-SOD level in response to *X. oryzae* infection. (C) Semiquantitative analysis of Apx1 and Cu/Zn-SOD transcriptional regulation in response to *M. oryzae* infection. (D) Western blot analysis of secreted Apx1 and Cu/Zn-SOD level in response to *M. oryzae* infection.

To understand the phylogenetic relationship among ascorbate peroxidases of Rice and *Arabidopsis*, APx protein sequences of 9 isoforms of rice and 8 isoforms of *Arabidopsis* were aligned by Bio-Edit software. The phylogenetic tree showed that *OsAPx1* protein has the close relationship with AtAPx1 (Fig. 4A), and shares 79.3% homology with each other. To further understand the function of *APx* gene, we studied the function of ascorbate peroxidase gene in *Arabidopsis* along with the rice. Therefore, we identified an *AtAPx1* knockout line (*apx1*) from the SALK collection, containing T-DNA insertions in the *AtAPx1* gene. We named it *apx1*. The insertion is located in the 3′-UTR in *apx1*. We use PCR method which described in 2.1 to selected homology apx1 plant. Western blot analysis showed that the expression of *OsAPx1* gene in these apx1 plants was eliminated (data not shown).

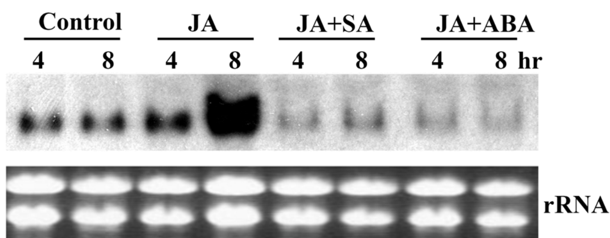

Fig. 3. Northern blot analyses of *OsAPx1* gene in treatment of plant hormones. Total RNA was extracted from rice callus after different plant hormone treatment which described in pictures. RNA was extracted. Each RNA sample (20 μg) was blotted onto nylon membranes, hybridized with ^{32}P-labeled probes, and washed at high stringency. Equal loading was verified by staining rRNA with ethidium bromide. Blots were exposed to X-ray film at －70℃ for 1 day. Abbreviations: ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid.

H_2O_2 treatment and Superoxide anion, hydrogen peroxide detection

Wild type and *apx1* seeds were planted on the Phytagel solid MS medium contains 2.5 mM H_2O_2. H_2O_2 showed an inhibitory effect on the growth of both wild type and *apx1* lines were. However, the degree of inhibition was higher in the *apx1* lines as compared with the wild type (Fig. 4B). The effect of H_2O_2 was further tested on the mature plants. The wild type and *apx1* root were dip into MS medium which contains 10 mM H_2O_2. The *apx1* lines showed early senescence just after 3 days treatment with H_2O_2 (Fig. 4B). H_2O_2 not only acts as a signal for biosynthesis and development, but it also acts as a signal for PCD when its concentration is increased over a threshold value. Therefore, we tried to examine whether the growth inhibit and early senescence is caused by the oxidative damage to cell. To detect the accumulation of O_2^- and H_2O_2 levels in Arabidopsis leaves, we stained it with NBT and DAB. The Arabidopsis

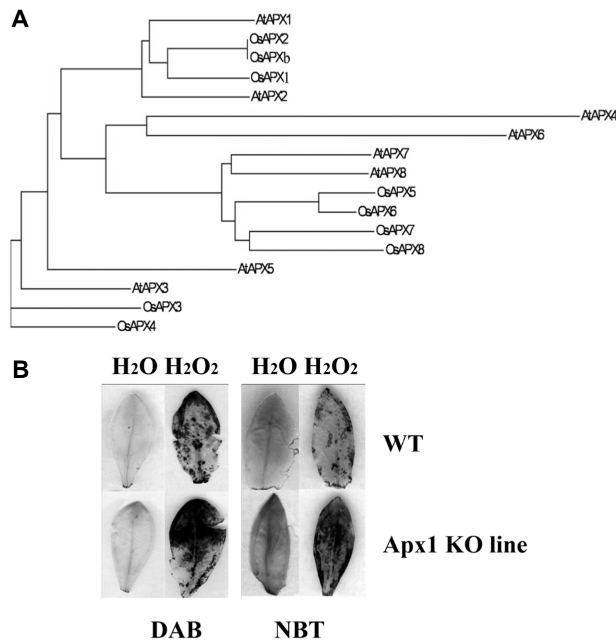

Fig. 4. Phylogenetic relationship of APx protein from Rice and Arabidopsis and Detection of O^{2-} and H_2O_2 in *Arabidopsis* leaves (A) The dendrogram was constructed using BioEdit program. Bootstrap values are shown at the branch. Protein sequences were identified by the accession numbers in the NCBI database. (B) Wild type and *apx1* line were infiltrated with 10mM H_2O_2. After 4 hr, plant leaves were stained with nitro blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) solution, respectively, for 2 hr. Leaves was washed with 98% ethanol at 70℃ for 10 min, and then washed with 10% Lactic Acid or 70% ethanol.

leaves were infiltrated with 10 mM H_2O_2 for 4 hr, then stained and washed as described in Materials and methods section. In *apx1* lines, the accumulation of both O_2^- and H_2O_2 were higher than the wild type (Fig. 4B). These results indicated that the high concentration of exogenous H_2O_2 cause the intercellular superoxide anion and hydrogen peroxide accumulation in *AtAPx1* knockout plant, and also lead to growth suppression and early senescence than wild type. Therefore, ascorbate peroxidase may have the function of scavenging H_2O_2 to reduce the oxidative damage of cell.

Conclusion

The fact that *OsAPx1* expression is activated both by pathogens (*M. oryzae* and *Xoo*) and JA raised the possibility that signal transduction leading to *OsAPx1* transcription occurs through a shared pathway. However, we first report that SA and ABA had antagonistic effects on pathogen responsive *OsAPx1* expression. In a similar way, PR genes including *OsGlu1*, *OsGlu2*, *OsTLP*, *OsRLK*, and *OsPR-10* transcription is induced by JA but inhibited by SA and ABA [19]. Taken together, these results suggest that the induction of *OsAPx* is differentially regulated by the signaling molecules, that is, positively by JA and negatively by SA and ABA.

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초록 : 벼 ascobate peroxidase 단백질의 병원균 및 식물호르몬에 대한 발현 분석

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본 논문에서 벼 ascorbate peroxidase (*OsAPx1*) 유전자의 발현 분석을 Northern과 Western 분석을 통하여 유묘에서는 뿌리, 정단분열조직(shoot apical meristem, SAM), 잎 보다는 잎집에서 더 많이 발현되는 것을 확인하였다. 성숙된 조직에서는 *OsAPx1* 유전자가 잎을 제외하고는 뿌리, 줄기, 꽃에서 강하게 발현되었다. 또한 이 *OsAPx1* 유전자는 벼 곰팡이 병원균인 벼 도열병 및 세균성 병원균인 흰빛잎마름병에도 반응하였고 특히 흥미있게도 *OsAPx1* 유전자는 식물호르몬에 대해서 서로 다르게 발현 양상을 보였다. 이 유전자는 자스몬산(JA)에 대해서는 강한 발현을 보였지만 반대로 살리실산(SA) 및 ABA와 같이 처리된 세포에서는 강한 발현 억제를 보였다. 이는 이 유전자가 JA에는 반응하지만 SA와 ABA하고는 서로 길항작용을 하는 것으로 보인다. 근연관계분석을 통하여 *OsAPx1*유전자가 애기장대의 AtAPx1 와 거의 유사하여 AtAPx1 결손 라인을 가지고 표현형 조사를 실시하였다. 그 결과, 외부에서 H_2O_2를 처리하였을 때에 O_2^- 와 H_2O_2의 축적이 wild type과 비교하여 AtAPx1 결손 라인에서는 현저히 높았다. 따라서 본 연구를, 통하여 *OsAPx1* 유전자는 벼에서 산화 환원 균형 유지를 통하여 다양한 세포 분화발달 및 병원균 방어에도 관여하며 이 유전자의 발현은 JA의 신호전달에 의해서 매개되는 것으로 예상이 된다.