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Regulation of Ascorbate Peroxidase Gene Expression in Response to Stresses and Phytohormone in *Rehmannia glutinosa*

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ABSTRACT: Ascorbate peroxidase (APX) plays a crucial role in the detoxification of hydrogen peroxide. APX activity is maintained significantly higher in the paraquat-treated leaves of the paraquat-tolerant *Rehmannia glutinos*. This study was conducted to understand structural and regulatory characteristics of APX gene in *R. glutinosa*. A putative APX cDNA clone (*RgAPX1*) was isolated from a leaf cDNA library using a partially sequenced expressed sequence tag clone. *RgAPX1* is consisted of 1148 bp nucleotides and contains an open reading frame encoding a 250 amino acid-long polypeptide. Deduced RgAPX1 amino acid sequence shares higher sequence similarity to cytosolic APXs. *RgAPX1* expression was higher in the leaf than in the flower and root. Southern blot result indicates the presence of one or two *RgAPX1*-related genes in *R. glutinosa* genome. *RgAPX1* transcription was affected differentially by various stresses and phytohormone. The results indicate that *RgAPX1* is constitutively expressed in most tissues and its expression is modulated for the immediate and efficient detoxification of H_2O_2 under normal and stress conditions.

Keywords: ascorbate peroxidase (APX), oxidative stresses, paraquat, *Rehmannia glutinosa*

Rehmannia glutinosa (Gaertn.) Libosch. is a perennial herb belonging to the familly *Scrophulariacea*. It has been grown in Asian countries and its roots have been used for traditional medicinal purposes. Despite its long history of cultivation and utilization, little is known about the botanical and agronomical characteristics of the species. Recently, it has been reported that *R glutinosa* plants that have not been exposed to the non-selective herbicide paraquat (1,1'-dimethyl-4,4'-bipyridilium) show very high levels of tolerance to the herbicide (Kim & Chun, 1992; Chun *et al*, 1997).

Paraquat is widely used for broad-leaf weed control. It is a fast acting, non-selective compound which destroys tissues of green plants on contact and by translocation within the plant. Divalent paraquat cation produces free radicals in the presences of molecular oxygen in the chloroplast during photosynthesis. Free radicals combine with molecular oxygen to form reactive oxygen species (ROS; Dodge, 1994). These ROS bring about disruption of photosynthetic activity and cause peroxidation of the fatty acid side chains of membrane lipids, eventually leading to plant death (Scandalios, 1993).

Antioxidant enzyme levels are significantly higher in paraquat tolerant *R glutinosa* than in paraquat-susceptible soybean (Choi *et al*, 2004; Chun *et al*, 1997). Among the antioxidant enzymes, the level of ascorbate peroxidase (APX, EC 1.11.1.11) activity was most highly increased in paraquat-tolerant *R. glutinosa* (Choi *et al*, 2004). Increased antioxidant enzyme levels correlate with paraquat resistance in plant species, like *Lycopersicum* species (Thomas & Pratt, 1982), *Lolium perennae* (Harper & Harvey, 1978), *Conyza banariensis* (Shaalteil & Gressel, 1986), and *Pisum sativum* (Donahue *et al*, 1997).

APX isozymes are localized both in the organelles, including chloroplasts and mitochondria, and cytosol (Shigeoka *et al*, 2002). Chloroplastic APXs help prevent light-induced damage to photosynthesis systems by scavenging H_2O_2 which is finally detoxified to H_2O and O_2 without producing another ROS. Since H_2O_2 freely diffuses through membranes (Willekens *et al*., 1997), cytosolic H_2O_2-scavanging enzymes can also help protect chloroplasts and cytosolic membranes from harmful levels of H_2O_2.

In our previous comparative studies, we observed significantly higher total APX activity in *R glutinosa* over that in soybean without and with paraquat treatments. Thus, the objective of this study was to characterize the APX system in *R.glutinosa* at molecular levels by cloning APX genes and examining their regulatory patterns in response to various stresses and stress-related phytohormones.

MATERIALS AND METHODS

Plant materials, bacterial strains, enzymes and chemicals

R glutinosa (accession Kemsan) plants were grown in a greenhouse at Chonbuk National University, Korea. The

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plants were raised from the tubers harvested in previous growing season. Three-month-old leaves collected by cutting the upper part of petioles with a razor blade were used for the treatment of stresses and hormone. The leaves with petioles were immersed in the following sterile solutions for 24 h at 20 °C under light: H_2O, 0.1 mM paraquat, 5 mM H_2O_2, 5 mM ethephon, or 25 mg/ml yeast extract (Chung *et al*, 2003). For wounding treatments, the leaves were punched with fine pins and floated on the sterile water. For UV treatment, plants were irradiated under UV lamps at 1.35 μE/m^2/s for 2 h and returned to the dark glasshouse. The irradiated leaves were collected 12 h after irradiation (Chung *et al*, 2003). All tissue samples were frozen in liquid nitrogen and stored at -70 °C until used.

Escherichia coli strain XL1 Blue (Stratagene, USA), JM109 (Promega, USA) and Qiagen EZ (Qiagen, USA) were used for cloning according to the standard techniques (Sambrook & Russell, 2001). DNA modifying enzymes were purchased from TaKaRa (Japan) and Promega (USA), reagents for plant DNA and RNA extractions from MRC (USA), kits for plasmid DNA purifications from Bioneer (Korea) and other chemicals from Sigma (USA) unless otherwise indicated.

Cloning and DNA sequence analysis

A cDNA library of *R glutinosa* leaf was constructed using Lamda ZAP cDNA library construction Kit (Stratagene, USA) according to the manufacturer's instruction. Expressed sequence tags (ESTs) were generated by the random sequencing of the insert DNAs of the plasmids excised from the lamda cloning vector. An EST showing sequence homology to the known plant APXs was used as a probe to screen the library. An APX-positive clone was isolated by screening 5.6×10^9 *pfu* of the cDNA library, and was designated *RgAPX1* (GenBank accession number, AY462246). The insert DNA of the clone was subcloned into pBluscript SK(+) for nucleotide sequence determination. The nucleotide sequences of both strands of the insert DNA of *RgAPX1* were determined by the dideoxy chain termination method. Homology searches of databases were performed using the BLAST programs (Altschul *et al*, 1997) against DNA and protein sequences. Nucleotide and deduced amino acid sequence analyses were performed using DNASIS (Hitachi, USA) and the programs and databases offered by the National Center for Biotechnology Information (NIH, USA) and European Bioinformatics Institute (EBI, UK). Multiple sequence analysis was performed using the program AliBee (GeneBee, SU).

Southern and northern blot analysis

Genomic DNA was prepared from the leaves using the plant DNA extraction kit (DNA zol ES, MRC, USA) according to manufacturer's instruction. DNA concentration was estimated by subjecting samples to 0.8% agarose gel electrophoresis and staining with ethidium bromide. Staining intensities of the total DNA were compared visually with a DNA molecular weight marker. Southern blot analysis was carried out with genomic DNA as described (Sambrook & Russell, 2001). Genomic DNA digested to completion with the restriction enzymes, *Bgl* II, Spe I, *Nde* I , and *Hind* III was separated by agarose gel electrophoresis and transferred onto a nylon membrane (Hybond-N$^+$, Amersham, UK). Labeling and detection were conducted with AlkPhos Direct system (Amersham Pharmacia biotech, UK). Hybridization, washing, signal generation and detection were performed with a chemiluminescent system (CDP-*Star*, Amersham Pharmacia biotech, UK) (Cho *et al*., 2005).

Total RNA was extracted using the TRI reagent procedure (MRC, USA). For northern blot analysis, total RNA (20 μg) was denatured, separated on a 1% formaldhyde-gel, and transferred onto a nylon membrane (Hybond-N$^+$, Amersham, UK). The membrane was prehybridized for 1 h at 60 °C in the AlkPhos Direct hybridization buffer (500 mM NaCl, 0.4% blocking reagent) and incubated for 16 h at 60 °C after adding the labeled *RgAPX* probe in the prehybrization solution. After hybridization the membrane was washed and mRNA on the membrane was detected as in Southern blot.

RESULTS AND DISCUSSION

Sequence analysis of *RgAPX1*

A full-length cDNA clone encoding putative APX polypeptide (*RgAPX1*) was isolated and its nucleotide and deduced amino acid sequences are shown in Fig. 1. *RgAPX1* is consisted of 1148 bp nucleotides and the ORF encodes a 250 amino acid-long polypeptide. The deduced amino acid sequence of RgAPX1 contains the active site sequence and proximal heme-ligand motif at position 33 to 44, and 155 to 161, respectively. There is no predicted signal peptide at its N-terminus, indicting that the clone is for a cytosolic isoform

The deduced amino acid sequence encoded by *RgAPX1* shows significant homology and a close phylogenetic relationship to plant APX sequences, indicating that the cDNA isolated from *R glutinos* corresponds to an mRNA encoding an APX enzyme (Fig. 2). The deduced amino acid sequence of RgAPX1 shows 90% similarity to a hot pepper APX (*CaAPX1*) (Yoo *et al*., 2002) and higher similarity to the group I cytosolic APXs (Shigeoka *et al*., 2002). In plants, at least four different forms of APX have been found in the

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1    T CGG GAA ACC GCG CCA TTG TGT TGG TAC CCG GGA AAT TCG GCC TTA CGG CCG GGG AGC TCA CTG CTT GTG CTT TCC ACA CTC   82
83   GCA TTT TGA GGG TTT TAT TAT ATT TTA GTC CAT CTC CAC TTT TCC AAC TCT CTC TAT CTT CAG AAA GCC GTG AAA ATG GTG AAA  166
1                                                                                                   M  V  K     3
167  AAC TAT CCA ACT GTG AGT GAG GAG TAC CTG AAT GCC GTT GAG AAA TGC AAG AAG AAG CTC CGG GGT TTG ATC GCT GAA AAG AAC  250
4    N  Y  P  T  V  S  E  E  Y  L  N  A  V  E  K  C  K  K  K  L  R  G  L  I  A  E  K  N                      31
251  TGT GCT CCG ATC ATG CTC CGC CTT GCT TGG CAC TCT GCT GGT ACA TTC GAT CAA TGC AGC AAG ACT GGA GGT CCT TTT GGA ACC  334
32   C  A  P  I  M  L  R  L  A  W  H  S  A  G  T  F  D  Q  C  S  K  T  G  G  P  F  G  T                      59
335  ATG AGA TTC AAG GCT GAG CAG GGT CAT GCT GCT AAC AAT GGT CTT GAC ATT GCT CTT AGG CTC TTG CAG CCA ATC AGG GAG CAA  418
60   M  R  F  K  A  E  Q  G  H  A  A  N  N  G  L  D  I  A  L  R  L  L  Q  P  I  R  E  Q                      87
419  TTC CCT ATC CTT TCT CAT GCT GAC TTC TAT CAG TTG GCC GGA GTT GTC GCT GTT GAA GTT ACC GGA GGA CCT GAA GTT CCA TTC  502
88   F  P  I  L  S  H  A  D  F  Y  Q  L  A  G  V  V  A  V  E  V  T  G  G  P  E  V  P  F                      115
503  CAC CCA GGA AGA CCG GAC AAG GAG GAG CCA CCA GTT GAA GGT CGC TTG CCT GAT GCT ACC AAG GGG TCT GAC CAC CTG AGG GAT  586
116  H  P  G  R  P  D  K  E  E  P  P  V  E  G  R  L  P  D  A  T  K  G  S  D  H  L  R  D                      143
587  GTG TTT ACC AAA CAA ATG GGT TTG AGT GAC CAG GAC ATC GTT GCA CTC TCT GGT GCC CAC ACT CTG GGA AGA TGC CAC AAG GAA  670
144  V  F  T  K  Q  M  G  L  S  D  Q  D  I  V  A  L  S  G  A  H  T  L  G  R  C  H  K  E                      171
671  CGT TCC GGG TTT GAG GGA CCA TGG ACT GCA AAC CCT CTC ATC TTC GAC AAT TCT TAC TTT AAG GAG CTT CTG AGT GGA GAA AAA  754
172  R  S  G  F  E  G  P  W  T  A  N  P  L  I  F  D  N  S  Y  F  K  E  L  L  S  G  E  K                      199
755  GAA GGG CTT TTG CAG TTG CCA TCA GAC AAA GCT CTT CTT GCC GAC CCT TCA TTC CGC CCA CTT GTT GAG AAA TAT GCT GCG GAC  838
200  E  G  L  L  Q  L  P  S  D  K  A  L  L  A  D  P  S  F  R  P  L  V  E  K  Y  A  A  D                      227
839  GAG GAT GCC TTC TTT GCT GAT TAT GCA GAG GCT CAC TTG AAG CTC TCT GAG TTG GGA TTT GCT GAT GCT TAA GCC ACA GAG CGA  922
228  E  D  A  F  F  A  D  Y  A  E  A  H  L  K  L  S  E  L  G  F  A  D  A                                        250
923  TCT GCA TGA AGC AAA AGG ATT GAT GGC CGA TTT TTG TCA AAT TTA TTT AGT ATT TTT GGA AAA AAA CTT GTT GGT TGT GTT AAG 1006
1007 GCT TTA GTA CTT CTT TTC ATT GAT GTG TTG GAA CTT GGT TTT GTT GTT AAC CAG TAA AAC AAT ATA TGG TCA TCA TCA CCT TCC 1090
1091 AAT AAA ATT AGT CGA TAC GTT TCT GCG CAA AAA AAA AAA AAA AAA AAA AAA AAA AAA A                                1148
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Fig. 1. The nucleotide and deduced amino acid sequences of *RgAPX1* cDNA clone (GenBank accession number, AY462246). The active site (amino acids 33 to 44) and proximal heme-ligand motif (amino acids 155 to 161) are underlined.

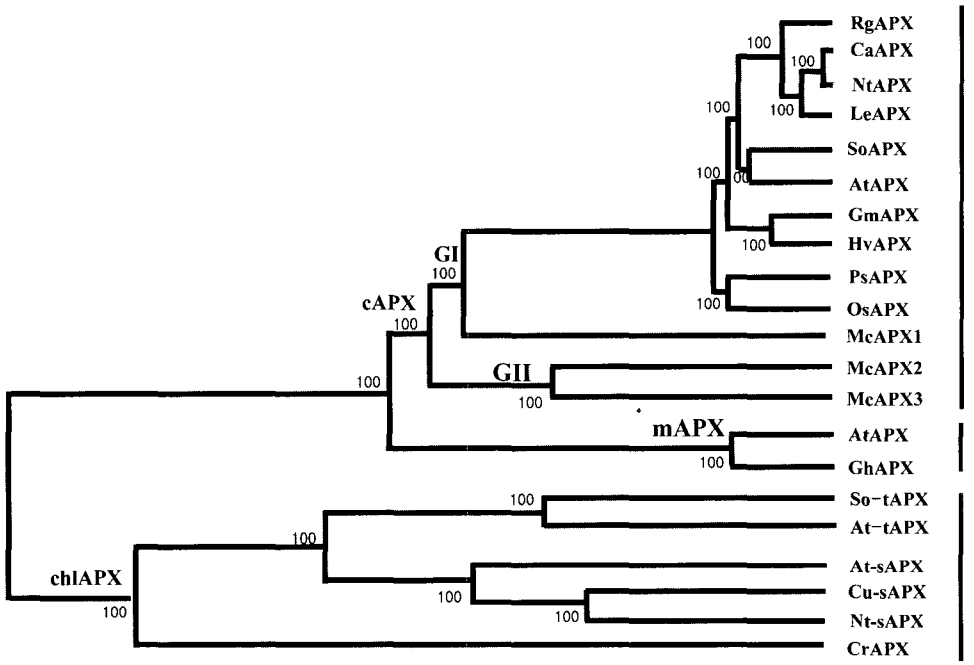

Fig. 2. Phylogenetic relationship of RgAPX1 to other plant APXs. Amino acid sequences were aligned and the phenogram was constructed using the Phylip program of GeneBee. Sequences were aligned with 100 bootstraps. Percent bootstrap values are placed at each node. cAPX, cytoplasmic APX; GI and GII, group I and group II of cAPX; chlAPX, chloroplastic APX; mAPX, membrane-bound APX The numbers below the line indicate the relative distance from the near node. Amino acid sequences were deduced from APX cDNA clones from *Arabidopsis thaliana* (AtAPX1, X98276; AtAPX2, X59600, At-sAPX, X98925; At-tAPX, X98926), *Capsicum annuum*, (CaAPX, AY078080.1), *Chlamydomonas reinhardtii* (CrAPX, AJ223325), *Cucurbita maxima* (Cu-sAPX, D88420), *Gossypium hirsutum* (GhAPX, U37060), *Glycine max* (GmAPX, AF127804), *Hordeum vulgare* (HvAPX, AJ006358), *Lycopersicaon esculentum* (LeAPX, Y16773), *Mesembryanthemum crystallinum*, (McAPX1 AF079512, McAPX2, U43561, McAPX3, AF079513), *Nicotiana tabacum* (NtAPX, D85912; Nt-sAPX, AB022274), *Oryza sativa* (OsAPX, AB050724), *Pisum sativum* (PsAPX, X62077), *Rehmannia glutinosa* (RgAPX1, AY462246), and *Spinacia oleracea* (SoAPX, D85864; So-tAPX, D77997). GenBank accession number for each clone is indicated.

cytosol, thylakoid membrane, stroma, and glyoxisomal membrane (Bunkelmann & Trelease, 1996; Mittler & Zilinskas, 1991, Miyake *et al.*, 1993; Shigeoka *et al.*, 2002).

Genetic constitution of *RgAPX1*

The genomic constitution of *RgAPX1* gene was estimated by Southern blot analysis with genomic DNA digested with the restriction enzymes *Bgl* II, *Spe* I, *Nde* I, and *Hind* III. The one or two major bands on Southern blot indicate *RgAPX1* is present as one gene or a member of a small gene family (Fig. 3). APX cDNA clones for at least three different forms of APXs, cytosolic, chloroplastic, and membrane-bound APXs have been isolated from plants, indicating that APX isoforms are encoded by a muligene family (Shigeoka *et al.*, 2002).

Regulation of *RgAPX1* expression

RgAPX1 mRNA was detected in the leaf, flower and root of healthy plants. The relative expression levels were higher in the leaf and lower in the root, indicating differential regulation of *RgAPX1* gene expression among the tissues (Fig. 4A). Similar differential expression among the tissues was reported in *CaAPX1* (Yoo *et al.*, 2002).

To better understand the regulation of *RgAPX1* gene expression, its expression in response to stresses and hormones were investigated in *R glutinosa* leaves. *RgAPX1* mRNA expression was increased a little by yeast extract and H_2O_2, and remained unchanged by UV. However, its expression was reduced slightly by paraquat and significantly by ethylene treatment (Fig. 4B). The results are in line with our APX activity observations in *R glutinosa*: APX activity is increased by yeast extract but decreased by paraquat and ethylene (Choi *et al.*, 2004; Moon *et al.*, 2004). Thus, the results suggest that regulation of *RgAPX1* expression is generally modulated at the transcriptional level. Transcriptional regulation of *APX* gene expression has been reported in several plant species, such as pea (Donahue *et al.*, 1997) and hot pepper (Yoo *et al.*, 2002). Interestingly, cytosolic *APX* mRNA expression was increased in paraquat-resistant pea leaves (Donahue *et al.*, 1997) and paraquat-treated spinach leaves (Yoshimura *et al.*, 2000). Furthermore, ectopic overexpression of cytosolic or chloroplastic *APX* mRNA reduced paraquat-induced damage and photoinhibition, supporting the role of APX in paraquat resistance (Allen *et al.*, 1997; Kwon *et al.*, 2003; Rizhsky *et al.*, 2002). However, no association of APX activity or gene expression levels was observed in paraquat resistance in *Lolium rigidum* (Yu *et al.*, 2004) and tolerance to paraquat-mediated oxidative stress in maize (Iannelli *et al.*, 1999).

No clear indication on the role of APX in paraquat tolerance in *R glutinosa* was observed from the expression response of *RgAPX1* mRNA to paraquat and other stresses. It is evident, however, that *RgAPX1* mRNA was decreased only slightly by paraquat treatment (Fig. 4B). It is noteworthy that paraquat-induced decrease in APX activity is much higher in paraquat-susceptible soybean than in paraquat-tolerance *R glutinosa* (Choi *et al.*, 2004). Thus, it can not be

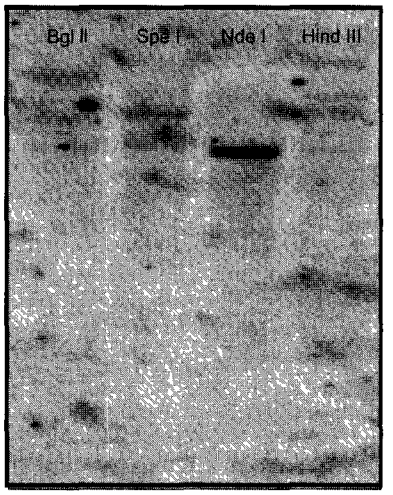

Fig. 3. Southern blot analysis of *RgAPX1* genomic DNA digested with restriction enzyme *Bgl* II, *Spe* II, Nde I, and *Hind* III. Ten micrograms of DNA was separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane, then hybridized with the alkaline phosphatase labeled *RgAPX1* insert DNA. The blot was washed to a final stringency of 0.1% SDS and 0.2% blocking reagent at 60 °C for 20 min.

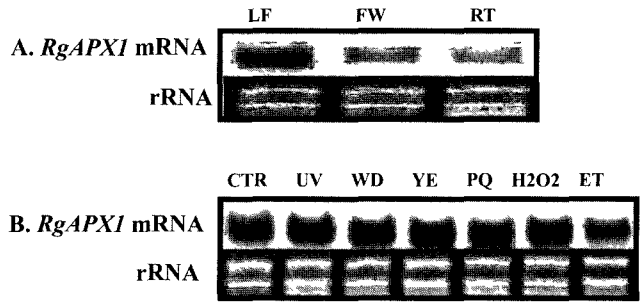

Fig. 4. A. Expression of *RgAPX1* mRNA in leaf (LF), flower (FL) and root (RT), respectively. B Expression of *RgAPX1* mRNA in response to various stresses and stress-related phytohormone. CTR, control; UV, ultraviolet; WD, wounding, YE, yeast extract; PQ, paraquat; H_2O_2, hydrogen peroxide, and ET, ethylene. Twenty micrograms of total RNA was resolved on a 1 0% (w/v) agarose/formaldehyde gel and transferred to a nylon membrane, then hybridized with the alkaline phosphatase labeled *RgAPX1* insert DNA. The blot was washed to a final stringency of 0.1% SDS and 0.2% Blocking reagent at 60 °C for 20 min. Ethidium bromide-stained rRNA bands as an indicator of equal loading (rRNA).

ruled out that low inhibition of *RgAPX1* gene expression by paraquat could help reduce oxidative damage caused by paraquat. Direct evidence on the role of *RgAPX1* in paraquat tolerance in *R glutinosa* could become available through further investigation on relationship between *RgAPX1* gene expression and paraquat tolerance.

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