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Induction of Rice Cytosolic Ascorbate Peroxidase mRNA by Oxidative Stress; the Involvement of Hydrogen Peroxide in Oxidative Stress Signalling

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The oxidative stress response of rice cytosolic ascorbate peroxidase (APX) was examined. The transcript level of cytosolic APX was significantly increased when suspension cultures of germinating rice embryos were treated with paraquat (7.9-fold) or H_2O_2 (6.1-fold). Induction by paraquat reached a maximum at 8 h. Induction by H_2O_2 peaked earlier at 4 h of treatment. This result suggests that the induction by paraquat might be caused by the H_2O_2 generated from superoxide. Treatment with a superoxide dismutase inhibitor, diethyldithiocarbamate, which is supposed to decrease the cellular H_2O_2 level, reduced the paraquat induction of cytosolic APX. In contrast, when APX and catalase were inhibited by hydroxyurea or aminotriazole, cellular H_2O_2 content was elevated and cytosolic APX mRNA was markedly increased without paraquat or H_2O_2 treatment. This suggests that cytosolic APX is regulated by the H_2O_2 level within cells. An increase in H_2O_2 content was observed in the paraquat treated embryos, suggesting that paraquat induction of cytosolic APX was caused by H_2O_2 generated through superoxide dismutation. These results indicate that H_2O_2 is involved in oxidative stress signalling, leading to the induction of cytosolic APX.

Key words: Ascorbate peroxidase (EC 1.11.1.11) — Hydrogen peroxide — Oxidative stress — Rice (*Oryza sativa*).

Active oxygen species that are inevitably produced as byproducts of oxygen metabolism have deleterious effects which cause cellular damage. As do other aerobic organisms, higher plants have active oxygen-scavenging systems consisting of multiple defense enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX: EC 1.11.1.11) and catalase (Bowler et al. 1992).

APX is a H_2O_2-scavenging enzyme found in higher plants, algae, and some cyanobacteria (Asada 1992). It is localized in chloroplasts (stroma and thylakoid), microbodies, mitochondria, and cytosol (Asada 1992, Miyake and Asada 1992, Yamaguchi et al. 1995, Jiménez et al. 1997).

Recent molecular cloning of APX revealed that cytosolic APX (including putative extraplastidic isoforms) are encoded by a multigene family (Ishikawa et al. 1995, Santos et al. 1996, Morita et al. 1997, Jespersen et al. 1997, Caldwell et al. 1998).

The importance of APX in stress defense was demonstrated in APX-antisense transgenic tobacco which was highly susceptible to ozone injury compared with the wild type (Örvar and Ellis 1997). Many authors have reported the enhanced expression of APX in response to environmental and biotic stresses such as drought, salt, high light, chilling, UV radiation, ozone, SO_2, oxygen tension, wounding, and pathogen attack (Mittler and Zilinskas 1994, Hernández et al. 1995, Mishra et al. 1993, Karpinski et al. 1997, Schöner and Krause 1990, Rao et al. 1996, Tanaka et al. 1985, Willekens et al. 1994, Kubo et al. 1995, Conklin and Last 1995, Ushimaru et al. 1992, Örvar et al. 1997, El-Zahaby et al. 1995). To date, several factors have been demonstrated to affect APX expression: ABA (Mittler and Zilinskas 1992), paraquat (Mittler and Zilinskas 1992, Pastory and Trippi 1992, Donahue et al. 1997), H_2O_2 (Pastory and Trippi 1992, Lappartient and Touraine 1997), glutathione (Lappartient and Touraine 1997), methyl jasmonate (Örvar et al. 1997), phytochrome (Thomsen et al. 1992), and redox status of the plastoquinone pool (Karpinski et al. 1997). Recently, the heat-shock element existing on the promoter of cytosolic APX gene (*APX1*) in *Arabidopsis* is revealed to contribute to the heat shock induction and partially to the paraquat induction of the gene (Storozhenko et al. 1998). However, the molecular mechanisms of APX gene regulation are not fully understood.

In order to examine the detail of APX gene regulation, we investigated the oxidative stress response of rice cytosolic APX using a recently cloned (Morita et al. 1997) cDNA encoding rice cytosolic APX. Our results suggest the involvement of H_2O_2 in oxidative stress signalling.

Materials and Methods

Plant materials—Rice (*Oryza sativa* L. cv. Nipponbare) embryos were prepared from bran by passing it through a sieve with an aperture size of 0.5 mm. The rice bran, which had been ob-

Abbreviations: APX, ascorbate peroxidase; DDC, *N,N*-diethyldithiocarbamate; SOD, superoxide dismutase; TCA, trichloroacetic acid.

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tained from matured rice grains, was provided by Gekkeikan Shuzou (Gekkeikan Brewery) Co. Ltd (Kyoto, Japan). The embryos were soaked in 70% ethanol for 3 min, then sterilized with sodium hypochlorite (0.4% active chlorine) for 15 min, and thoroughly washed with sterilized distilled water. The embryos were suspended in N6 medium and cultured for 6–7 d on a rotary shaker at 110 rpm at 28°C in the dark. On the second day, the medium was replaced with fresh solution. Five to six day-old cultures were then divided into aliquots and cultured for another day with fresh medium. Chemical treatments were performed by adding concentrated stock solutions into the medium.

Northern blot analysis—The total RNA was prepared by the phenol/SDS method. The RNA (15–20 μg) was denatured with formamide, fractionated through 1.2% agarose gels, and transferred to nitrocellulose membranes (Hybond C extra, Amersham Pharmacia). The blots were probed with [^{32}P]-labeled full length cDNA encoding rice cytosolic APX, *APXa* (Morita et al. 1997). They were subsequently hybridized with rice actin cDNA, C982 (accession No D15628, provided by the Rice Genome Research Program, National Institute of Agrobiological Resources, Tsukuba, Japan) as an internal control. The probes were labeled with a *Bca*Best Random Primer Labelling Kit (Takara, Kyoto, Japan). Hybridization was carried out at 42°C and washing was performed twice at 42°C for 20 min, with 2 × SSC, 0.1% SDS for the APX probe and 0.5 × SSC, 0.1% SDS for the actin probe. Hybridization signals were visualized by an autoradiogram and a bio-image analyzer (Molecular Imager, Bio-Rad). Quantification of transcript levels was performed by measuring the radioactivity of the signal bands using a Molecular Imager (Bio-Rad).

Measurement of H_2O_2 content—The cellular H_2O_2 content was measured by a fluorometrical assay with homovanillic acid according to Ishikawa et al. (1993) or by luminol chemiluminescence following the method described in Warm and Laties (1982) with some modifications. Samples (0.4 g) were homogenized in 1.5 ml of ice-cold 5% TCA, and the homogenate was passed through a Dowex 1x8 (Dow Chemical) spin column (1 ml of bed volume) by centrifugation at 400 × *g* for 5 min. The eluate was made to 3 ml with 5% TCA and used for H_2O_2 measurement.

For fluorometrical measurement, 100 μl of the extract prepared as described above was added to an equal amount of 0.5 M potassium phosphate buffer (pH 7.5). The reaction mixture (1 ml) contained 1.25 mM homovanillic acid, 1 unit of horseradish peroxidase (Wako, Osaka, Japan), 25 mM potassium phosphate buffer (pH 7.5), and 200 μl of buffered extract. The fluorescence yield was measured at an excitation of 315 nm and an emission of 425 nm.

For luminol chemiluminescence measurement, 50 μl of the extract was added to 50 μl 0.2 M NH_4OH and then mixed with 50 μl 0.5 mM luminol, 0.2 M NH_4OH. Chemiluminescence was started by injecting 50 μl of 1 mM $K_3Fe(CN)_6$, 0.2 M NH_4OH. The emitted photons were counted for 5 s with a luminometer (Lumat LB 9507, EG&G Berthold, Wildbad, Germany). In order to evaluate the H_2O_2-independent luminescence, 50 μl of the extract was buffered with an equal volume of 1 M Tris-HCl (pH 8.0) and then mixed with catalase (250 units, Sigma). After 10 min incubation at room temperature, the mixture was analyzed for chemiluminescence by adding luminol and ferrycyanide solutions as described above. The difference between the two measurements is presented as H_2O_2-specific chemiluminescence.

Results

We used germinating rice embryos which had been cultured in liquid medium because suspension cultures are suitable for chemical treatments. It is possible that, because of their dedifferentiated state, the responses seen in callus cells might not reflect the physiological responses in normal cells. Therefore, we assumed that germinating embryos would be more adequate materials for physiological analysis of stress response than callus cells.

To investigate the response of cytosolic APX to oxidative stress, we treated the cultured rice embryos with either paraquat (10 μM) or H_2O_2 (1 mM). Northern blot analysis was performed using full length cDNA of rice cytosolic APX as a probe. As shown in Figure 1, paraquat and H_2O_2 treatments both caused significant transient induction of rice cytosolic APX. The transcript level was increased to 7.9 times of the initial level by the paraquat treatment and 6.1 times by the H_2O_2 treatment. However, the induction kinetics differed between the two treatments. In the paraquat experiment, a maximum transcript level was observed at 8 h of treatment. In the H_2O_2 experiment, the mRNA level peaked at 4 h. The increase in the mRNA level was 2.1-fold at 2 h of the paraquat treatment and 4.2-fold at 2 h of the H_2O_2 treatment. Thus cytosolic APX induction by H_2O_2 occurs earlier than the induction by paraquat. It appears that the induction by paraquat is not a direct result but rather a secondary effect of paraquat application. It is probably caused by H_2O_2 generated from superoxide. Rice contains two cytosolic APX genes (*APXa* and *APXb*, Morita et al. 1997). In these experiments, the APX probe was cross-hybridized with both transcripts but not with mRNAs of other APX isozymes. Using gene specific probes, both *APXa* and *APXb* were similarly shown to be induced by either paraquat or H_2O_2 treatment (data not shown).

The paraquat and H_2O_2 dose responses of rice cytosolic APX were examined. The rice cultured embryos were treated with 0.1–10 μM paraquat for 4–8 h. As shown in Figure 2A, cytosolic APX induction was enhanced in parallel with the concentration of paraquat. In the H_2O_2 treatment, exposure to 0.01 mM H_2O_2 for 4 h resulted in a significant increase in cytosolic APX mRNA. Increasing the concentration of H_2O_2 also caused an enhanced stimulation of cytosolic APX expression in a dose dependent manner (Fig. 2B).

The result shown in Figure 1 suggests that paraquat induction of rice cytosolic APX might be mediated by H_2O_2 generated from superoxide via an SOD-catalyzed reaction. To test this hypothesis, we tried to manipulate the H_2O_2 level within the cells. Expecting a decrease in the cellular H_2O_2 level, we applied a CuZn-SOD inhibitor, DDC, together with paraquat. When treated with DDC, the paraquat induction of APX was significantly inhibited

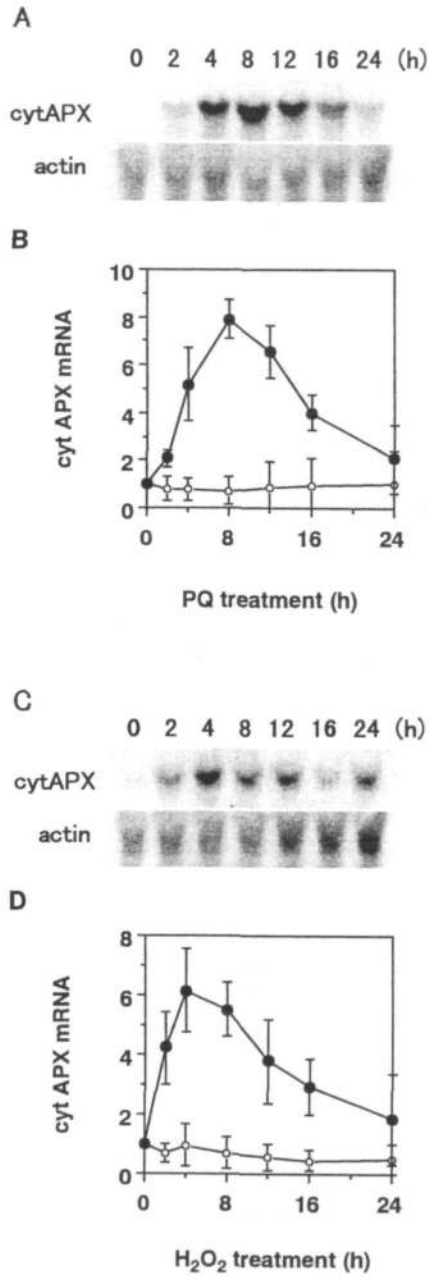

Fig. 1 The response of rice cytosolic APX mRNA to oxidative stresses. Rice cultured embryos were treated with 10 μM paraquat (PQ: A and B) or 1 mM H_2O_2 (C and D) for 2 to 24 h. The total RNA (15 μg per lane) was analyzed by Northern blotting using full length cDNA encoding rice cytosolic APX as a probe (A and C). The same blots were hybridized with a rice actin probe as a control. The results of the Northern analysis were quantitated, and the mRNA levels of cytosolic APX were normalized by actin mRNA levels. The results (the mean $\pm$ SD, n = 3) are presented in (B) and (D). Closed circles, paraquat or H_2O_2 treated samples; open circles, non-treated control.

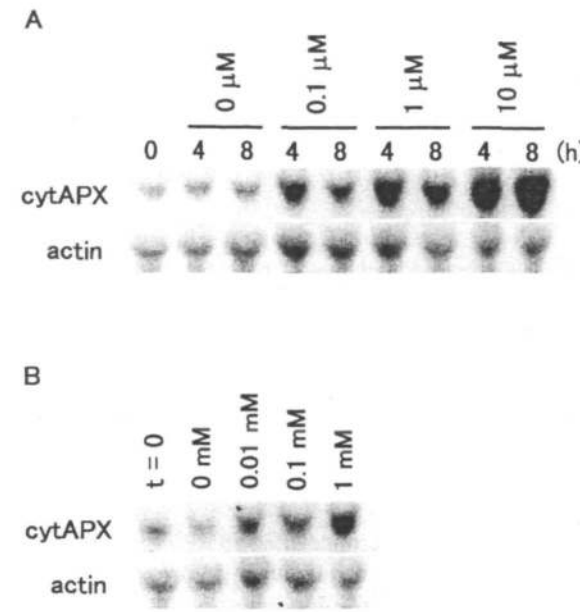

Fig. 2 The dose response of rice cytosolic APX mRNA to paraquat (A) and H_2O_2 (B). Rice cultured embryos were treated with 0.1–10 μM paraquat for either 4 or 8 h (A) or 0.01–1 mM H_2O_2 for 4 h (B). The cytosolic APX mRNA was analyzed by Northern blotting as described in Figure 1. Each lane contains 15 μg total RNA. In (B), t = 0 denotes the sample at the initial time. The representative results of triplicated experiments are presented.

(Fig. 3). The increase in cytosolic mRNA by paraquat treatment was suppressed to 48% under the DDC-treated conditions. We also used inhibitors of the H_2O_2scavenging enzyme for the purpose of increasing the level of H_2O_2. The addition of either 1 mM 3-aminotriazole (catalase inhibitor) or 0.1 M hydroxyuea (APX inhibitor, Chen and Asada 1989) resulted in a marked increase in cytosolic APX mRNA within 4 h without paraquat or H_2O_2 treatment (Fig. 4). We further measured the H_2O_2 content of the embryos treated with 3-aminotriazole or hydro-

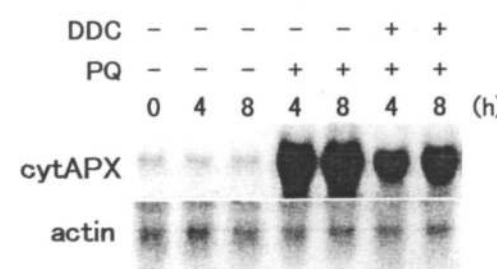

Fig. 3 The effect of the superoxide dismutase inhibitor on rice cytosolic APX induction by paraquat. Rice cultured embryos were treated with 10 μM paraquat (PQ) together with 1 mM DDC for either 4 or 8 h. The total RNA (15 μg per lane) was analyzed by Northern blotting as described in Figure 1. The representative results of triplicated experiments are presented.

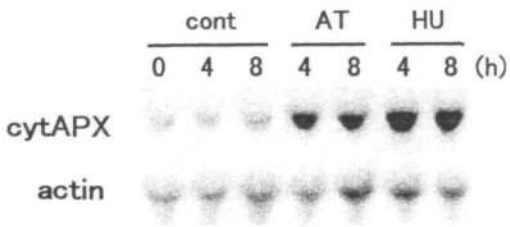

Fig. 4 The effect of inhibitors of H_2O_2-scavenging enzymes on rice cytosolic APX expression. Rice cultured embryos were treated with either 10 mM 3-aminotriazole (AT: catalase inhibitor) or 0.1 M hydroxyurea (HU: APX inhibitor) for either 4 or 8 h. The total RNA (20 μg per lane) was analyzed by Northern blotting as described in Figure 1. The abbreviation "cont", represents samples with no treatment. The representative results of triplicated experiments are presented.

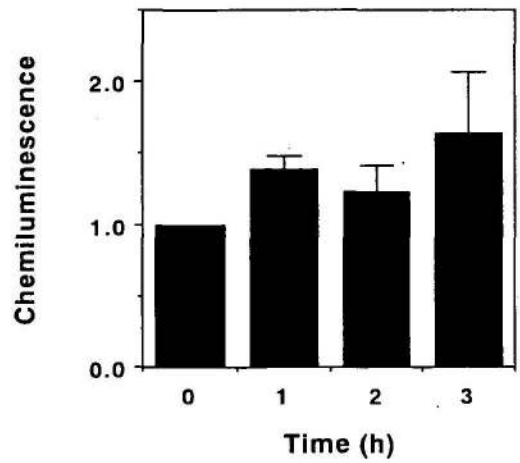

Fig. 5 The changes in the cellular H_2O_2 level within paraquat (PQ)-treated rice embryos. Rice cultured embryos were treated with 10 μM paraquat, and the cellular H_2O_2 levels were measured by luminol chemiluminescence. The data (mean $\pm$ SD, n=4) are presented as relative chemiluminescence compared with the initial time.

xyurea. H_2O_2 levels were elevated to 123 or 139% by 4 h treatment with 3-aminotriazole or hydroxyurea, respectively (Table 1). These results indicate that cytosolic APX responds to endogenous H_2O_2 levels.

We then examined whether the cellular H_2O_2 level was elevated by paraquat treatment by luminol chemiluminescence. The changes in the H_2O_2-dependent chemiluminescence of paraquat-treated embryos are presented in Figure 5. The H_2O_2 level was elevated to 139% by paraquat treatment within 1 h. An increased chemiluminescence (123%–164%) was observed for up to 3 h. This result indicates that paraquat-induced oxidative stress caused an increased production of H_2O_2 in vivo.

Discussion

In this study, we demonstrated cytosolic APX induction by paraquat and H_2O_2 at the RNA level (Fig. 1). Our results for paraquat treatment are consistent with previous observations of cytosolic APX induction by paraquat in pea and maize (Pastory and Trippi 1992, Mittler and Zilinskas 1992, Donahue et al. 1997). Although an increase in APX activity by H_2O_2 (Pastory and Trippi 1992, Lappartient and Touraine 1997) has been previously reported, this is the first report examining the H_2O_2 response of cytosolic APX at the RNA level. Both paraquat and H_2O_2 caused a significant induction of cytosolic APX in germinating embryos (Fig. 1). In our system of heterotrophic cultures in the dark, photosynthesis is not involved in the production of active oxygen. Similar induction of rice cytosolic APX by paraquat and H_2O_2 was observed in green seedlings (unpublished data).

The induction of cytosolic APX by paraquat occurred later than induction by H_2O_2 (Fig. 1). This suggests that paraquat application does not directly cause the induction of cytosolic APX. This result led us to the hypothesis that cytosolic APX induction by paraquat might be mediated by H_2O_2 generated from superoxide. When SOD activity was lowered by inhibitor treatment, the induction of cytosolic APX by paraquat was suppressed (Fig. 3). This result seems to be attributable to the decreased H_2O_2-producing capacity. The inhibition of H_2O_2-scavenging enzymes also resulted in the increase in the cellular H_2O_2 level and the up-regulation of cytosolic APX (Fig. 4, Table 1). These results indicate that cytosolic APX is expressed in response to the cellular H_2O_2 levels. Enhanced expression of endogenous cytosolic APX was observed in CuZn-SOD over-expressing (Sen Gupta et al. 1993) and catalase-deficient tobacco (Willekens et al. 1997). This is supposed to be due to elevated H_2O_2 stress as a consequence of antioxidative enzyme manipulation. These observations are consistent with our results. As shown in Figure 5, the paraquat application also caused an elevation in the H_2O_2 level in vivo. Taken together, these findings suggest that the response of rice cytosolic APX to oxidative stress is mediated by intracellular H_2O_2.

The induction of cytosolic APX by paraquat was al-

Table 1 The cellular H_2O_2 level within rice embryos treated with inhibitors of H_2O_2-scavenging enzymes

Treatment (time)	H_2O_2 (nmol (g FW)$^{-1}$)	Fold
None (0 h)	238 $\pm$ 19	1.00
3-Aminotriazole (4 h)	291 $\pm$ 20	1.23
Hydroxyurea (4 h)	330 $\pm$ 12	1.39

Data are represented as mean $\pm$ deviation (n=2).

ready active at 2 h (Fig. 1), indicating that the delay between the H_2O_2 induction and the paraquat induction is less than 2 h. An accumulation of H_2O_2 was observed at 1 h of paraquat treatment (Fig. 5). Considering that there might be a time lag while the effects of H_2O_2 lead to the cytosolic APX expression, induction at 2 h seems reasonable. In our experiments, the maximum mRNA level in paraquat treatment occurred at 8 h of treatment, 4 h after the peak of the induction by H_2O_2. We observed that cytosolic SOD was induced by paraquat up to 8 h under the conditions of the present study (unpublished result). SOD induction may lead to an increased H_2O_2 generation and to a further enhancement of cytosolic APX induction. This enhancement could account for the substantial increase in APX mRNA level up to 8 h of paraquat treatment.

It has been suggested that H_2O_2 is involved in the signalling of chilling (Prasad et al. 1994a), heat (Dat et al. 1998), and pathogen defense (Levine et al. 1994, Mehdy 1994). H_2O_2 is a membrane-permeable molecule that has been demonstrated to function as a diffusible intercellular signal (Levine et al. 1994). It is known to induce a number of genes and proteins involved in stress defenses (catalase: Prasad et al. 1994a, Scandalios et al. 1997. peroxidase: Prasad et al. 1994b. glutathione peroxidase and glutathione *S*-transferase: Levine et al. 1994. SOD: Pastori and Trippi 1992. pathogenesis-related (PR) protein: Chen et al. 1993. alternative oxidase: Vanlerberghe and McIntosh 1996). Our findings suggest that H_2O_2 may function in the signalling of oxidative stresses which lead to the induction of cytosolic APX genes, as well as the stresses described above.

Environmental stresses are known to cause oxidative stress within plant cells. The accumulation of H_2O_2 in planta has been observed in response to chilling (Okuda et al. 1991, Prasad et al. 1994a, O'Kane et al. 1996, Fadzillah et al. 1996), UV radiation (Murphy and Huerta 1990), heat (Foyer et al. 1997, Dat et al. 1998), and excess light (Karpinski et al. 1997). In these cases, the time range of H_2O_2 accumulation varied from only a few min to 4 to 8 d. The extent of the increase in H_2O_2 level observed within hours was 40 to 50% in mustard and tobacco seedling during heat acclimation (Foyer et al. 1997, Dat et al. 1998), about 3 to 4 fold by cold treatment in wheat and maize (Okuda et al. 1991, Prasad et al. 1994a) and 1.5 fold by excess light in *Arabidopsis* (Karpinski et al. 1997). Our data indicate that H_2O_2 elevation of 23 to 39% is sufficient for inducing cytosolic APX (Fig. 4 and Table 1). Although regulatory signals of cytosolic APX expression other than H_2O_2 (i.e. ABA, glutathione and redox status of the plastoquinone pool) have been suggested (Mittler and Zilinskas 1992, Karpinski et al. 1997, Lappartient and Touraine 1997), it seems likely that H_2O_2 accumulation is involved in cytosolic APX regulation in some, if not all, of these stress conditions.

To date, putative *cis*-element motifs have been reported on the promoter sequences of cytosolic APX genes in pea and *Arabidopsis*. The promoter of the pea gene contains a putative ARE (antioxidant responsive element) motif (Mittler and Zilinskas 1992). Since the ARE in rat glutathione *S*-transferase gene is responsive to H_2O_2 (Rushmore et al. 1991), the induction of cytosolic APX gene by H_2O_2 might be mediated by this element. A heat-shock element is conserved between pea and *Arabidopsis* genes and is involved in oxidative stress response in *Arabidopsis* (Mittler and Zilinskas 1992, Santos et al. 1996, Storozhenko et al. 1998). Whether the promoters of the cytosolic APX genes in rice have a conserved heat-shock element will be clarified by further gene analysis. It is also probable that there are H_2O_2-responding redox regulators in higher plants similar to those reported in bacteria (OxyR: Christman et al. 1985) and mammals (NF-κB and AP-1: Schreck et al. 1991, Meyer et al. 1993). The elucidation of the regulatory mechanisms of APX genes remains for future analysis.

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