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Activity of Antioxidant Enzymes during Senescence in Rice Seedlings

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ABSTRACT: Activity of senescence-induced antioxidant enzymes in the detached rice seedlings (*Oryza sativa* L. cv. Dongjin) was examined. The levels of H_2O_2 content and peroxidase (POD) activity were gradually increased during leaf senescence, whereas catalase activity was decreased. The activity of superoxide dismutase (SOD) was increased, and ascorbate peroxidase (APX) and glutathione reductase (GR) were slightly increased until 3d and 4d of dark induced-senescence, and thereafter were decreased. The activation of all SOD isoforms showed a significant decrease after 6d and 7d. After 4d to 7d of dark senescence, there was a significant effect in enhancing the activity of APX-12 and -13 isoforms as compared with light, despite similar levels in total APX activity. GR-8 and -10 isoforms were more effective in leaf senescence at 4d to 7d, particularly with respect to dark-induced senescence. These results suggest that the metabolism of active oxygen species such as H_2O_2 is dependent on various functionally interrelated antioxidant enzymes such as catalase, peroxidase, SOD, APX and GR.

Keywords: *Oryza sativa*, senescence, H_2O_2, oxidative stress, antioxidant enzyme.

During leaf senescence in many plant species, leaf photosynthetic capacity is diminished through a destruction of chlorophylls, a proteolytic attack on the abundant CO_2-fixing enzymes and a decline in *de novo* synthesis of the photosynthetic apparatus (Gepstein, 1988). Although visible symptoms of senescence are the result of a chlorophyll breakdown during chloroplast disassembly, many other catabolic events, such as protein, lipid and nucleic acid degradation also occur (Lohman *et al.*, 1994). Leaf senescence is associated with increased oxidative damage to cellular macromolecules by active oxygen species (AOS) (Ye *et al.*, 2000). Plants have evolved protective scavenging systems in response to these AOS. Susceptibility to oxidative stress depends on the overall balance between the production of oxidants and the antioxidant capability of the cell (del Rio *et al.*, 1998). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), as well as the enzymes of the ascorbate-glutathione cycle (Halliwell-Asada cycle): ascorbate peroxidase (APX) and glutathione reductase (GR) provide an endogenous defense against the accumulation of harmful concentrations of AOS (Hodges and Forney, 2000). Also, SOD produces H_2O_2, which may lead to further free radical production. H_2O_2 is reduced by catalase (CAT) or ascorbate peroxidase (APX) (Bailly *et al.*, 2001). APX might be responsible for the fine modulation of AOS for signaling, whereas CAT might be responsible for or the removal of excess AOS during stress (Mittler, 2002). The early processes of senescence seem neither related to diminished free radical scavenging by SOD, APX and catalase, nor to diminished GR activity. Leaf senescence in general is associated with higher oxidative stress and a decline in antioxidant activity towards maturity. Mn-SOD has a major role in the scavenging of superoxide radicals during leaf senescence (Prochazkova *et al.*, 2001). SOD isoforms present in the mitochondria increased in activity as senescence progressed, and isoforms of CAT in peroxisomes responded in various ways during monocarpic senescence (Srivalli and Khanna-Chopra, 2001). The different APX isoforms are also regulated differentially in response to stress and development. APX increase is associated with declines in lipid peroxidation and with the onset of visible senescence symptoms (Ye *et al.*, 2000).

The aim of this study was to examine the activities of various enzymes in order to identify any biochemical and physiological changes under leaf senescence in rice plants. We wished to monitor the antioxidant enzymes in response to the oxidative stress that induced the overproduction of active oxygen species during leaf senescence. We describe the changes of H_2O_2 contents, as well as the biochemical and physiological changes both in the activation of antioxidant enzymes and the induction of the enzyme isoforms regulated during the progress of senescence. Therefore, these physiological and biochemical analyses will provide important new insights into the protective mechanisms against senescence processes.

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MATERALS AND METHODS

Plant material and growth and senescence conditions

Rice plants (*Oryza sativa* L. cv. Dongjin) were germinated in the water for 5 days at 25°C under dark conditions and then planted in a pot containing vermiculite that was moistened with tap water, and then cultivated in growth chamber for 21 days. The environmental conditions in the growth chamber were 70% humidity, a temperature of 25°C, and a light intensity of 200 μmol m^{-2} s^{-1} with 16 h photoperiod. The detached leaves of 21-day-old rice plants were used as the materials. The detached leaves, which placed in a test vessel with a solution of 50 mM MES medium (pH 5.6), were constantly exposed to light or dark according to the previously mentioned conditions for 7 days. The biochemical and physiological changes of senescence were studied at one-day intervals. All of the experiments were repeated at least three times.

Measurement of RWC

Leaf relative water content (RWC) was estimated according to the following equation by recording the turgid weight of 1 g fresh leaf samples, which were hashed from the 1st cotyledons and were 90 mm length. They were kept in water for 24 hours, followed by drying in hot air oven until a constant weight was achieved for 48 hours (Whetherley, 1950).

$$RWC = \frac{\text{Fresh wt.–dry wt.}}{\text{Turgid wt.–dry wt.}} \times 100$$

Measurement of H_2O_2 content

For assay of the H_2O_2 content, 1 g of leaves was homogenized in 3 mL of 100 mM sodium phosphate buffer (pH 6.8). To remove cellular debris the homogenate was filtered through four layers of cheesecloth and then centrifuged at 18,000 g for 20 min at 4°C. The supernatant was collected for assay of the H_2O_2 content. Measurement of the H_2O_2 content was performed according to the modified method of Bernt and Bergmeyer (1974) using the peroxidase enzyme. To initiate the enzyme reaction an aliquot of 0.5 mL of supernatant was mixed with 2.5 mL of a peroxide reagent consistng of 83 mM sodium phosphate, pH 7.0, 0.005% (w/v) o-dianisidine, 40 μg peroxidase/mL, and incubated for 10 minutes at 30°C in a waterbath. The reaction was stopped by adding a 0.5 mL of 1 N perchloric acid and centrifuged at 5,000 g for 5 min. The resultant supernatant was read at 436 nm and its absorbance was compared to the extinction of a H_2O_2 standard.

Preparation of enzyme extracts

For determination of antioxidant enzyme activities, leaves (1 g) were homogenized in 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM ethylenediamine-tetraacetic acid (EDTA), 1% (w/v) polyvinyl-pyrrolidone (PVP) and 0.5% (v/v) Triton X-100 at 4°C. However, in the case of APX activity leaves were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate and 1 mM EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 18,000 g for 20 min at 4°C. The resultant supernatant was collected for determination of antioxidant enzyme activities and stored at -80°C for further analyses. Protein content was measured according to the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as a standard.

Enzyme assay

Catalase activity was determined by monitoring the decomposition of H_2O_2 (extinction coefficient 39.4 mM cm^{-1}) at 240 nm following the method of Aebi (1974). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0) and plant extract in a 3 mL volume. The reaction was initiated by adding 10 mM H_2O_2. One unit of catalase is defined as the amount of enzyme which liberates half the peroxide oxygen from 10 mM H_2O_2 solution in 100 second at 25°C. Peroxidase activity was determined by monitoring the formation of guaiacol dehydrogenation product (extinction coefficient 6.39 mM cm^{-1}) at 436 nm following the method of Pütter (1974). 3.18 mL of the reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0) and 0.3 mM guaiacol and plant extract. The reaction was initiated by adding 0.1 mM H_2O_2. One unit of peroxidase specific to guaiacol is defined as the oxidation of μmol of guaiacol from 0.3 mM guaiacol and 0.1 mM H_2O_2 per minute at 25°C at pH 7.0. Determination of SOD activity was performed by the method of Beyer and Fridovich (1987). 30.25 mL of the reaction mixture was composed of 50 mM potassium phosphate buffer (pH 7.8), 9.9 mM methionine, 57 μM nitroblue tetrazolium (NBT) and the appropriate volume of plant extract. The reaction was initiated by light illumination. One unit of SOD is defined as the amount of enzyme that causes a 50% decrease of the SOD-inhibitable NBT reduction. APX activity was determined by following the decrease of absorbance at 290 nm (extinction coefficient 2.8 mM cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.2 mM H_2O_2 and the suitable volume of enzyme extract (Chen and Asada, 1989). GR activity was determined by the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM cm^{-1}) as

described by Rao *et al.* (1996). The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) and the appropriate volume of enzyme extract in a 1 mL volume. The reaction was initiated by the addition of NADPH at 25°C.

Activity of gel analysis

Plant extracts containing equal amounts of protein, with the addition of bromophenol blue and glycerol to a final concentration of 12.5%, were subjected to discontinuous PAGE under nondenaturing, nonreducing conditions essentially as described by Laemmli (1970), except that SDS was omitted and the gels were supported by 10% glycerol. Electrophoretic separation was performed at 4°C for 4 h with a constant current of 30 mA per gel. For the analysis of APX activity, 2 mM ascorbate was added to the electrode buffer and the gel was pre-run for 30 minutes before the samples were loaded (Mittler and Zilinskas, 1993). SOD activity was detected by following the modified method of Beauchamp and Fridovich (1971). After completion of electrophoresis the gel was incubated in a solution containing 2.45 mM NBT for 25 minutes, followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 28 μM riboflavin and 28 mM tetramethyl ethylene diamine (TEMED) under dark conditions. Expression of SOD was achieved by light exposure for 10 to 20 minutes at room temperature. Identification of SOD isoforms was achieved by incubating the gels in 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM KCN or 5 mM H_2O_2 for 30 minutes before staining for SOD activity. APX activity was detected by the procedure described by Mittler and Zilinskas (1993). The gel equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 30 minutes was incubated in a solution composed of 50 mM sodium phosphate (pH 7.0), 4 mM ascorbate and 2 mM H_2O_2 for 20 minutes. The gel was washed in the buffer for 1 minute and submerged in a solution of 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 10 to 20 minutes with gentle agitation. GR activity was detected by incubation of gel in 50 mL of 0.25 M Tris-HCl buffer (pH 7.5) containing 10 mg of 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 10 mg of 2,6-dichlorophenolindophenol, 3.4 mM GSSG and 0.5 mM NADPH (Rao *et al.*, 1996). These SOD, APX and GR isoforms were identified 7, 14 and 11 isoenzymes in the rice leaves, respectively. In SOD No. 1, 3, 6 and 7 isoenzyme were identified as Cu/Zn-SOD, both 2 and 4 were identified Mn-SOD (Fig. 1).

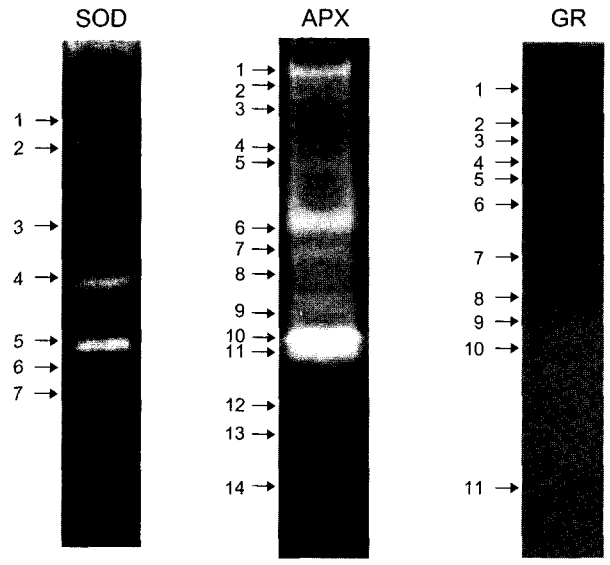

Fig. 1. All of isoforms were identified SOD, APX and GR by native gel stained for its activity, respectively. Each number indicates different isoforms in the leaves of rice plants.

RESULTS AND DISCUSSION

There has been little detailed study concerning the responses of various antioxidant enzymes in a single species during leaf senescence, although senescence has been shown to induce one or more antioxidant enzymes (Srivalli

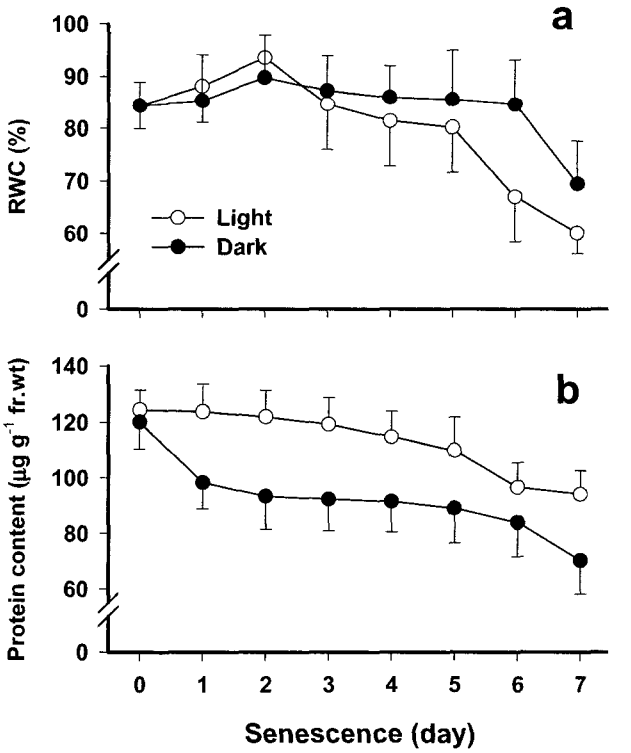

Fig. 2. Changes of RWC and total protein activity in the rice leaves during leaf senescence. The data presented are mean values of three independent replicate assays.

and Khanna-Chopra, 2001; Jimenez *et al.*, 1998). Hence, the responses of antioxidant enzymes against the generation of active oxygen species during leaf senescence were examined in the rice plants. The relationship between oxidative process and antioxidant enzymes were investigated and were expected to clarify the physiological and biochemical changes between light- and dark-induced senescence when early experimental events were designed.

Relative water content (RWC) levels somewhat increased from the initial measurement to 2d of leaf senescence, then decreased afterwards. Compared with the RWC in dark-induced senescence, RWC in light-induced senescence was rapidly decreased by withholding water from 3d to 7d of senescence (Fig. 2a). The changes of protein content in the leaves of rice seedlings during senescence are shown in Fig. 2b. The total protein content was slowly decreased during senescence and also maintained its level for a longer period than it held its total chlorophyll content, and in opposition to RWC the pattern of protein levels in dark-induced senescence compared with protein levels in light-induced senescence was rapidly decreased.

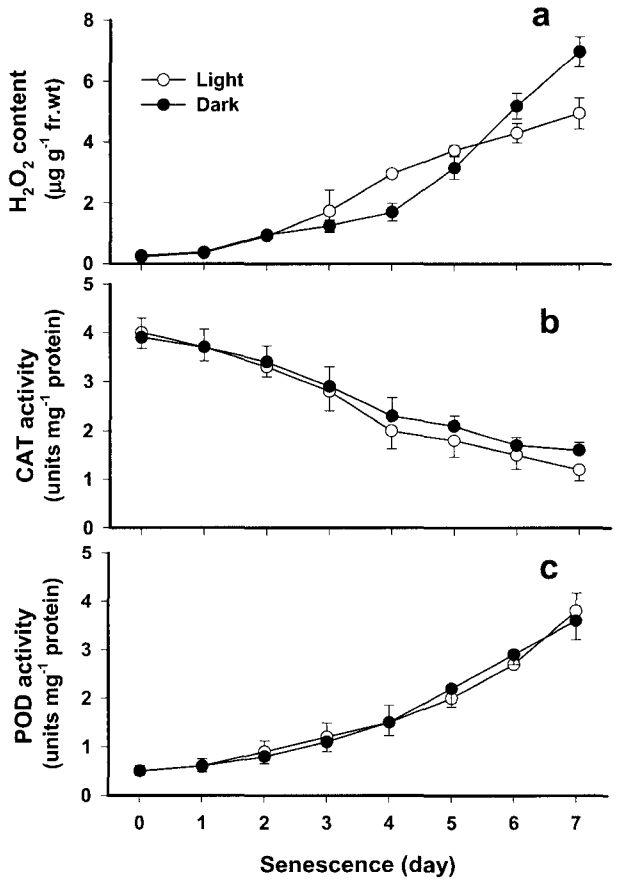

Fig. 3. H_2O_2 levels and the activities of catalase and peroxidase in rice leaves during senescence. a, measurement of the H_2O_2 ; b, activity of catalase; c, activity of peroxidase.

The enhancement of H_2O_2 levels resulting from senescence would be alleviated through the combined activity of catalase and APX (Bailly *et al.*, 2001). In the present study, however, the levels of catalase activity were gradually decreased even after 7d of senescence (Fig. 3b). A marked decline in catalase activity has also been reported in the leaf of wheat (Srivalli and Khanna-Chopra, 2001) and in cucumber cotyledons (Kanazawa *et al.*, 2000) during senescence. The levels of H_2O_2 and peroxidase activity were gradually increased, whereas the catalase activity was gradually decreased in the progression of senescence (Fig. 3). Peroxidases (POD), which occur as multiple isozymes, are known to utilize substrates to metabolize H_2O_2. When guaiacol was used as a substrate, peroxidase activities were enhanced during leaf senescence. Peroxidase is also an important antioxidant enzyme in scavenging or utilizing H_2O_2.

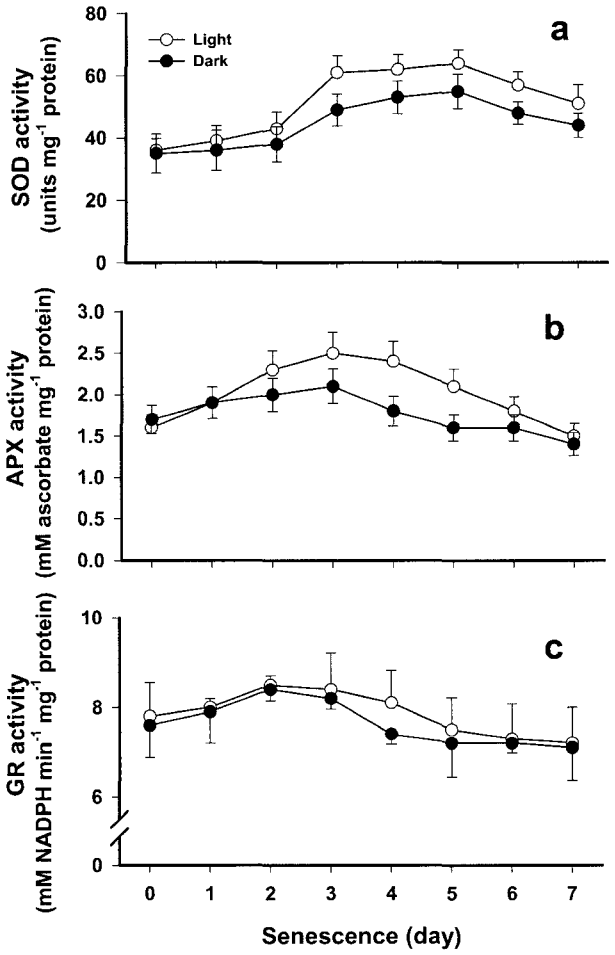

Fig. 4. Activity of SOD, APX and GR in rice leaves during senescence. One unit of SOD is defined as the amount of enzyme that causes a 50% decrease of the SOD-inhabitable NBT reduction (a). APX activity was determined by following the decrease of absorbance at 290 nm (b). GR activity was determined by the oxidation of NADPH at 340 nm (c).

The activity of total SOD during leaf senescence was still maintained at a higher level, The activity of total SOD was slightly elevated after the 3d senescence, after the 6d and 7d of senescence the activity of the leaves even sustained almost the same activity as the initially measured leaves did (Fig. 4a). With catalase deactivation in senescence stressed-leaves, there is little detailed study on the metabolic role of APX together with other antioxidant enzymes in an H_2O_2 scavenging metabolism. Thus, we examined the changes of APX activity in the leaves of rice plants during senescence (Fig. 4b). The elevated APX activity was sustained until 3d and then its activity gradually decreased until the final stage of sampling on 7d. In this experiment the pattern of changes in the APX activity was very similar to that of changes in the SOD activity. Our results indicated that senescence, particularly dark-induction, caused an early slight enhancement of total APX activities. Also, there were no significant differences in levels of total APX activity from 3d to 7d of senescence. Expression of APX activity may have an even more dramatic effect on the protective mechanism against leaf senescence as compared with catalase (Fig. 3b). Because H_2O_2 is generated in the intercellular space of the plant during environmental stress, it appears to diffuse first into the cytosol, where cytosolic APX is localized. Only afterwards it diffuses into peroxisome where catalase is typically found, and because cytosolic APX has a higher affinity for H_2O_2 than catalase does (Asada, 1992), changes in the relative distribution of APX isoforms could contribute to stress tolerance or to the plants response under stress conditions (Yoshimura *et al.*, 2000). In this experiment, the results suggest a possibility that cytosolic APX in the rice seedlings may be a key enzyme for the decomposition of hydrogen peroxide under catalase deactivation due to senescence. GR is an essential catalyzer in the conversion of H_2O_2 in order to maintain the redox state of ascorbate and glutathione (Foyer *et al.*, 1994). We examined the changes of GR activity in senescing leaves (Fig. 4c). GR activity did not create significant changes in the progression of senescence at the overall stage. GR is known to act in conjunction with APX to metabolize H_2O_2 into water through an ascorbate-glutathione cycle (Jimenez *et al.*, 1998). GR activity was enhanced somewhat early by senescence from the initial measurement to the 2d. After that, the level of enzyme activity was lower than before senescence (Fig. 4c). These increases in GR activity help to partially eliminate the toxic levels of H_2O_2 in the cell while converting oxidized glutathione (GSSH) into reduced glutathione (GSH). This indicates that the glutathione/ascorbate cycle commonly plays an important role in detoxifying H_2O_2, a result that is supported in research by Bailly *et al.* (2001).

The accumulation of H_2O_2 was induced by the increase of

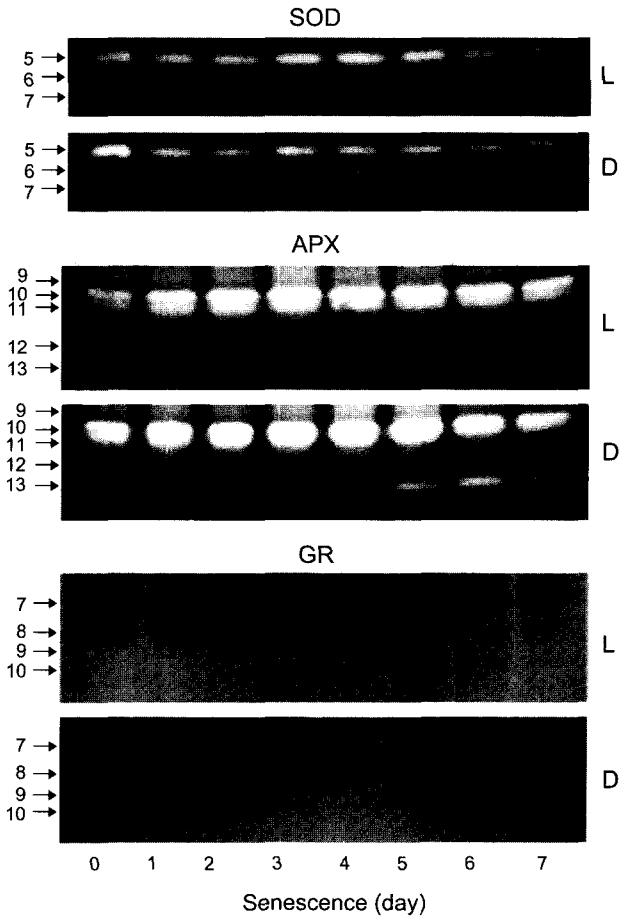

Fig. 5. Native gel stained for the activity of SOD, APX and GR in rice leaves. Each number indicates different isoforms in the leaves of rice plants. L and D were constantly exposed to light and dark for 7 days, respectively.

total SOD activity or alterations in the relative distributions of SOD isoforms (Figs. 4a and 5), and in turn the newly accumulated H_2O_2 might be triggering a mechanism that increased the activity of several enzymes such as peroxidase, APX and GR or induced alterations in the relative distributions of several enzyme isoforms under catalase deactivation (Figs. 3, 4 and 5). There was no significant difference in total SOD activity between light- and dark-induced senescence (Fig. 4a), and it no longer further enhanced SOD isoenzymes in gel analysis. In gel assay of SOD the 4 isoforms of Cu/Zn-SOD were observed on the activity gels in the rice seedlings (Fig. 1) and showed slight differences in different isoforms after 3d to 5d, particularly Cu/Zn-SOD-5 (Fig. 5). It was especially interesting that light-induced senescence was observed higher Cu/Zn-SOD-5 than in dark-induced senescence. In the present study senescence caused the enhancement of total SOD activities and appeared to be due to preferential induction of all SOD isoforms, particularly the Cu/Zn-SOD isoforms (Fig. 5). Although there were

little changes in the total SOD activities after the 3d to 6d of senescence, increases in the relative distributions of Cu/Zn-SOD-5 could contribute to a response against leaf senescence, particularly under light. As shown in the results of the expression of SOD isoforms, the induction of different SOD isoforms may be regulated differently upon exposure to various environmental stressors (Prochazkova *et al.,* 2001; McKersie *et al.,* 2000; Wu *et al.,* 1999).

APX activity assays were performed on natural leaves and senescence leaves using nondenaturing gels. The 14 isoforms of APX were visible on the activity gels (Fig. 1). There was a somewhat detectable difference in the activity of APX-12 and 13 between light- and dark-induced senescence, despite similar levels in total APX activity (Fig. 4b). After 4d to 7d of senescence in the dark there was a significant effect in enhancing these activations as compared with light-induced senescence. Thus, the expression of these isoforms was significantly increased according to senescence in the dark. As shown in Fig. 1, the 11 isoforms of GR were visible on the activity gels, of which GR-8 and -10 isoforms were more effective in leaf senescence at the 4d to 7d, particularly dark-induced senescence. All expressions of GR isoforms were little changed during senescence, whereas GR-7 isoforms was significantly decreased after 3d of senescence.

Active oxygen species, which are thought to be primarily responsible for oxygen toxicity in the plant cells, were produced under stress conditions of plant cell. SOD catalyzes the first step in the AOS scavenging system. Thus, the dismutation of superoxide radicals into H_2O_2 and oxygen is an important step in protecting the cell. The metabolism of active oxygen species such as H_2O_2 is dependent on various functionally interrelated antioxidant enzymes such as catalase, peroxidase, SOD, APX and GR. The results obtained in the present experiment agree with the reports that senescence induces an antioxidative defense response against oxidative damage and that it significantly increases the levels of O^{2-} and H_2O_2, followed by an increase in activities of SOD, CAT, APX and GR in maize leaves (Prochazkova *et al.,* 2001).

In summary, the findings in the present study suggest that high cellular levels of H_2O_2 can induce the activation of a defense mechanism against programmed cell death by leaf senescence. The metabolism of active oxygen species such as H_2O_2 is dependent on various functionally interrelated antioxidant enzymes such as catalase, peroxidase, SOD, APX and GR. The accumulation of H_2O_2 was induced by the increase of total SOD activity or alterations in the relative distributions of SOD isoforms, and in turn the newly accumulated H_2O_2 might be triggering a mechanism that increased the activity of several enzymes such as peroxidase, APX and GR or it might have induced alterations in the relative distributions of several enzyme isoforms under catalase deactivation. These results on the leaf senescence suggest that the response timing of enzyme isoforms against various environmental stresses on the antioxidant enzyme system may not be the same for all isoforms of antioxidant enzymes. The response of timings of antioxidant enzyme isoforms to leaf senescence is little known and is an area for fruitful future research.

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