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SHORT COMMUNICATION

CHANGES IN ANTIOXIDANT DEFENSE COMPONENTS ASSOCIATED WITH SENESCENCE OF DETACHED RICE LEAVES

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Different components of the antioxidant defense system were studied during the course of dark-induced senescence of detached primary leaves of rice (*Oryza sativa* L. cv. IR36) seedlings. The activities of ascorbate peroxidase, peroxidase (o-dianisidine) and superoxide dismutase were increased in senescing leaves as compared to control leaves (freshly excised), while catalase activity showed drastic loss during senescence. Among the non-enzymic antioxidants, glutathione content was elevated and ascorbate content was decreased in senescing leaves. In contrast to other antioxidants, peroxidase activity showed striking elevation even at the advance stages of senescence. Isozyme analysis of peroxidase on native PAGE also revealed higher synthesis of the enzyme from senescing leaves. Determination of peroxidase activity with function-specific substrates such as syringaldazine (for lignification) and NADH (for H_2O_2 generation) also revealed large increases at the final stages of senescence. The activity of IAA oxidase remained unchanged at this time period. Probable role of antioxidants along with peroxidase(s) during senescence had been discussed.

Key words: **Ascorbate peroxidase, catalase, glutathione, peroxidase, senescence, superoxide dismutase**

One of the earliest events that occur during the process of senescence in plants is the formation of reactive oxygen species (ROS) in large quantities which leads to the oxidative damage and ultimately cell death (Leshem 1988, del Rio *et al*. 1998). Leakage of electrons from different electron transport chains of chloroplast, mitochondria, ER into dioxygen results in the formation of superoxide radical ($O_2^{\cdot-}$), which is converted by superoxide dismutase (SOD) to yield less toxic H_2O_2 (Dalton *et al*. 1987). The concurrent accumulation of $O_2^{\cdot-}$ and H_2O_2 may prove deleterious to the cellular constituents because of their ability to initiate cascade reactions that lead to the production of more reactive intermediates like hydroxyl radicals (Polle 1997, Noctor and Foyer 1998). The key defense mechanisms against such oxidative damage include ascorbate-glutathione mediated scavenging of ROS through Foyer-Halliwell-Asada cycle, which operates not only in different compartments of a cell but different plant parts/organs as well (Nakano and Asada 1981, Dalton *et al*. 1987, Polle *et al*. 1990, del Rio *et al*. 1998, Pereira *et al*. 2005, Palma *et al*. 2006). Ascorbate peroxidase (APX) is an important enzymic component of this cycle. The cycle assumes greater importance particularly in the chloroplast, one of the earliest sites of catabolism in leaf senescence (Smart 1994), where even very low

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concentrations of H_2O_2 might cause damage to the photosynthetic machinery of a cell (Polle *et al*. 1990, Foyer 1996). Hydrogen peroxide is also removed through the action of catalase in peroxisomes and mitochondria (Scandalios 1994). Peroxidase (PX), ubiquitous among plant enzymes, catalyzes the oxidation of a diverse group of organic compounds using H_2O_2 as the ultimate electron acceptor (Lagrimini 1991).

In the present paper we have investigated the behaviour of different antioxidant parameters during senescence of detached rice leaves with a view to understanding adaptive responses or degenerative changes involving each of the parameters. Since formation of ROS serves as a trigger for both stimulation as well as depletion of antioxidants, it would be relevant to study such changes within a specific timeframe and attempted to correlate both approaches. We have also tried to highlight the role of peroxidase(s) during senescence by measuring its' activity with substrates that signify specific functions *in vivo*.

Fresh and viable seeds of rice (*Oryza sativa* L. cv. IR-36) were collected from State Rice Research Institute, Chuchura (W.B.). The details on the raising of rice seedlings have already been described in our previous communication (Banerjee *et al*. 2002). Ten centimeter tips from the primary leaves of 14 days old seedlings were excised randomly, washed with distilled water and floated in Petri dishes (15 cm diameter) containing 40 ml of double distilled water @ 10 excised leaves per Petri dish at a temperature of 30±1°C in complete darkness (Kar and Mishra 1976). Three such Petri dishes were incubated in the dark to promote senescence in the excised leaves. First sampling at 0 hr was considered as freshly excised or control leaves and subsequent samplings were carried out at 24 hrs interval up to a period of 96 hrs. Immediately after sampling, the leaves were washed again with distilled water and blotted properly to remove excess moisture. The crude enzyme fractions from leaf samples were prepared according to Banerjee *et al*. (2008) with following modifications. Leaf samples weighing 400 mg were homogenized in 4 ml of 100 mM ice cold potassium phosphate buffer (pH 7.2) containing 0.1% Triton x-100 and centrifuged at 12000 g for 25 minutes at 4°C. The pellets were discarded and supernatants were used for determining enzyme activities and soluble protein content.

Soluble protein content was measured by the method of Lowry *et al*. (1951). Superoxide dismutase activity was assayed in terms of its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm (Beauchamp and Fridovich 1971). One unit of enzyme activity is defined as the quantity of enzyme that reduces the photoreduction of NBT by 50% with respect to control system. Activities of APX, catalase (CAT) and non-specific PX (using o-dianisidine as the electron donor) were analyzed following the methods of Nakano and Asada (1981), Klapheck *et al*. (1990) and Chen and Asada (1989) respectively. Peroxidase activity with regard to function specific substrates like syringaldazine, NADH and IAA was determined only at 96 hrs time point following standard methods. Syringaldazine peroxidase was estimated by measuring increase in absorbance at 530 nm (Polle and Chakrabarti 1994). NADH oxidase activity was determined by measuring the breakdown of NADH at 340 nm (Ishida *et al*. 1987). IAA oxidase was measured by recording the increase in absorbance at 250 nm (Jacob and Bopp 1990). The specific activities for all the enzymes were expressed as unit per mg of protein.

The analysis of PX isozymes was performed on non-denaturing (native) Polyacrylamide gel electrophoresis (PAGE) without SDS following the method of Mittal and Dubey (1991). Separation of isozymes was carried out using a stacking gel (5% polyacrylamide gel containing 0.5 M TRIS-HCl, pH 6.8) and a separating gel of 7.5% polyacrylamide (containing 1.5 M TRIS-HCl, pH 8.8) at 4°C at 30 miliampere for 4 hours. Requisite amount of crude enzyme extracts from control and senescing leaves (96 hours) were mixed with equal volume of glycerol and loaded in each lane of the gel using bromophenol blue as the marker dye. For activity staining the gels were immersed in an incubation mixture containing 10 mM K-Phosphate buffer (pH 6.0), 20 mM o-dianisidine and 5 mM H_2O_2. Dark brown bands appeared within two hours in the sites of PX activity.

For determining ascorbate (total) and glutathione (total) contents, leaf samples weighing 500 mg were

homogenized in 5 ml of 5% metaphosphoric acid and sulfosalicylic acid respectively containing 0.5 mM EDTA and centrifuged at 12000 g for 25 minutes. The clear supernatants were used for analysis of ascorbate (total) and glutathione (total) content. Ascorbate (AsA) was estimated by hydrazine formation with 2, 4-dinitrophenyl hydrazine (Shigeoka *et al*. 1980). Glutathione (GSH) was estimated by the recycling assay involving pure glutathione reductase (obtained from Sigma chemicals) and DTNB at 412 nm following the method of Smith (1985). Chlorophyll was estimated according to Arnon (1949).

Each data in tables and figures represent mean±SD of at least three separate experiments. The significance of difference was determined by Student t-test. Significance levels have been expressed as $^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$.

In this paper we have followed two approaches for analyzing our results. The first approach was based on the observation of changes in the selected parameters relative to control leaves at fixed intervals of 24-, 48-, 72- and 96 hrs. In this approach the control leaves have been considered as senescence 0 hr stage (freshly excised leaves). The other approach focused on the comparison of these parameters between two consecutive intervals expressed as a percentage of the values at the starting point viz. $(T_i \sim T_f/T_i) \times 100$, where T_i and T_f were values at the initial and final time points.

The process of senescence is mainly characterized by extensive loss of chlorophyll and proteins, chloroplasts being the earliest sites of catabolism in leaf senescence (Smart 1994, del Rio *et al*. 1998). In the present study, the excised rice leaves also showed yellowing with passage of time owing to the progressive loss of chlorophyll and protein contents. The decreases of chlorophyll pigments were 23%, 49%, 72% and 84% with respect to control leaves after 24, 48, 72 and 96 hrs of senescence (Table 1). The corresponding decrease in protein content was 17%, 26%, 50% and 57% respectively. Chlorophyll degradation was apparently of higher magnitude than the rate of protein loss. This is due to the fact that under the dark, the process of chlorophyll degradation is irreversible in angiosperm species as the final enzyme in the

Table 1. Changes in chlorophyll and protein content during senescence of detached primary rice leaves (values are means±SD).

Period of Senescence	Chlorophyll (mg/g FW)	Protein (mg/g FW)
0 hour	3.57 ± 0.23	35.04 ± 1.14
24 hours	2.75^{**} ± 0.15	29.32^{*} ± 1.09
48 hours	1.82^{***} ± 0.12	25.91^{**} ± 1.01
72 hours	1.01^{***} ± 0.07	17.39^{***} ± 0.78
96 hours	0.572^{***} ± 0.03	15.17^{***} ± 0.37

Values significant at $^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$

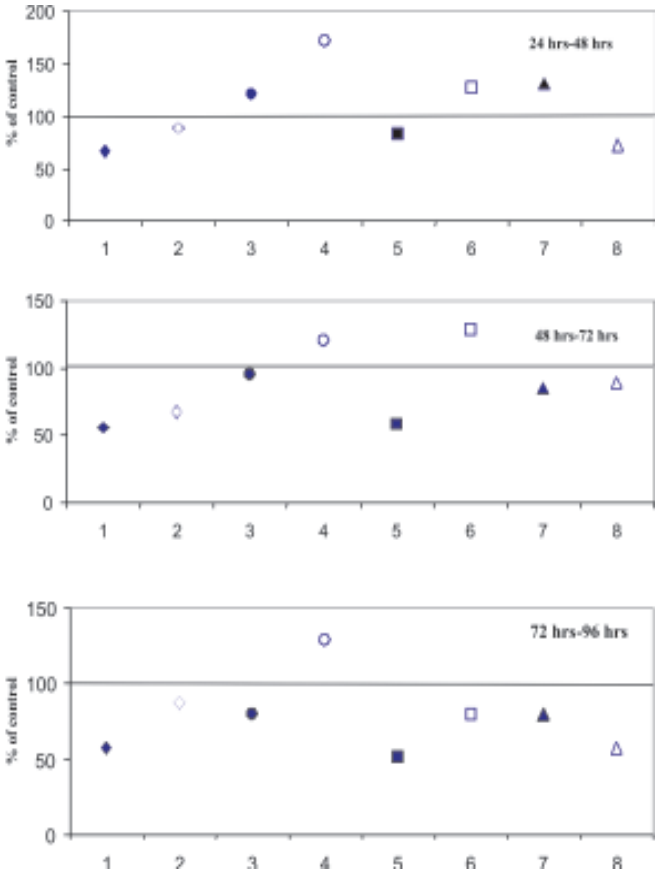

Fig. 1. Changes in the levels of physiological and antioxidant parameters between consecutive time points of detached leaf senescence

1. Chlorophyll, 2. Protein, 3. Ascorbate peroxidase, 4. Peroxidase, 5. Catalase, 6. SOD, 7. Glutathione (total), 8. Ascorbate (total)

biosynthetic pathway viz. protochlorophyllide reductase is light dependent (Mariani *et al*. 1990). However, protein synthesis can also proceed in the dark. The decreased rate in protein loss in detached rice leaves might be indicative of a "positive turnover" (such as synthesis coupled with slow loss) of protein as against chlorophyll content. This was also evident from the comparative changes of chlorophyll and protein between consecutive intervals (Fig. 1). Chlorophyll loss was higher (44%) between 48-72 hrs and 72-96 hr intervals as compared to that of protein loss which was 33% and 13% for the same time period. An interesting hypothesis is that proteins or at least a part of it are "resistant" to the catabolic processes of senescence, which could account for its relatively slow rate of decay between the consecutive time intervals.

The activity profiles of the scavenger enzymes viz. APX, SOD, PX and CAT during senescence have been shown in Table 2. The activity of APX in senescing leaves showed increases of 20% and 45% over controls after 24 hrs and 48 hrs of incubation. Though this trend persisted over the entire experimental period, the extent of increase was only 39% (72 hrs) and 11% (96 hrs). A close look at the comparative activity of APX in senescing leaves between the time points showed that the rise (21%) was localized only during the 24-48 hrs interval (Fig. 1). A marginal decrease (5%) in the net activity in senescing leaves between 48-72 hrs was followed by a higher decrease (20%) between 72-96 hrs. The activity of SOD was higher in senescent leaves at all the time points, the peak being after 72 hrs of incubation (91.5%). An increase in the comparative activity of SOD (in the range of 27%) in senescing leaves was observed between 24-48 hrs and 48-72 hrs periods. The net activity actually declined by 20% during 72-96 hrs, which was similar as observed for APX during the same time period (Fig. 1).

The activity of PX exhibited an increase over control during senescence, but the extent and pattern was somewhat different from that of APX and SOD. After 24 hrs, a marginal increase (23%) gave rise to a large increase of 92% after 48 hrs of incubation. The increase was further enhanced to 132% over control at 72 hrs of incubation. This upswing in activity continued even at 96 hrs when an increase of 198% was observed. The comparative activity of PX in senescing leaves also showed remarkable increase at each of the intervals that were examined. The comparative increase was highest during the initial phase e.g. 24-48 hrs. During the later phases viz. 48-72 hrs and 72-96 hrs, the increases were 20% and 29% respectively (Fig. 1).

The isozyme analysis of PX on native PAGE (using o-dianisidine as the staining agent) also validated our conception regarding higher synthesis of the enzyme (Fig. 2). The band pattern of the enzyme from control and senescing leaves (96 hours) was found to remain unaltered, although the intensity of the bands pertaining to senescing leaves was higher than that of control leaves.

Table 2. Changes in the activities of enzymic antioxidants during senescence of detached primary rice leaves (values are means±SD).

Period of Senescence	Ascorbate peroxidase (µ mol ascorbate oxidized/min/mg protein)	Peroxidase (µ mol o-dianisidine oxidized/ min/mg protein)	Catalase (µ mol H_2O_2 decomposed/ min/mg protein)	Superoxide dismutase (Unit/min/mg protein)
0 hour	0.44± 0.02	1.99±0.09	141.45±4.92	15.72±0.13
24 hours	0.53**±0.01	2.44*±0.12	114.58**±2.13	18.47*±0.42
48 hours	0.64***±0.02	3.83***±0.14	94.77***±1.01	23.42***± 0.56
72 hours	0.61***±0.02	4.61***±0.13	55.16***±1.17	30.10***±0.62
96 hours	0.49*±0.01	5.93***± 0.15	28.29***±0.09	24.05***±0.29

Values significant at $^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$

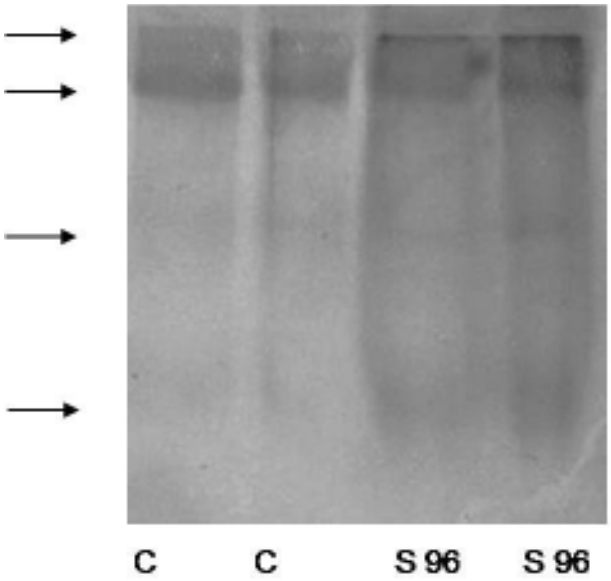

Fig. 2. Pattern of peroxidase isozymes on native PAGE from control and senescing leaves. (C-Control, S 96-Senescence 96 hours; ⟶ indicates position of isozymes on gel matrix)

In contrast to the trends exhibited by the other H_2O_2 scavenging enzymes viz. APX and PX, CAT activity in senescing leaves showed steady decrease throughout the experimental period (Table 2). The extent of this decrease varied from 19% (after 24 hrs) to 80% (after 96 hrs). The comparative activity also suffered a steady loss in the senescing leaves. The extent of loss gradually increased from the 24-48 hrs phase (17%) to the 72-96 hrs phase (49%) (Fig. 1).

Table 3. Changes in the levels of non-enzymic antioxidants during senescence of detached primary rice leaves (values are means±SD)

Period of Senescence	Ascorbate (total) (µg/g FW)	Glutathione (total) (µg/g FW)
0 hour	353.67 ± 9.59	224.57 ± 5.31
24 hours	275.86** ± 6.13	295.31*** ± 6.48
48 hours	199.82*** ± 5.48	388.51*** ± 8.94
72 hours	176.84*** ± 6.27	330.12*** ± 5.31
96 hours	100.16*** ± 3.15	264.99* ± 3.25

Values significant at $^{*}P\leq0.05$, $^{**}P\leq0.01$ and $^{***}P\leq0.001$

Among the non-enzymic antioxidants, ascorbate content progressively declined with senescence (Table 3). The decline was 22%, 43.5% and 50% after 24-, 48- and 72 hrs of incubation respectively. The decline was so severe that after 96 hrs, only 28% of the initial control level persisted. The comparative decline in AsA was 28%, 11% and 43% between 24-48 hrs, 48-72 hrs 72-96 hrs of interval (Fig. 1). In contrast to AsA, glutathione (total) content showed increases of 31.5% and 73% after 24 hrs and 48 hrs of incubation (Table 3). During the later periods the extent of increase was reduced to such a way that it approached control levels after 96 hrs of incubation. Comparative levels of GSH also showed that the rise was localized up to 48 hrs of time period. The levels of GSH, however, recorded decrease of 15% and 20% between 48-72 hrs and 72-96 hrs of intervals (Fig. 1).

Several authors (Asada and Takahashi 1987, Bowler *et al*. 1992) have reported higher activities of the antioxidant enzymes under conditions of stimulated ROS synthesis. We have also observed remarkable increases in the specific activities of APX, SOD and PX. Higher GSH levels were also observed during initial phase of senescence. An increase in the activities of these antioxidants was probably indicative of improved protection against the surge of ROS that usually accompany the process of senescence (Leshem 1988). Pastori and del Rio (1994a, b, 1997) observed increased activities of $O_2^{\cdot-}$ producing xanthine oxidase, H_2O_2-generating Mn-SOD and urate oxidase, with a concomitant decrease in catalase activity in peroxisomes isolated from dark-induced senescent pea leaves. In a previous work, we have also observed remarkable stimulation of SOD, APX and PX in etiolated rice leaves (Banerjee *et al*. 2002). Increased SOD activity indicated that there was greater conversion of $O_2^{\cdot-}$ to H_2O_2. Superoxide radicals in combination with H_2O_2 may give rise to more lethal hydroxyl radicals (Polle 1997, Noctor and Foyer 1998). The initial increases in APX, PX and GSH might have been an effective measure to prevent these cascade reactions. The decrease in AsA levels in senescing leaves during the entire experimental period might be due to a stimulation of the breakdown pathway viz. oxidized ascorbate being converted to oxalate and

threonate, and/or impairment in the biosynthetic pathways (Loewus and Loewus 1987).

Glutathione synthesis was reportedly stimulated under conditions that enhanced the formation of H_2O_2 (Smith 1985). Many herbicides that stimulate GSH levels do so by inhibiting CAT activity, which ultimately may lead to higher concentrations of H_2O_2 (Smith 1985). In the present experiment, we observed a steady decrease in CAT activity, and a simultaneous increase in SOD activity that might have resulted in an accumulation of H_2O_2. The increase in the GSH content during initial phases of senescence might be due to a stimulation of its biosynthetic pathways to counteract the threat of H_2O_2 or other ROS. Maintenance of the biosynthetic pathways requires optimal functioning of the key enzymes e.g.γ–glutamylcysteine synthase and glutathione synthase along with a constant supply of ATP (Rennenberg and Lamoureux 1990). The biosynthetic enzymes of GSH might be reduced during the later phase of senescence. The reduction in the level of GSH between 48-72 hrs and 72-96 hrs might be the consequence of such interference in the biosynthetic processes. Further the decrease in GSH levels might also result from an enhancement in the catabolic pathways as compared to the synthetic pathways.

The activity profile of PX in the present study deserves special mention. Kar and Mishra (1976), working with 8-weeks old rice plant, observed an increase in PX activity during detached leaf senescence. These workers also reported that the increase in PX activity after 48 hrs was, however, found to be relatively low. In contrast to the work of Kar and Mishra, we observed a steady increase of PX in senescing leaves, excised from 2-weeks old rice plants, both in terms of net activity as well as the activity with respect to control leaves right up to the final point of incubation. Peroxidase is involved in a very broad spectrum of plant metabolism including regulation of cell elongation, polysaccharide cross-linking, lignification, wound-healing, phenol oxidation and pathogen defense (Lagrimini 1991). Although utilization of H_2O_2 is a general aspect of PX function in many of these metabolic events, generation of H_2O_2 that acts as a co-substrate in the lignin biosynthesis is also a unique aspect of this enzyme function (Elstner and Heupel 1976). The *in vivo* regulation of PX regarding its role in the utilization and generation of H_2O_2 in these events is not well understood (Lagrimini 1991). We have used o-dianisidine as the substrate, which is a non-specific substrate for measuring the *in vitro* PX activity. Apart from the non-specific PX, we have also measured the activities of syringaldazine peroxidase (lignifying PX), NADH oxidase and IAA oxidase at the final time point from both control and senesced leaves (Table 4). The extent of increase of syringaldazine peroxidase and NADH oxidase in senesced leaves was more or less similar (177% and 183% respectively) as compared to that of o-dianisidine PX (198%), while IAA oxidase remained unchanged during senescence. The increase in NADH oxidase in the present study indicates a pro-oxidant threat by virtue of its ability to generate H_2O_2. It is also pertinent to note that during final phase of senescence e.g. between 72-96 hrs (Fig.1), activities of all the antioxidants including APX, SOD and GSH were reduced, in sharp contrast to PX, which was still showing substantial increase (29%) in its activity. So it might be possible, therefore, that there was a "preferential induction" of non-specific and lignifying PX along with NADH oxidase over other antioxidants, which would probably favor the senescence process as well as yellowing in detached rice leaves at final time point. An increase in the NADH-dependent generation of $O_2^{\cdot-}$ radicals and the H_2O_2 concentration along with SOD was observed in peroxisomes of senescing pea leaves (Pastori and del Rio 1997).

Table 4. Changes in the specific activities of substrate-specific peroxidase(s) during final stages of senescence of detached primary rice leaves (values are means±SD)

Period of Senescence	Peroxidase(o-dianisidine)	Syringaldazine peroxidase	NADH oxidase	IAA oxidase
0 hour	1.99±0.09	0.588 ± 0.02	4.32 ±0.05	4.35 ±0.06
96 hours	5.93^{***}± 0.15(198%)	1.63^{***} ± 0.04(177%)	12.24^{***} ± 0.08(183%)	4.36 ± 0.05(0%)

Values significant at *** $P \leq 0.001$

It is apparent from the above discussion that initially there was an increase in the capacity of the antioxidant defense systems during induced senescence of young leaves from two weeks old rice seedlings that might be construed as 'adaptive'. This increase was all the more significant in the sense that degenerative conditions prevail during senescence, as evident from the steady decrease in protein and chlorophyll contents. The enhancement in the capacity of antioxidant defense was one of the major life-saving measures under a pro-oxidant environment. During the final phases, however, detached leaves could not support the synthetic processes to maintain the levels of antioxidants. This was evident from the substantial reduction of all the antioxidants between 72-96 hrs period, except for PX(s), which were still showing a net increase of 29% (Fig. 1). The levels of PX(s) were thus "preferentially" induced during this period. The age of the detached leaves also seems to have a determining role in the extent and pattern of increase of antioxidants including PX(s). In view of the multi-faceted character of PX *vis a vis* its ability to carry out diverse functions including H_2O_2 generation, in addition to its scavenging function, further in-depth study is required to predict the actual role of this enzyme *in planta* under senescing conditions.

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