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RESEARCH PAPER

Location and effects of long-term NaCl stress on superoxide dismutase and ascorbate peroxidase isoenzymes of pea (*Pisum sativum* cv. Puget) chloroplasts

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Abstract

The present work describes the intrachloroplast localization and the changes that took place in the thylakoid and stroma-located superoxide dismutases (SOD, EC 1.15.1.1) and ascorbate peroxidases (APX, EC 1.11.1.11), in response to long-term NaCl stress in *Pisum sativum* L. cv. Puget plants. Native PAGE using high chloroplast protein concentrations pointed to the presence of the two main Fe-SODs, together with CuZn-SODs, both in thylakoids and in the stroma. Western blot and immunogold labelling using the antibodies against chloroplastic Fe-SOD from *Nuphar luteum* also confirmed the chloroplastic localization of a Fe-SOD. Thylakoidal Fe-SOD activity was induced by a NaCl concentration as low as 70 mM, while CuZn-SOD was induced at 90 mM, although in severe stress conditions (110 mM) both activities were similar to the levels at 90 mM NaCl. NaCl stress also induced stromatic Fe-SOD and CuZn-SOD activities, although these inductions only started at higher NaCl concentration (90 mM) and were significant at 110 mM NaCl. The increase in activity of both Fe-SODs was matched by an increase in Fe-SOD protein. Chloroplastic APX isoenzymes behaved differently in thylakoids and stroma in response to NaCl. A significant increase of stromal APX occurred at 70 mM, whereas the thylakoidal APX activity was significantly and progressively lost in response to NaCl stress (70–110 mM). A significant increase in the H_2O_2 content of chloroplasts during stress and a reduction in the ascorbate level at 90 mM NaCl also took place, although the oxidized ascorbate pool at the highest NaCl concentration did not show significant changes. These results suggest that the loss of thylakoidal APX may be an important factor in the increase in chloroplastic H_2O_2, which also results from the increased thylakoid and stroma-located Fe-SOD and CuZn-SOD activities. This H_2O_2 may be involved in the induction of stromal APX. The up-regulation of the above enzymes in the described stress conditions would contribute to the adaptation of cv. Puget plants to moderate NaCl stress.

Key words: AOS, ascorbate peroxidase, chloroplast, *Pisum sativum*, salt stress, superoxide dismutase.

Introduction

Even under optimal conditions, active oxygen species (AOS), such as the superoxide radical ($O_2^{\cdot-}$), H_2O_2 and the hydroxyl radical ($^{\cdot}OH$), are generated as by-products of normal metabolism in different subcellular compartments including the chloroplasts, mitochondria, peroxisomes, and plasma membrane-linked electron transport system (Elstner, 1991; del Río *et al.*, 1998; Asada, 1999). These AOS can damage DNA, proteins, chlorophyll, and membrane functions. Furthermore, the imposition of biotic or abiotic stress may give rise to an excessive concentration

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Abbreviations: AOS, activated oxygen species; Mn-SOD, manganese-superoxide dismutase; Fe-SOD, iron-superoxide dismutase; CuZn-SOD, copper-zinc superoxide dismutase; APX, ascorbate peroxidase; sAPX, stromal APX isoform; tAPX, thylakoid-bound APX isoform; ASC, ascorbate, reduced form; DHA, ascorbate, oxidized form (dehydroascorbate); TPI, triose phosphate isomerase; SDS, sodium dodecyl sulphate; CHAPSO, 3[(3-cholamidopropyl)dimethylammonio]-2-hydroxyl-1-propanesulphonate; MDA, malondialdehyde; $O_2^{\cdot-}$, superoxide radical; PAGE, polyacrylamide gel electrophoresis; NBT, nitroblue tetrazolium; H_2O_2, hydrogen peroxide; MV, methyl viologen; DCMU, dichlorophenyldimethyl urea; CF1a, alpha-subunit coupling factor-1 of ATP synthase; RbcS, ribulose-1,5-*bis*phosphate carboxylase small subunit.

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of AOS, resulting in oxidative damage at a cellular level. To mitigate and repair damage initiated by AOS, plants have developed a complex antioxidant system (Foyer and Mullineaux, 1994; del Río *et al*., 2002).

Superoxide dismutase (SOD) is the first line of defence against injury caused by AOS, catalysing the dismutation of $O_2^{\cdot-}$ to H_2O_2 and molecular oxygen. According to the metal co-factor used by the enzyme, three major SOD types have been described: iron SOD (Fe-SOD), manganese SOD (Mn-SOD) and copper-zinc SOD (CuZn-SOD), which are located in different compartments of the cell. In plants, CuZn-SOD is the most abundant and has been localized in the cytosol, chloroplast, peroxisome, and apoplast (Bueno *et al*., 1995; Ogawa *et al*., 1997), although a thylakoid-associated form has also been reported in spinach and wheat (Ogawa *et al*., 1995; Navari-Izzo *et al*., 1998; Meneguzzo *et al*., 1998). Mn-SOD is predominantly found in the mitochondrial matrix and has also been found in peroxisomes (Sandalio *et al*., 1987). Biochemical studies have revealed the presence of Fe-SOD in taxonomically distant plant species (Bridges and Salin, 1981; Sevilla *et al*., 1984; Van Camp *et al*., 1996; Donahue *et al*., 1997), although it has only been characterized in dicotyledonous plants (Salin and Bridges, 1980; Kwiatowski *et al*., 1985; Almansa *et al*., 1991). Fe-SOD was thought to be absent in monocotyledonous plants because such activity had not been found by SOD activity staining. Recently, however, the presence of Fe-SOD activity has been reported in rice and barley and the sequence of Fe-SOD cDNA for rice has been obtained (Kaminaka *et al*., 1999). Fe-SOD has been located in chloroplasts (Salin and Bridges 1982; Van Camp *et al*., 1996; Meneguzzo *et al*., 1998), and it has been suggested that Fe-SOD is associated with thylakoid membranes in tobacco (Van Camp *et al*., 1996).

Ascorbate peroxidases (APX) comprise a family of isoenzymes with different characteristics and play a crucial role in the detoxification of cellular H_2O_2, the toxic product of superoxide dismutation. APX isoenzymes are distributed in different cellular compartments: chloroplast stroma and membrane-bound to the thylakoid, microbody, mitochondria and cytosol (Mittler and Zilinskas, 1992; Bunkelmann and Trelease, 1996; Jiménez *et al*., 1997*a*, *b*; Ishikawa *et al*., 1998). Recent cloning of APX from *Arabidopsis thaliana* revealed that cytosolic APXs (including putative extrachloroplastic isoforms) are encoded by a multigene family (Santos *et al*., 1996; Caldwell *et al*., 1998), while in spinach and other plants it has been shown that stromal and thylakoid-bound APX isoenzymes arise from a common pre-mRNA through alternative splicing of its 3′-terminal exons (Yoshimura *et al*., 1999). The subcellular compartmentation of defence mechanisms is, therefore, crucial for the efficient removal of $O_2^{\cdot-}$ and H_2O_2 at the sites where they are generated throughout the cell, thus avoiding oxidative stress in some organelles (Mittler and Zilinskas, 1994; Jiménez *et al*., 1998*a*; del Río *et al*., 1998; Asada, 1999). However, the physiological role of individual SOD and APX isoenzymes in plant responses to oxidative stress is inconclusive. The importance of both antioxidant enzymes in stress defence has been demonstrated in transgenic plants overexpressing APX and SOD, which showed enhanced oxidative stress tolerance (McKersie *et al*., 1996; Van Breusegem *et al*., 1999; Allen, 1997). The degree of enhanced tolerance to oxidative stress varies with external conditions, and some interesting differences between the effects of overexpression of Fe-SOD and Mn-SOD in plants have been reported. These differences seem to be related to their differential subcellular locations, which might provoke different protective effects against oxygen radicals (Van Camp *et al*., 1996; Van Breusegem *et al*., 1999). Similarly, protection provided by the *Arabidopsis thaliana* peroxisomal APX gene in tobacco seems to be specific against the oxidative stress originated in peroxisomes, but not in chloroplasts (Wang *et al*., 1999).

As regards salt stress, evidence accumulated from various plant systems shows that this type of stress alters the amount and the activities of some enzymes involved in scavenging AOS and their corresponding steady-state levels of mRNA (Hernández *et al*., 1993, 2000; Gosset *et al*., 1994; Olmos *et al*., 1994; Gueta-Dahan *et al*., 1997). Previously, a detailed study was made of the effect of long-term salt stress on both the NaCl-tolerant pea (*Pisum sativum* L.) cv. Granada and the NaCl-sensitive cv. Challis, and a significant role was found for the antioxidant defences of chloroplasts, mitochondria and cytosol in NaCl tolerance (Hernández *et al*., 1993, 1995, 2000). Using a different pea cultivar, Puget, the presence of two different SOD activity bands in chloroplasts was reported, which were identified as Fe-SOD and CuZn-SOD, respectively (Hernández *et al*., 1999). Moreover, when the response of cv. Puget plants to NaCl stress conditions was characterized, it was found that, in agreement with previous results, the degree of enhanced tolerance to mild NaCl stress seemed to require the induction of specific isoforms of antioxidant enzymes (SOD, APX, MDHAR, DHAR, and GR), depending on whether they are located in the mitochondria, chloroplasts or apoplast (Gómez *et al*., 1999; Hernández *et al*., 2001). Although substantial information now exists on the response to salt stress of the AOS-scavenging system in pea cv. Puget chloroplasts, questions still remain, including, for example, the intraorganellar localization of the different SODs (CuZn-SOD and Fe-SOD) as well as their specific responses and those of chloroplast APX to NaCl stress. In this paper, the localization of Fe-SOD, CuZn-SOD and APX in the stroma and thylakoid of cv. Puget leaves is shown. The effect of NaCl stress on the stromal and thylakoid-bound APX, CuZn-SOD and Fe-SOD, as well as on the chloroplastic ascorbate and H_2O_2 contents was studied.

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This information may contribute towards elucidating the regulation mechanisms of SOD and APX isoenzymes in response to NaCl stress and also help to understand the oxidative stress response of pea plants.

Materials and methods

Growth of plants in salt stress conditions

Pea (*Pisum sativum* L. cv. Puget) seeds, supplied by Sharpes International Seeds Ltd. (Sleaford, England), were surface-sterilized (sodium hypochlorite, 10% v/v) for 2 min, germinated and grown in vermiculite with 0.5 mM $CaSO_4$ for 1 week. Vigorous seedlings were selected for hydroponic culture in pots containing aerated distilled water, in a growth chamber (ASL) under optimal conditions for 7 d, as previously described (Hernández *et al*., 1999). The plants were then transplanted to aerated Hoagland nutrient. After 7 d, 0, 70, 90 or 110 mM NaCl was added to the nutrient solution and plants were left for 2 weeks.

All the experiments were run in quadruplicate at least, and each sample was analysed twice. Statistical analysis of the results from the different experiments was made according to Duncan's multiple range test.

Isolation and purification of chloroplasts

All operations were performed between 0 °C and 4 °C. Intact purified chloroplasts were obtained from pea leaves by differential and density-gradient centrifugation in discontinuous Percoll gradients according to published protocols (Hernández *et al*., 1995). Approximately 80 g of pea leaves were homogenized at high speed for 2–3 s in a 1:4 (w/v) ice-cold isolation medium, using an Osterizer cycle blend homogenizer. The isolation medium consisted of 0.3 M mannitol, 4 mM cysteine, 1 mM EDTA, and 0.2% BSA in 30 mM MOPS buffer pH 7.3. Homogenates were filtered through four layers of nylon cloth and centrifuged at 2200 *g* for 30 s. The pellet was suspended in 0.3 M mannitol, 10 mM MOPS buffer (pH 7.2), 1 mM EDTA, and 0.1% BSA. Chloroplasts purified with this method showed between 70–80% intactness and, on the basis of the specific activities of cytochrome *c* oxidase and catalase, mitochondrial and peroxisomal contaminations were about 1% and 4%, respectively (Hernández *et al*., 1995).

To study APX activity, an independent organelle-isolation procedure was used (20 mM sodium ascorbate added to the extraction medium), while all other solutions contained 2 mM ascorbate to prevent the possible inactivation of APX.

Thylakoid and stromal fractions were separated by centrifugation at 100 000 *g* for 30 min after the intact chloroplasts had been disrupted by osmotic shock in a hypotonic medium (10 mM HEPES-KOH, pH 7.2, 0.1 mM EDTA, 1 mM $MgCl_2$, 10% glycerol). 1 mM sodium ascorbate was added if APX was to be measured. After centrifugation, thylakoid membranes were carefully washed twice with the isolation medium to remove stromal proteins. The supernatants resulting from washing were eliminated and not combined with the stromal fraction. All the steps of this procedure were carried out at 4 °C (Navari-Izzo *et al*., 1998).

Solubilization of thylakoid-bound enzymes

The washed thylakoids were further solubilized at 4 °C, by adding 0.1% CHAPSO to the hypotonic medium described above, and incubated for 30 min with continuous stirring The suspension was ultracentrifuged at 100 000 *g* for 15 min (Miyake and Asada, 1992).

Enzyme assays

Unless otherwise indicated, the activities of all enzymes were assayed in organelle samples diluted 2–5-fold with 50 mM phosphate buffer, pH 7.8, containing 0.1% (w/v) CHAPSO. Triose phosphate isomerase activity was determined according to Feierabend (1975). Total APX (EC 1.11.1.11) and SOD (EC 1.15.1.1) were assayed spectrophotometrically in each sample as described previously (Jiménez *et al*., 1997*a*). APXt was spectrophotometrically determined in the CHAPSO solubilized thylakoids fraction (Jiménez *et al*., 1997*a*). SOD isozymes (CuZn-SOD, Mn-SOD and Fe-SOD) were separated by PAGE in 10% acrylamide gels using 150 μg of chloroplast protein and 100 μg of stromal and thylakoid protein, respectively. SOD activity bands were detected in gels by the photochemical NBT stain of Beauchamp and Fridovich (1971). The isozyme activity was quantified by recording the transmittance of gels on a Shimadzu CS-9000 densitometer, as reported previously (Almansa *et al*., 1994). To detect the stromal APX isoform, native PAGE was carried out as described by Mittler and Zilinskas (1993), and APX activity was detected in the gels by staining with NBT (Jiménez *et al*., 1998*b*), using 200 μg protein of chloroplasts and 100 μg of stromal and thylakoid protein respectively.

Other analytical methods

The H_2O_2 content in purified intact chloroplasts was determined immediately after purification by a peroxidase-coupled assay, using 4-aminoantipyrine and phenol as donor substrates (Hernández *et al*., 1995). Proteins were quantified by using Bradford's method (Bradford, 1976).

Determination of ascorbate and dehydroascorbate

Total ASC was extracted, at 0 °C, from chloroplasts previously isolated using media without ASC or Cys. ASC was extracted from purified chloroplasts by mixing the samples with an equal volume of 10% *m*-phosphoric acid and incubating for 30 min. The mixture was diluted with distilled water to give a final concentration of 2% *m*-phosphoric acid and centrifuged at 12 000 *g* for 10 min. ASC in the supernatant was determined immediately by HPLC, as described by Castillo and Greppin (1988). DHA was quantified from ASC data by incubating the samples for 24 h at room temperature with 1 mM DTT (final concentration). The DHA concentration was measured as ASC after rechromatography (Jiménez *et al*., 1997*a*, *b*).

Western blot analysis

Detergent-solubilized thylakoid and stromal proteins were heated at 95 °C for 5 min in 125 mM TRIS-HCl, pH 6.8, containing 0.5% (w/v) SDS, 0.1% (v/v) glycerol, 100 mM DTT, and 0.05% bromophenol blue. Polypeptides were separated by SDS-PAGE in 12% minigels according to Laemmli (1970), using Bio-Rad Mini Protean II slab cells. Kaleidoscope prestained standards (Bio-Rad) were used to estimate the molecular mass of electrophoresed polypeptides. For immunodetection, SDS polypeptides were electroblotted on nitrocellulose sheets from Schleicher & Schuell (Dassel, Germany) at 15 V for 25 min, using a Trans-Blot semi-dry transfer cell (Bio-Rad). For immunodetection of electrotransferred polypeptides, the membranes were immersed for 1 h at room temperature in TBS (25 mM TRIS-HCl, pH 7.5, 150 mM NaCl) containing 5% (w/v) non-fat dry milk, and then in polyclonal antibody raised against water lily (*Nuphar luteum*) Fe-SOD (Salin and Bridges, 1982) diluted 1:1000 in TBSTA (1%) for 1 h. Bound antibody was detected using alkaline phosphatase-conjugated anti-rabbit immunoglobulin G antibodies (Roche) diluted 1:10 000 in TBSTA. The immunoreactive proteins were visualized using CDP-Star TM (Roche) as the chemiluminescent substrate.

Immunolabelling

Small pieces (1×1 mm) of leaves from pea plants were fixed in 2% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 M

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phosphate buffer (pH 7.2) for 2 h at 4 °C, rinsed in the same buffer and dehydrated in an ethanol series. Samples were embedded in LR White. Thin sections (60 nm) for immunolabelling were collected onto nickel grids covered with formvar. The grids were placed in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin (BSA) for 30 min at room temperature and then incubated overnight at room temperature with Fe-SOD antibody diluted (1:1000) in PBS. The sections were washed three times in PBS and incubated with the secondary antibody (goat anti-rabbit immunogold conjugate, gold particle 10 nm, BioCell International), diluted (1:50) in PBS with 0.5% BSA, for 1 h. Finally, samples were stained with uranyl acetate followed by lead citrate before being observed by Zeiss EM10 and Zeiss EM109 electron microscopy. Data were analysed quantitatively using image analysis (Leica Q500MC). At least 15 micrographs at ×25 000 magnification were used in each determination.

Results

At least four clear SOD isoforms were observed after crude leaf extract proteins were electrophoresed on 10% native PAGE. These isoforms were identified as one Mn-SOD, two CuZn-SODs, named I and II in order of increasing migration, and one Fe-SOD, by using KCN and H_2O_2 (Fig. 1A). When 12% gels were used, the Fe-SOD band appeared to be composed of two different but not clearly-defined bands.

In purified chloroplasts lysed by osmotic shock in the presence of CHAPSO, native PAGE analysis revealed three well-defined SOD activity bands (Fig. 1B), which were identified as two Fe-SODs and one major CuZn-SOD II. In addition, a minor SOD band with similar mobility to that of the CuZn-SOD I of the crude extracts was sometimes apparent, especially when a high concentration of purified chloroplasts was subjected to PAGE. This SOD band showed higher electrophoretic mobility than Fe-SODs and was resistant to KCN, but sensitive to H_2O (Fig. 1C, D), indicating the inhibitor response characteristic of Fe-SOD. Figure 2 shows the suborganellar localization of the chloroplast SOD isoenzymes using isolated stroma and thylakoid preparations subjected to native PAGE analysis. Both CuZn-SOD and Fe-SOD isoenzymes were located in the stroma and in thylakoids (Fig. 2B, C). The minor SOD activity band sensitive to H_2O_2 appeared mainly in the stroma fraction after PAGE (Fig. 2B).

Since the quantification of SOD isozymes activity was carried out by densitometric analysis of the gels, which were always loaded with the same amount of thylakoid and stromal proteins, the specific activity values in each compartment were used to calculated the distribution percentages of Fe-SOD and CuZn-SOD (Table 1). These percentages were obtained by dividing the specific activity of each enzyme in each fraction by the sum of its specific activity in the stroma and solubilized thylakoids, after considering TPI activity as a stromal marker. The analysis showed that around 60–65% of Fe-SOD specific activiy was localized in thylakoids and 35–40% in the stromal fraction. Similarly, CuZn-SOD was mainly (70–73%) recovered in the thylakoids, whilst about 25–27% of CuZn-SOD specific activity was found in the stromal fraction.

To confirm the identification of Fe-SOD obtained by determining enzyme activity, the proteins extracted with CHAPSO from thylakoids and those of the stromal preparations were subjected to immunoblot analysis. It was assumed that the antibodies raised against chloroplastic Fe-SOD in *Nuphar luteum* would react with a pea homologue. Thus, the immunoblot analysis presented in Fig. 3 revealed a cross-reacting band in extracts of pea leaves in the same location where the activity was exhibited, although some minor non-specific association with the antibodies was also observed. Western blot analysis following denaturing gel electrophoresis of stromal and thylakoid proteins, with the antiserum raised against chloroplastic Fe-SOD from *Nuphar luteum,* revealed a polypeptide of about 42 kDa in the stroma and a polypeptide of about 44.6 kDa in the thylakoids

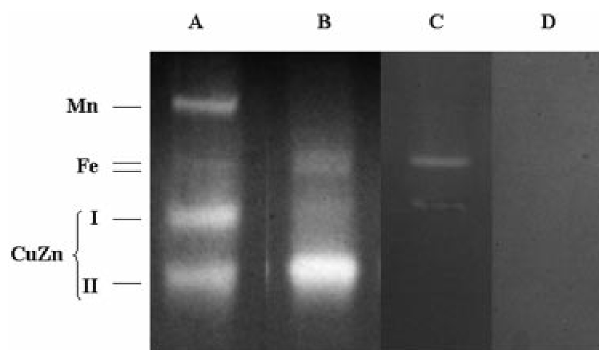

Fig. 1. Separation on non-denaturing activity gels of SOD isoforms occurring in purified chloroplasts from pea leaves. (A) Total activity in crude extract; (B) total activity in purified chloroplasts; (C) staining in the presence of 2 mM KCN; (D) staining in the presence of 5 mM H_2O_2. Samples applied to the gel contained (A) 250 µg and (B, C, D) 150 µg of proteins, respectively.

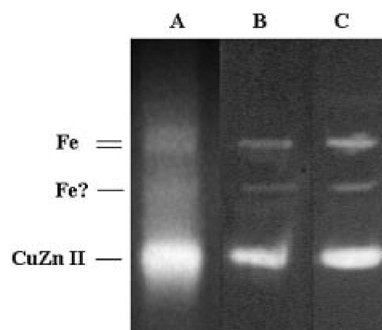

Fig. 2. Suborganellar localization of SOD isoforms in chloroplasts from pea leaves. (A) Total chloroplasts; (B) stromal fraction; (C) thylakoid fraction. Samples applied to the gel contained (A) 150 µg and (B, C) 100 µg of proteins, respectively.

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Table 1. *Intraorganellar distribution of Fe-SOD, CuZn II-SOD and APX in chloroplasts purified from pea leaves*

The activity of each isozyme was expressed as units mg^{-1} protein for SOD and as nmol min^{-1} mg^{-1} protein for APX and TPI. The percentage of activity was calculated by dividing the specific activity of each isoenzyme in the thylakoids or stroma by the total activity, considered as the sum of its specific activity in solubilized thylakoids plus stroma, as described in the Materials and methods. Triose phosphate isomerase (Triose P I) was used as a stroma marker enzyme. Data are the means of at least four different experiments.

Fraction	% Intraorganellar distribution			
	Fe-SOD	CuZn II-SOD	APX	Triose P I
Stroma	35–40	27–30	75–80	93–97
Thylakoids	60–65	70–73	20–25	3–7

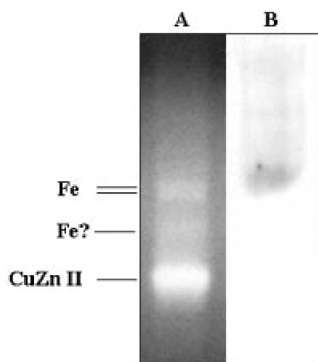

Fig. 3. Activity staining of chloroplast SODs (A) and western blot analysis from native PAGE of chloroplast Fe-SOD (B). Samples applied to the gel contained (A) 150 µg and (B) 400 µg of proteins, respectively.

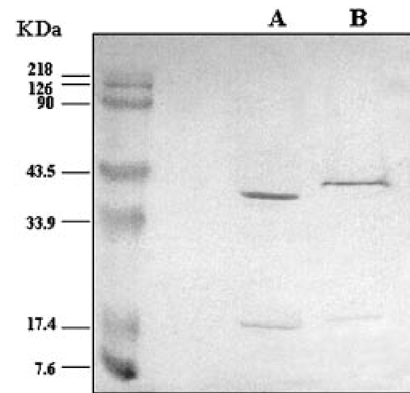

Fig. 4. Western blot analysis from SDS PAGE of stromal (A) and thylakoid (B) Fe-SOD in chloroplasts from pea leaves. Stroma and thylakoid samples applied to the gels contained 200 µg of proteins. The numbers on the left indicate the masses of standard proteins (kDa).

(Fig. 4A, B). In both chloroplastic fractions, a faster-migrating minor band was also recognized, which corresponded to an 18–20 kDa polypeptide. The intensity of the 18–20 kDa immunoreactive band was lower in the thylakoidal membrane fraction (Fig. 4B). The results obtained with antiserum raised against Fe-SOD from *Chlamydomonas reinhardtii* were similar to those obtained with the antibodies against Fe-SOD of *Nuphar luteum* (data not shown). Immunogold labelling was also localized within the chloroplasts, with relative densities of about 60% located in the thylakoids and 40% in the stroma after the quantitation of gold particles μm^{-2} (Fig. 5).

Analysis of the relative distribution of APX activity in the stroma and thylakoids, based on determining its specific enzyme activity spectrophotometrically and after correcting for thylakoid contamination by stromal proteins (TPI activity), showed that the percentage of specific APX activity recovered in the stromal fraction, at least 75–80%, was higher than that in thylakoids, which represented between 20–25% of the total chloroplastic APX activity (Table 1).

Native PAGE analysis of the stromal and thylakoid fractions indicated that the stromal APX appeared as one well-resolved wide band (Fig. 6B) with the same mobility as that in total chloroplasts (Fig. 6A). Although the medium was never depleted of ascorbate and all the samples were immediately run on the same gels, it was difficult to identify the thylakoid-bound APX isoform. Occasionally, a slight APX activity band with the same mobility as the stromatic APX isoform was apparent in thylakoid preparations after native PAGE (Fig. 6C). In addition, two white bands were also evident in the stroma and were faint, but visible, in the thylakoids. However, it is not clear whether these bands represent activity that results from aggregated enzymes or whether it is the outcome of enzymes of very low mobility (Mittler and Zilinskas, 1993), so they were not considered as APX isozymes.

The response of chloroplast antioxidant enzyme activity to NaCl stress was analysed in both thylakoid and stromal fractions. As regards the SODs, together with CuZn-SOD only the two bands cross-reacting with the antibodies were analysed for the quantification of Fe-SOD activity in the different electrophoretic runs. In general, NaCl produced an increase of Fe-SOD and CuZn-SOD isozymes. The specific activity of thylakoidal Fe-SOD started to increase at 70 mM NaCl and continued to increase together with thylakoidal CuZn-SOD up to 90 mM (1.94- and 1.83-fold, respectively), both showing similar activity values at 110 mM NaCl (Fig. 7A, B). NaCl treatment also induced stromatic Fe-SOD and CuZn-SOD specific activities. In the case of Fe-SOD, the induction started at a higher NaCl concentration (90 mM) than that needed for inducing the thylakoidal isoforms, and this increase was significant at 110 mM NaCl (2.56-fold). Similar to that described for thylakoidal CuZn-SOD, the stromatic CuZn-SOD isozyme was induced at 90 mM NaCl and was increase further at 110 mM (1.5- and 2.56-fold, respectively) (Fig. 7A, B).

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The increase in thylakoid- and stroma-located Fe-SOD activity was accompanied by an enhanced Fe-SOD protein content, as detected by western blot of thylakoids and stromal preparations (Fig. 8). Chloroplastic APX isoenzymes behaved differently in thylakoids and stroma in response to NaCl treatment. A significant increase in stromal APX activity occurred at 70–90 mM NaCl, and plants grown at 110 mM showed APX activity similar to control plants (Fig. 9A), whereas a decrease began early in tAPX specific activity (53% at 70 mM NaCl) and it decreased further at 90 mM and 110 mM (2.8-fold) (Fig. 9B). A substantial increase in the chloroplast H_2O_2 content also occurs under salt stress conditions (being 12.6 nmol mg^{-1} protein in controls), which also started at 70 mM NaCl and was 3.5-fold higher in plants at 110 mM (Table 2). The ascorbate content was examined in chloroplasts from control and NaCl-treated plants. The pool of chloroplast ascorbate was relatively insensitive to 70 mM NaCl, but was slightly diminished by 90 mM. This involved a decrease in the ASC and an increase in the DHA contents, which resulted in a lower chloroplast ASC/DHA ratio. Further intensification of the NaCl stress (110 mM) resulted in a 19.6% decrease in total ascorbate although the ASC/DHA ratio was higher than in chloroplasts from 90 mM NaCl-plants (Table 2).

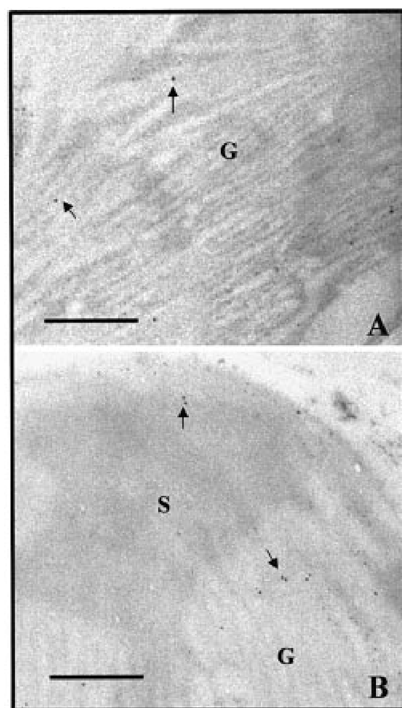

Fig. 5. Immunolocalization of Fe-SOD in chloroplasts of pea plants. (A) Details of thylakoids. (B) Details of stroma and thylakoids. G: grana, S: stroma. Arrows: gold particles. Bar=0.5 µm.

Discussion

The difficulty involved in identifying Fe-SOD in different species of higher plants is related to their low enzymatic activity and low expression (Salin and Bridges, 1980; Almansa *et al.*, 1991; Gueta-Dahan *et al.*, 1997). In pea leaves, constitutive Fe-SOD activity is low, but the use of an improved protocol with a higher amount of chloroplast proteins and high gel concentrations revealed that the band initially identified as Fe-SOD was constituted by two different Fe-SOD isozymes. In addition, a rapidly-migrat-

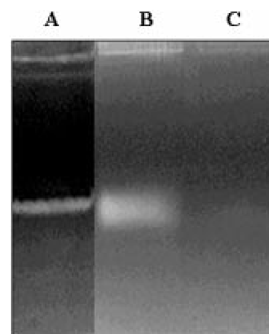

Fig. 6. Activity staining of stromal and thylakoid ascorbate peroxidase isozymes after native-PAGE of pea chloroplasts. (A) Chloroplasts; (B) stromal fraction; (C) thylakoid fraction. Samples applied to the gel contained (A) 200 µg and (B, C) 100 µg of proteins, respectively.

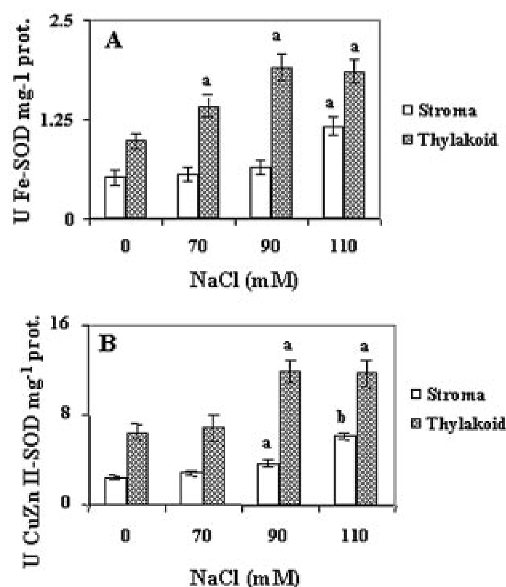

Fig. 7. Effect of NaCl concentration in the culture medium on the level of Fe-SOD (A) and CuZn II-SOD (B) isoenzyme activity in the stroma and thylakoids from pea plant chloroplasts, after correction for stromal contamination. The activity of the enzymes was calculated by means of densitometric analyses of the bands that have been detected by native-PAGE. Values are the means of at least four different experiments ±SE. Differences from control values were significant at: (a) P <0.05, (b) P <0.01, according to Duncan's Multiple Range Test.

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ing minor SOD enzyme was identified; this appears to be a Fe-SOD (resistant to KCN and sensitive to H_2O_2), in spite of it co-migrating with the soluble CuZn-SOD. A similar overlapping between cytosolic CuZn-SOD and chloroplastic Fe-SOD was reported in control and transgenic tobacco plants (Van Camp *et al*., 1996).

In this work, the approach used to obtain stromal and thylakoid fractions agrees well with that widely described for the analysis of stromatic, peripheral and lumenal thylakoid proteins, including SOD and APX isozymes (Navari-Izzo *et al*., 1998; Miyake *et al*., 1993; Peltier *et al*., 2000). Since the pea thylakoid fraction is not further fractionated into thylakoid lumen and membranes there is no reason to worry about potential contamination of the stromal fraction by thylakoid proteins.

Native PAGE of solubilized thylakoids and stroma clearly localized both Fe-SOD and CuZn-SOD in both fractions. Immunoblot analysis in parallel with the activity gel analysis indicated that the antibodies against *Nuphar luteum* Fe-SOD recognize the two main native enzymes of pea, but not the faint (putative) Fe-SOD. Similar non-recognition results were reported for Fe-SOD activity bands observed in MV and DCMU-treated *Arabidopsis thaliana* plants. Thus, it appears that members of the Fe-SOD family seem to be sufficiently distinct in sequence so as not to cross-hybridize (Alscher *et al*., 2002). On the other hand, the low activity shown by this Fe-SOD presumably reflects a low protein content, that would be reduced considerably after electroblotting, which could well be hindering its recognition by the antibodies. However, the possibility that the antibodies recognized epitopes that were less accessible in this Fe-SOD could also be considered. The use of this antibody to localize Fe-SOD in pea chloroplastic fractions confirmed the presence of this enzyme both in thylakoids and in the stroma (Fig. 4A, B). Immunogold labelling also showed the localization of Fe-SODs in both chloroplast fractions. Reports on Fe-SOD localization in thylakoids are scarce. In wheat chloroplasts, immunoblot analysis confirmed the presence of an Fe-SOD in the PSII membrane and also showed that, in addition to the previously-found CuZn-SOD (Navari-Izzo *et al*., 1998), an Fe-SOD was also present in thylakoids (Navari-Izzo *et al*., 1999). In chloroplasts from tobacco a preferential membrane location for Fe-SOD has been suggested (Van Camp *et al*., 1996).

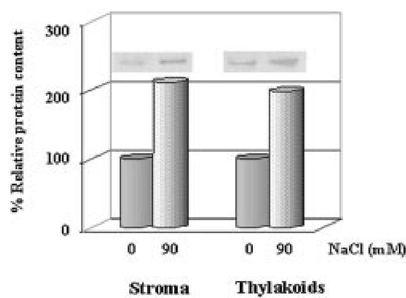

Fig. 8. Fe-SOD protein content determined by densitometric analysis after western blot of stroma and thylakoid Fe-SOD from chloroplasts of control and NaCl-treated pea plants, using polyclonal antibodies against Fe-SOD from *Nuphar luteum*. The bands shown are dimeric forms of Fe-SOD of 42 and 44.6 kDa from stromal and thylakoid fractions, respectively.

From the overall results obtained by western blots with Fe-SOD antibodies, it seems that the thylakoid- and stroma-located Fe-SODs in pea chloroplasts have apparent molecular masses of about 44 and 42 kDa, respectively, in the range of the 40–48 kDa reported for other Fe-SODs, which are homodimers (Duke and Salin, 1985; Kwiatowski *et al*., 1985; Salin and Bridges, 1980, 1982; Almansa *et al*., 1994). Thylakoid Fe-SOD seems to be about 2 kDa larger than stromatic Fe-SOD and could reflect some additional differences in sequence. Similar differences were found also between tAPX and sAPX

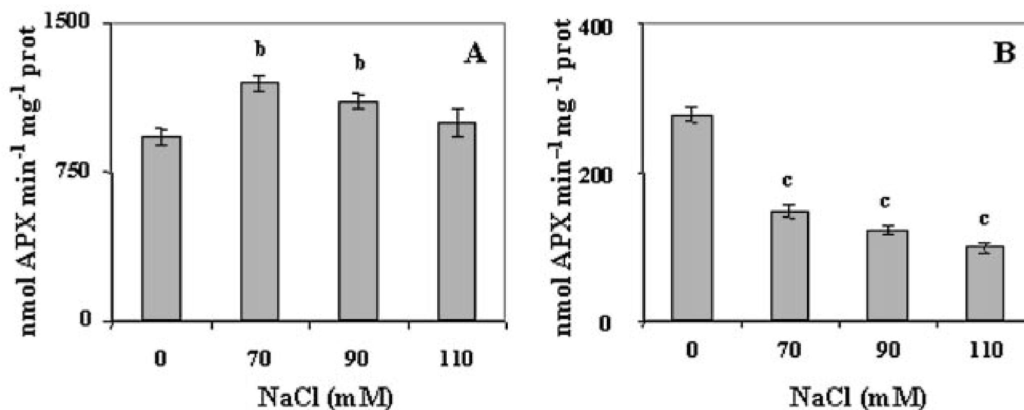

Fig. 9. Effect of NaCl concentration in the culture medium on the level of APX activity in the stroma (A) and thylakoids (B) of chloroplasts from pea plants, after corrections for stromal contaminations. Values are the means of at least four different experiments ±SE. Differences from control values were significant at: (b) $P < 0.01$ and (c) $P < 0.001$, according to Duncan's Multiple Range Test.

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Table 2. *Effect of NaCl concentration in the culture medium on ascorbate (ASC), dehydroascorbate (DHA) and H_2O_2 content in chloroplasts of the pea plants.*

For each parameter, chloroplasts obtained from two density gradients were combined and processed. Values are the means of at least four different experiments ±SE. Differences from control values were significant at (a) $P < 0.05$, according to Duncan's Multiple Range Test.

NaCl (mM)	ASC (μg mg^{-1} protein)	DHA (μg mg^{-1} protein)	ASC/DHA	H_2O_2 (nmol mg^{-1} protein)
0	0.43±0.01	0.11±0.01	3.91	12.59±1.54
70	0.41±0.02	0.10±0.02	4.10	15.08±1.34
90	0.34±0.04	0.15±0.01(a)	2.27	26.22±3.95(a)
110	0.29±0.02(a)	0.09±0.01	3.20	44.03±9.13(a)

(Chen and Asada, 1989; Shigeoka *et al*., 2002). The appearance of two polypeptide bands after SDS-PAGE of the enzyme suggests that pea Fe-SODs are resistant to dissociation by SDS since they are not completely converted into the monomeric form. This resistance to SDS denaturation has been reported for other SODs from different organisms, including Mn-SOD from pea leaves (Sevilla *et al*., 1980).

As regards APX, it was reported that the tAPX/sAPX ratio varies according to plant species and, possibly, leaf age. In pea chloroplasts, a lower tAPX/sAPX ratio than in spinach was found (Miyake and Asada, 1992), although these results agree with those of Mittova *et al*. (2000) in tomato. The lack of an apparent tAPX activity band agrees with previous results and with the low stability described for chloroplast APX, especially for the tAPX activity isoform (Miyake and Asada, 1993, 1996; Van Camp *et al*., 1996; Meneguzzo *et al*., 1998). As regards its stability, tAPX from pea seems to be more related to the peroxisomal isozyme (perAPX) than to the mitochondrial (mitAPX) isoform previously reported (Jiménez *et al*., 1998*b*).

The modification of AOS-scavenging systems can lead to considerable changes in oxidative stress tolerance (Alscher *et al*., 2002; Hernández *et al*., 2001). NaCl-stress conditions led to an oxidative stress in the chloroplasts of pea leaves. This NaCl-induced oxidative stress, which has been observed in other pea cultivars (Hernández *et al*., 1995), is manifested as increases in the MDA and H_2O_2 contents and is accompanied by an up-regulation of both thylakoid and stromal CuZn-SODs and Fe-SODs, as well as of stromal APX. The analysis by western blot indicated that the increases in thylakoidal and stromal Fe-SOD activities were accompained by a higher amount of Fe-SOD protein (Fig. 8). The fact that this increase in protein levels was clear at 90 mM NaCl suggests that it was a result of the oxidative stress induced by NaCl treatment. Thus, it seems that NaCl might have induced a general response in the biosynthesis of both thylakoidal and stromal Fe-SODs. A similar suggestion was reported for thylakoid- and stroma-located CuZn-SOD in wheat treated with Cu (Navari-Izzo *et al*., 1998**.** This idea is lent weight if it is considered that Fe-SODs are sensitive to H_2O_2, and that, under NaCl stress, the chloroplast H_2O_2 content was significantly enhanced (Table 2). This inactivating effect would be even more evident in thylakoids than in the stroma due to the NaCl-dependent increase in the SOD/APX ratio found in thylakoids, which may upset the equilibrium between $O_2^{\cdot-}$ radicals and H_2O_2. However, a comparable increase in protein amount and activity occurs for the thylakoidal Fe-SOD under NaCl, whereas at 90 mM NaCl, a higher rise in protein amount than in activity occurred for the stromal Fe-SOD (Fig. 7). This difference could be due to NaCl-induced post-translational inactivation of the stromal Fe-SOD and may reflect differing characteristics of the two Fe-SOD isozymes, although a putative differential regulation of both Fe-SODs at the transcriptional or translational levels cannot be discarded (Mittler and Zilinskas, 1994). A senescence-induced inactivation of mitochondrial Mn-SOD, has been reported, whereas a possible post-translational activation of the peroxisomal Mn-SOD during senescence was suggested (del Río *et al*., 2003).

The unavailability of polyclonal antibodies against chloroplast CuZn-SODs meant that there was no certainty about what was happening to the protein content during NaCl stress. In a different pea cultivar, it was previously observed that 70 mM NaCl-mediated increases in chloroplast CuZn-SOD mRNA levels were even greater than the correponding increases in CuZn-SOD activity (Hernández *et al*., 2000). It has been reported that the rise in CuZn-SOD mRNA levels could be due to its *de novo* synthesis or to its decreased degradation. It has been suggested that mRNA stability may be an important factor in regulating the response to salt stress, as shown in pea plants subjected to drought or chilling stress (Mittler and Zilinskas, 1994; Stevens *et al*., 1997).

Recent results obtained in transgenic tobacco chloroplasts containing *Escherichia coli* catalase and maintained under high light and drought conditions, as well as those reported in paraquat-treated spinach plants, suggest that chlAPX isoenzymes are more inactivated by oxidative stress than is phosphoribulokinase, one of the thiol-modulated enzymes that is most sensitive to H_2O_2

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(Shikanai *et al*., 1998; Mano *et al*., 2001). Similar to the reported results, in pea chloroplasts tAPX seems to be the primary target under NaCl-induced oxidative stress. The important decrease in tAPX activity (53%) was already apparent at 70 mM NaCl (Fig. 9B), when the rise in H_2O_2 content in intact chloroplasts was only 19% (Table 2) and no significant changes in the total ascorbate or in the ASC/DHA ratio had taken place, although a decrease in the ascorbate/H_2O_2 ratio seems to have taken place (Table 2). These results differ from the analysis of Meneguzzo *et al*. (1998), in chloroplasts from seedlings of two NaCl-treated wheat cultivars, that showed a sudden increase in tAPX activity and a decrease in sAPX with 50 mM NaCl in the more sensitive cultivar, whereas a slight increase of both sAPX and tAPX was induced by salt in the more tolerant cultivar. Since the data concerning the H_2O_2 and ascorbate contents refer to whole chloroplasts (Table 2), it may be assumed that the changes in both will be especially pronounced at sites where thylakoid enzymes are found, that is, near the site of $O_2^{\cdot-}$ and H_2O_2 formation. The level of ASC in the vicinity of tAPX may decrease due to the increase in AOS generated by NaCl, which would cause irreversible damage to the tAPX, similar to that reported for tAPX from tobacco (Shikanai *et al*., 1998; Miyagawa *et al*., 2000). In pea chloroplasts, the initial excess of H_2O_2 near tAPX may be favoured by the early induction of thylakoid Fe-SOD at 70 mM NaCl (Fig. 7A) and 90 mM NaCl also for that of thylakoid CuZn-SOD (Fig 7B), although the slight decrease in stomatal conductance previously observed (Hernández *et al*., 1999) may also contribute to the increases in AOS, by limiting photosynthesis. However, it is worth nothing that, since both chloroplastic APX activity and H_2O_2 content are NaCl-dependent, and that the results shown are a long-term response to NaCl, a differentiation between cause and effect cannot be established clearly. On the other hand, the NaCl-induced loss of pea tAPX activity, which participates in the Mehler ascorbate peroxidase cycle (Asada, 1999), may be identified as the principal source of chloroplastic H_2O_2 since it permits H_2O_2 diffusion away from thylakoid membranes to the stroma.

A dual function of AOS ($O_2^{\cdot-}$ and H_2O_2) in exacerbating damage and signalling the activation of defence responses has been described in pathogenesis and also in plant responses to several abiotic stresses (Dat *et al*., 2000; Schraudner *et al*., 1998; Meneguzzo *et al*., 1999). Recently, it was also suggested that NaCl acted as an abiotic elicitor in pea plants, including cv. Puget, in which the benefical role that apoplastic AOS might have in the induction of some antioxidant enzymes under NaCl-stress was reported (Hernández *et al*., 2001). Similarly, the induction of sAPX by NaCl may be mediated by the level of H_2O_2 in chloroplasts. This increase in H_2O_2 and the NaCl-induced lipid peroxidation previously reported (Gómez *et al*., 1999) may affect H_2O_2 efflux from the chloroplasts into the cytosol. This suggestion, and the increase in both chloroplast Fe-SOD activities, agrees with the reported H_2O_2-mediated induction of one of the chloroplast soluble APXs in Fe-SOD-transformed tobacco plants under salt-stress conditions (Van Camp *et al*., 1996). Similar H_2O_2-mediated inductions of both the *Apx 2* gene in *Arabidopsis* and the cytosolic *Apx* gene in rice were reported (Karpinski *et al*., 1999; Morita *et al*., 1999).

Regarding ASC, it was found that, although the chloroplast ASC content decreased at 110 mM NaCl (Table 2), the DHA levels did not increase, which explains why the ASC/DHA ratio was higher at this concentration than at 90 mM NaCl and fits well with the progressive and significant rise (around 4.5-fold) of DHAR previously reported in chloroplasts at 110 mM NaCl (Gómez *et al*., 1999). The increase in DHAR activity may be required to sustain the activity of oxidized ascorbate when the flux through the ASC-GSH cycle is increased, as may occur in transgenic plants with enhanced Fe-SOD (Arisi *et al*., 1998). In pea chloroplasts, NaCl stress seems to induce a similar situation. Thus, these data sustain previous suggestions concerning the important function of stromal APX, GR and DHAR cycling activities as being the main pathway in pea chloroplasts for ascorbate recycling. These activities, together with the induction of thylakoid-bound and stromal Fe-SODs and CuZn-SODs, indicate that the co-ordinated up-regulation of chloroplast antioxidant enzymes, in both thylakoids and stroma, is an important factor in the reported adaptation of cv. Puget to moderate NaCl stress (Hernández *et al*., 1999), since it allows a moderate level of AOS to be maintained in the chloroplasts. Another factor which would contribute to the NaCl response, and to the protection of cv. Puget, is the strong interaction between apoplast and symplast in the control of the apoplastic ASC content, and in the induction of apoplastic CuZn-SOD and symplast DHAR, GR and MDHAR, which was described under moderate NaCl stress (Hernández *et al*., 2001).

Further research is necessary to study the regulation of the expression of chloroplastic antioxidant enzymes during NaCl stress. However, as reported by Shigeoka *et al*. (2002), a major question which still needs to be answered is whether the instability of APX has a physiological role *in vivo*. The loss of tAPX in cv. Puget under NaCl stress might have a function related to stress signalling.

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