Activity and protein level of AO isoforms in pea plants (Pisum sativum L.) during vegetative development and in response to stress conditions

Edyta Zdunek-Zastocka2*, Rustem T. Omarov1, Tomokazu Koshiba3 and Herman S. Lips1

1 Biostress Research Laboratory, J Blaustein Institute for Desert Research and Department of Life Sciences, Ben-Gurion University of the Negev, Sede-Boqer 84990, Israel
2 Department of Biochemistry, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warsaw, Poland
3 Department of Biological Sciences, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan

Received 19 August 2003; Accepted 20 February 2004

Abstract

Among three AO isoforms detected in pea plants, the activity of PAO-1 was dominant in leaves of seedlings and young leaves of mature plants, while PAO-3 revealed the highest band intensity in old leaves and roots. PAO-1 and PAO-3 are homodimers consisting of 145 kDa and 140 kDa subunits, respectively, while PAO-2 is a heterodimer of one 145 kDa and one 140 kDa subunit. In leaves, the activity of PAO-1 disappeared gradually with leaf ageing, while in roots it was present only in seedlings but not in mature pea plants. PAO-3 could oxidize abscisic aldehyde, a precursor of abscisic acid, indicating the possible involvement of this isoform in ABA synthesis in pea. The ability of PAO-3 to oxidize abscisic aldehyde was higher in old leaves than in young ones and increased significantly both in roots and leaves of plants exposed to salinity and ammonium treatments. A marked increase of the AO protein level was observed after ammonium application but not under salinity. Interestingly, the activity of PAO isoforms may be transcriptionally and post-transcriptionally regulated during vegetative growth and in response to stress conditions, and such a regulation might be particularly important to adjust ABA levels to the recent requirements of the plant. The observations suggest that the AO isoforms have different metabolic roles and that the activity and protein level of each isoform is regulated not only by environmental conditions but also through plant developmental stages.

Key words: Aldehyde oxidase, AO, ammonium, Pisum sativum L., plant development, salinity, stress conditions.

Introduction

Aldehyde oxidase (AO; EC 1.2.3.1) belongs to the family of molybdenum hydroxylases and catalyses the oxidative hydroxylation of a number of diverse aldehydes and aromatic heterocyclic compounds in reactions that necessarily involve the cleavage of a C-H bond (Hille, 1996). AO has been extensively characterized in animals and micro-organisms, where it has been implicated in the detoxification of environmental pollutants and xenobiotics (Bauer and Howard, 1991). In plants, however, the information about AO is limited and focused particularly on an involvement of the enzyme in the final step of plant hormone biosynthesis, such as indole-3-acetic acid (IAA) and abscisic acid (ABA). The enzyme catalyses in the cytosol the oxidation of indole-3-acetaldehyde (IAAld) to IAA (Koshiba et al., 1996; Seo et al., 1998) and abscisic aldehyde (ABAld) to ABA (Taylor et al., 1988; Walker-Simmons et al., 1989; Seo et al., 2000b). The endogenous ABA level increases upon stress conditions, most notably under drought and salinity (Munns and Cramer, 1996). The application of exogenous ABA creates effects similar to plant-stress responses and increases plant tolerance to environmental stresses (Fedina et al., 1994; Welbaum et al., 1997). Elucidation of the regulation mechanisms of the ABA biosynthetic pathway is, therefore, essential to understand the adaptive stress-induced mechanisms in plants.

* To whom correspondence should be addressed. Fax: +48 22 853 09 50. E-mail: zdunek@delta.sggw.waw.pl
Two enzymes, involved in the early steps of ABA synthesis in chloroplasts, have recently attracted much attention as the enzymes having a putative key role in the modulation of ABA levels in the plant. Zeaxanthin epoxidase (ZEP) converts zeaxanthin to violaxanthin by a two-step epoxidation reaction and appears to be rate-limiting for ABA biosynthesis in non-photosynthetic tissues such as roots and developing seeds (Marin et al., 1996; Audran et al., 1998). The over-expression of ZEP in transgenic tobacco resulted in enhanced seed dormancy (Frey et al., 1999). Epoxycarotenoid dioxygenase (NCED) cleaves the 9-cis-isomers of both violaxanthin and neoxanthin to cis-xanthohin and a C25 epoxy apocarotenoid (Cutler and Krochko, 1999). Over-expression of the LeNCED1 in tomato (Thompson et al., 2000) and AtNCED3 in Arabidopsis (Iuchi et al., 2001) resulted in increased ABA levels. It was suggested that the epoxycarotenoid cleavage reaction might be the key regulatory step of drought-induced ABA biosynthesis (Qin and Zeevaart, 1999; Iuchi et al., 2000).

The oxidation of abscisic aldehyde to abscisic acid may constitute another important element in the regulatory mechanism of stress-induced ABA biosynthesis, most probably controlled at various steps of the pathway and in a tissue- and growth condition-specific manner. Plant AO has only been purified from coleoptiles of maize (Koshiba et al., 1996). Two AO cDNAs (zmAO-1 and zmAO-2) with a high homology to each other at both the nucleotide and amino acid sequence levels have been subsequently cloned for maize (Sekimoto et al., 1997). Four cDNAs (atAO-1, 2, 3, and 4) encoding AO proteins have been cloned for Arabidopsis (Sekimoto et al., 1998; Hoff et al., 1998), while three putative AO cDNAs have been isolated from tomato (Min et al., 2000). Information on AO regulation in plants is limited and little is known about its sensitivity to environmental factors. AO activity increased in ryegrass plants exposed to salinity and/or NH4+, although the salinity-enhanced activities were more pronounced in roots than in leaves (Sagi et al., 1998). Root, but not shoot, AO activity was enhanced by salinity in barley plants, especially in the presence of ammonium in the nutrient medium (Omarov et al., 1998). An increase of AO activity, particularly in old leaves, was observed in pea plants exposed to salinity, nitrogen deficiency as well as to ammonium treatment (Zdunek and Lips, 2001). Plant aldehyde oxidase exhibited broad substrate specificity with respect to different aromatic and aliphatic aldehydes (Koshiba et al., 1996; Akaba et al., 1998; Koiwai et al., 2000). However, AO isoforms that can efficiently oxidize abscisic aldehyde to abscisic acid have only been identified recently in Arabidopsis rosette leaves (Seo et al., 2000a) and in roots of barley (Omarov et al., 2003). The AOδ isoform, that could efficiently oxidize abscisic aldehyde in rosette leaves of Arabidopsis (Seo et al., 2000a), is encoded by AAO3 (formerly called atAO-3), one of four Arabidopsis AO genes previously cloned by Sekimoto et al. (1998). When the Arabidopsis rosette leaves were detached and exposed to dehydration, the expression of mRNA encoding AOδ increased rapidly, but the activity was unaffected (Seo et al., 2000a). Drought-induced increase of abscisic aldehyde oxidase activity was, however, observed in leaves of Arabidopsis thaliana by Bittner et al. (2001).

The detailed mechanisms of molecular and biochemical regulation of AO in plants, as well as the physiological function of AO isoforms are just starting to be unravelled. In the present study, AO activity changes and the protein level of each AO isoform following stress application as well as during plant development have been investigated. The roles of particular AO isoforms in stress-induced ABA synthesis and during plant development are discussed.

Materials and methods

Plant material and experimental conditions

Seeds of Pisum sativum L. (cv. Little Marvel) were germinated in moist vermiculite. After 14 d, the seedlings were transferred to 20 l containers containing aerated half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938) with an adequate nitrogen supply (4 mM NO3). The residual cotyledons were removed from the seedlings after 3 d of adaptation to hydroponics, and the treatments were initiated. Nitrogen was provided as 4 mM NaNO3 or 2 mM (NH4)2SO4. Salinity was supplied at a concentration of 100 mM NaCl. Plants grown with 4 mM NO3 in the absence of salinity were taken as controls. The pH of the medium (pH 6.5) was adjusted daily and the nutrient solutions were replaced weekly. Experiments were conducted in a greenhouse with temperatures ranging between 25–28 °C during the daytime and 15–18 °C during the night, with a 14 h photoperiod, and a midday photosynthetic photon flux density of c. 900–1000 μmol m−2 s−1.

For the ‘developmental’ experiments, seedlings were harvested 6 d after sowing while mature plants were collected at the age of 3 and 5 weeks. For the ‘stress treatments’ experiments, plants were harvested 7 d after application of the treatments. Plants were separated into roots and young (half expanded), mature (fully expanded), and old leaves. Fresh samples were used for immediate enzyme activity assays or frozen in liquid N2 and stored at −80 °C for other analysis.

Native PAGE and AO activity staining

Plant tissues were homogenized immediately after harvesting with acid-washed sand and an ice-cold extraction medium containing 250 mM TRIS-HCl, pH 8.5, 1 mM EDTA, 10 mM GSH, and 2 mM DTT. A ratio of 1 g tissue to 3 ml buffer (1:3 w/v) was used for leaves and 1 g tissue to 2 ml buffer (1:2 w/v) for roots. The homogenized plant material was centrifuged at 18 000 g and 4 °C for 15 min. The resulting supernatant was subjected to native polyacrylamide gel electrophoresis (PAGE) on 7.5% polyacrylamide gels in a Laemmli buffer system (Laemmli, 1970) in the absence of SDS at 4 °C. Each lane in the gel was loaded with 100 μg root proteins or 400 μg leaf proteins. After electrophoresis, AO activity staining was developed at room temperature in a mixture containing 0.1 M TRIS-HCl, pH 7.5, 0.1 mM phenazine methosulphate, 1 mM MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium-bromide), and 1 mM substrate (abscisic aldehyde or imidole-3-aldehyde). AO activity was estimated on the basis of MTT reduction, which resulted in the development of specific formazan.
bands. The relative intensity of formazan bands was directly proportional to enzyme activity (Rothe, 1974). Native PAGE was carried out with a Protean II xi Cell (Bio-Rad, USA).

**Western blot analysis**

Protein extraction was carried out essentially as described above with the exception that a ratio of 1 g tissue to 1 ml extraction buffer (1:1 w/v) was used for SDS-PAGE. The extracts were centrifuged at 18 000 g and 4 °C for 15 min. Aliquots of the supernatants containing 300 μg leaf proteins were combined with the same volume of sample loading buffer (125 mM TRIS-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue), and heated for 5 min at 100 °C before being subjected to SDS-PAGE.

Native-PAGE and SDS-PAGE were carried out in 7.5% polyacrylamide gels (Laemmli, 1970). The separated proteins were electrophoretically blotted onto a nitrocellulose membrane (0.2 μm pore size; Schleicher and Schüll, Dassel, Germany) at 2 mA cm⁻² for 2 h. Unbound sites on the membranes were blocked with 5% (w/v) non-fat dry milk (Blotting Grade Blocker, Bio-Rad, USA) at 25 °C before being subjected to SDS-PAGE. Western-blot analysis following protein separation by native PAGE revealed at least three AO proteins both in leaves (Fig. 1) and in roots (Fig. 2) of 6-d-old seedlings. In leaf extracts, a faint fourth protein band with the lowest mobility in gel was also observed. The location of immunologically detected proteins corresponded to the AO activity bands. In leaves, the protein level of PAO-1 was significantly higher than those of PAO-2 and PAO-3. Immunodetection of AO proteins was carried out with polyclonal rabbit antibodies raised against Arabidopsis AAO1 and AAO2 peptides (Akaba et al., 1999). For extracts of pea plants, both antibodies gave similar results and the immunoblots obtained with anti-AAO2 antibodies were chosen for presentation.

**AO isoforms in pea plants**

Staining of proteins extracted from leaves of 3-week-old plants with indole-3-aldehyde as the substrate revealed three AO activity bands (Fig. 1) corresponding to the location of the AO bands detected in pea seedlings. Only one band of AO activity was detected in root extracts (PAO-3, Fig. 2). The ability of leaf AO isoforms to oxidize indole-3-aldehyde varied between leaves at different developmental stages. In young leaves, PAO-1 showed the strongest activity, while in old leaves the highest ability to produce formazan bands corresponded to PAO-3. In fully expanded leaves, PAO-1 and PAO-3 were detected at a similar activity level. When abscisic aldehyde was used as substrate, two activity bands were detected in leaf extracts (PAO-2, PAO-3) and one in roots (PAO-3). In 5-week-old plants, three AO activity bands were detected with indole-3-aldehyde only in young leaves (Fig. 1). Two AO activity bands (PAO-2, PAO-3) were detected in fully expanded leaves, while only one band was visible in old leaves (PAO-3). When the gel was stained with abscisic aldehyde, fully expanded and old leaves revealed only the PAO-3 activity band. The level of PAO-3 activity, developed either with indole-3-aldehyde or abscisic aldehyde, was significantly higher in old leaves when compared with that of young and fully expanded leaves.

At least three leaf proteins cross-reacted with the anti-AO antibodies regardless of leaf developmental stage and the age of the plant (Fig. 1). However, PAO-1 was predominantly expressed in young leaves while the PAO-3 protein level was slightly higher in old leaves. In mature and old leaves the protein content of the PAO-1 and PAO-2 isoforms remained roughly at the same level throughout development.
plant vegetative development, thus suggesting a post-translational inactivation leading to a gradual disappearance of activity which was observed in fully expanded and old leaves of 5-week-old plants. Only one AO protein band was detected in the roots of 3- and 5-week-old plants (Fig. 2).

**AO isoforms as affected by stress conditions**
A significant increase in abscisic aldehyde (ABAld)-oxidase activity was observed both in leaf (Fig. 3) and root extracts (Fig. 4) after 7 d of exposure to salinity and ammonium. Within leaves of three different ages the most pronounced stress-induced enhancement of AO activity was observed in old leaves. No increase of ABAld-oxidase activity following stress application was observed in young leaves. Within two AO isoforms able to oxidize abscisic aldehyde (PAO-2, PAO-3), the lower one with the highest mobility in the gel (PAO-3) was particularly induced by unfavourable growth conditions both in leaves and roots. The increase in leaf and root ABAld-oxidase activity after 7 d of stress treatments was more pronounced in ammonium-treated plants than under salinity.

As detected by native western-blot analysis, the level of AO proteins, particularly of PAO-3, increased significantly both in roots and leaves of plants grown under ammonium treatment (Figs 3, 4). The salt-induced increase of the AO protein level was much less remarkable.

SDS-PAGE immunoblot analysis revealed, in leaf extracts, the presence of two polypeptides with molecular weights of about 145 kDa and 140 kDa each (Fig. 5). A polypeptide with a molecular weight of about 90 kDa, which was also observed in the analysed extracts, may be a proteolytic product of the 145 kDa subunit. Polypeptides with a similar molecular weight have been previously reported in maize coleoptiles (Koshiba et al., 1996) and barley roots (Omarov and Lips, 2000; Omarov et al., 2003). Stress treatments brought about a significant increase in the protein level of the 140 kDa subunit, especially in ammonium-treated plants (Fig. 5).

**Subunit composition of AO isoforms extracted from pea leaves**
Anti-AO antibodies that cross-reacted with the denatured PAO-1 isoform from pea leaves revealed a polypeptide with a molecular mass of 145 kDa, while the PAO-3 band was presented as a single 140 kDa polypeptide. The PAO-2 isoform consisted of one 145 kDa and one 140 kDa polypeptide (Fig. 6).
Discussion

Unlike animal species, where AO is present as a single gene copy (Li Calzi et al., 1995; Terao et al., 1998), in plants, the enzyme is represented by a group of several proteins with different electrophoretic mobility, substrate specificity, and subunit composition. Studies reporting organ distribution of aldehyde oxidases showed AO polymorphism of gel electrophoretic bands mainly in leaves of dicotyledones such as Arabidopsis (Schwartz et al., 1997), tomato (Sagi et al., 1999), and roots of monocots such as ryegrass (Sagi et al., 1998), barley (Omarov et al., 1999), and maize (Barabás et al., 2000). In Pisum sativum, the polymorphism of aldehyde oxidase isoforms, observed in both leaves and roots of seedlings, changes during plant vegetative development. The isoform with the lowest gel mobility (PAO-1) exhibited the highest intensity of activity band in leaves of seedlings and young leaves of mature pea plants, but disappeared gradually with leaf ageing. The protein content of this isoform in leaves remained roughly at the same level during vegetative development, thus indicating a post-translational inactivation taking place, especially in fully expanded and old leaves of 5-week-old plants (Fig. 1). In roots, the PAO-1 isoform was only detected in seedlings, but not in mature pea plants (Fig. 2). PAO-1 was unable to oxidize abscisic aldehyde to abscisic acid (Figs 1, 2, 3, 4). Thus, PAO-1 may play an important role in the development of pea plants that is unrelated to ABA production, but is linked to the yet undefined events in young organs. A unique function during plant development was also suggested for the products of each of the two maize AO cDNAs; zmAO-1 was expressed at a high level in roots, whereas zmAO-2 was expressed in coleoptiles (Sekimoto et al., 1997).

Nothing is known about the involvement of aldehyde oxidase in any of the developmental processes that occur in plants and, at the present stage, it is difficult to speculate about any specific role of the enzyme throughout the life cycle of pea plants. However, apical meristems of shoots, young leaves, and developing fruits are considered as the primary sites of indole-3-acetic acid (IAA) synthesis in higher plants (Bandurski et al., 1995), from where auxin moves basipetally toward the root system (Estelle, 1998). The high activity of PAO-1 in young leaves (Fig. 1) may therefore suggest the involvement of PAO-1 in IAA synthesis in these organs. Aldehyde oxidase has been
As determined by immunodetection following SDS-PAGE, the three AO isoforms found in leaves of *Pisum sativum* were dimers. PAO-1 and PAO-3 are homodimers consisting of 145 kDa and 140 kDa subunits, respectively, while PAO-2 is a heterodimer of one 145 kDa and one 140 kDa subunit (Fig. 6). Subunit composition similar to pea AOs was found for three AO isoforms detected in seedlings of *Arabidopsis thaliana* (AO\(\alpha\), AO\(\beta\), AO\(\gamma\), Akaba et al., 1999) and in roots of barley (AO2, AO3, AO4; Omarov et al., 2003). Two different polypeptides, 150 and 145 kDa, forming the three dimeric AO isoforms in *Arabidopsis* were products of two cDNAs, AAO1 and AAO2 (Akaba et al., 1999). Immunodetection of pea AO proteins was carried out with polyclonal rabbit antibodies raised against *Arabidopsis* 30 kDa AAO2 peptide. Each lane in the gel was loaded with 100 \(\mu\)g of root proteins. Nitrate was supplied as 4 mM NaNO\(\text{3}\), ammonium as 2 mM (NH\(\text{4}\))\(\text{2}\)SO\(\text{4}\), salinity as 100 mM NaCl. Plants grown with 4 mM NO\(\text{3}\) in the absence of salinity were the controls. The zymogram and immunoblot represent similar results obtained in five different experiments.

assumed to be implicated in IAA biosynthesis from tryptophan (Koshiba et al., 1996; Seo et al., 1998), although no direct evidence for this hypothesis has been reported in any plant.

As determined by immunodetection following SDS-PAGE, the three AO isoforms found in leaves of *Pisum sativum* were dimers. PAO-1 and PAO-3 are homodimers consisting of 145 kDa and 140 kDa subunits, respectively, while PAO-2 is a heterodimer of one 145 kDa and one 140 kDa subunit (Fig. 6). Subunit composition similar to pea AOs was found for three AO isoforms detected in seedlings of *Arabidopsis thaliana* (AO\(\alpha\), AO\(\beta\), AO\(\gamma\), Akaba et al., 1999) and in roots of barley (AO2, AO3, AO4; Omarov et al., 2003). Two different polypeptides, 150 and 145 kDa, forming the three dimeric AO isoforms in *Arabidopsis* were products of two cDNAs, AAO1 and AAO2 (Akaba et al., 1999). Immunodetection of pea AO proteins was carried out with polyclonal rabbit antibodies raised against *Arabidopsis* 30 kDa AAO2 peptide. Each lane in the gel was loaded with 100 \(\mu\)g of root proteins. Nitrate was supplied as 4 mM NaNO\(\text{3}\), ammonium as 2 mM (NH\(\text{4}\))\(\text{2}\)SO\(\text{4}\), salinity as 100 mM NaCl. Plants grown with 4 mM NO\(\text{3}\) in the absence of salinity were the controls. The zymogram and immunoblot represent similar results obtained in five different experiments.
AO isoforms in pea plants

thaliana by Bittner et al. (2001). These findings on stress-induced changes of ABAld-oxidase activity in pea plants are only the second to be published. In pea plants, there seems to be only one AO isoform, PAO-3, that might be responsible for stress-induced ABA production. PAO-3 activity was higher in old than in young leaves and increased significantly both in roots and leaves of plants exposed to salinity or ammonium treatment (Figs 3, 4). The increase of PAO-3 activity, developed with indole-3-aldehyde, but not abscisic aldehyde as the substrate, was observed previously in roots and leaves of pea plants exposed to salinity, ammonium, and low nitrogen treatment (Zdunek and Lips, 2001). In the same experiment the increase of root PAO-3 activity observed under stress was accompanied by the enhancement in ABA xylem delivery rate. The ABA accumulated in leaves of stressed pea plants might, therefore, originate in roots in view of the higher ABA xylem delivery rate or may be a result of in situ synthesis in leaves (Zdunek and Lips, 2001). It has been suggested previously that the old leaves are the main ABA producers in plant shoots both under control as well as stress conditions (Zeevaart and Boyer, 1984; Zhang and Zhang, 1994; Zdunek and Lips, 2001). In pea plants exposed to salinity and ammonium treatment, the ability of PAO-3 to oxidize abscisic aldehyde did not change in young leaves but increased significantly in old leaves (Fig. 3). Thus, the observed leaf PAO-3 activity changes indicate once more that, in pea shoots, the stress-induced synthesis of ABA takes place in old leaves but not in young ones.

The level of PAO-3 protein increased significantly in old leaves and roots of plants grown with ammonium in the nutrient solution, but not under salinity (Figs 3, 4). The changes in relative AO protein abundance observed after ammonium application may have contributed at least partially to the changes of PAO activity. Under salt treatment, however, the post-translational activation of a latent PAO protein should be taken under consideration. The activity of AO in the ABA mutants such as aba3 of Arabidopsis thaliana, aba1 of tobacco, and flaca of tomato could be restored by in vitro anaerobic sulphuration with dithionite and Na₂S (Schwartz et al., 1997; Akaba et al., 1998; Sagi et al., 1999). Thus, it was assumed that these mutants are defective in the gene encoding the sulphurase responsible for the final step of the AO molybdenum cofactor (Moco) maturation, the conversion of a dioxo/desulpho form of Moco to a mono-oxo/sulphid form. The enzyme catalysing this reaction, the Mo-hydroxylase sulphurase, has been recently cloned in Arabidopsis (Bittner et al., 2001; Xiong et al., 2001) and in tomato (Sagi et al., 2002). The gene expression of Arabidopsis Mo-hydroxylase sulphurase was induced by drought-stress (Bittner et al., 2001). Therefore, the sulphuration of the AO-Moco might be an important regulatory mechanism of AO activity involved in the regulation of ABA biosynthesis. The sulphuration step is reversible in vitro (Wahl and Rajagopalan, 1982; Schwartz et al., 1997), although, it is not known whether this reversibility has biological significance.

In spite of the extensive study of plant AO during recent years, it is still problematic to ascribe well-defined physiological roles to this enzyme and to explain the occurrence of its polymorphism in plant tissue. Apart from the implication of AO in plant hormones synthesis, the enzyme may also fulfil other physiological tasks. In fact, liver AO carries out biochemical detoxification function (Li Calzi et al., 1995). If AO has a similar detoxification function in plants, then plant environmental adaptability may require the maintenance of AO polymorphism to deal with a wide range of toxic substrates.

References


Taylor IB, Linforth RST, Al-Naieb RJ, Bowman WR, Marples BA. 1988. The wiltly tomato mutants *flacca* and *sittens* are impaired in the oxidation of ABA-aldehyde to ABA. *Plant, Cell and Environment* 11, 739–745.


