Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 338 (2005) 169–174
BBRC
www.elsevier.com/locate/ybbrc

Review

α-Dioxygenases

Mats Hamberg [a,*], Ines Ponce de Leon [b], Maria Josefa Rodriguez [c], Carmen Castresana [c]

[a] Department of Medical Biochemistry and Biophysics, Division of Physiological Chemistry II, Karolinska Institute, S-17177 Stockholm, Sweden
[b] Departamento Biología Molecular, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay
[c] Centro Nacional de Biotecnologia, Campus Universidad Autonoma, Cantoblanco, E-28049 Madrid, Spain

Received 1 August 2005
Available online 24 August 2005

Abstract

α-Dioxygenases constitute a family of fatty acid-metabolizing enzymes recently discovered in plants. The present paper gives a brief overview of the literature dealing with these enzymes and additionally reports the new finding of an α-dioxygenase in the moss, *Physcomitrella patens*, and some properties of this enzyme.

Keywords: α-Dioxygenase; Fatty acid; 2-hydroperoxide; *Physcomitrella patens*; α-Oxidation

Fifty years ago, Stumpf [1] found that palmitic acid was converted into pentadecanal when exposed to a preparation from peanut cotyledons. A stoichiometric amount of carbon dioxide was liberated during the conversion, which appeared to involve oxidation at the α-carbon (C-2) of the fatty acid chain and therefore named "α-oxidation." In subsequent work, α-oxidation of various C_n fatty acids into C_{n-1} aldehydes together with varying amounts of C_n-hydroxy acids and C_{n-1} fatty acids was studied in preparations from several plants. The putative α-oxidation enzyme(s) was suggested to operate together with aldehyde dehydrogenase and NAD^+ to provide a pathway for stepwise degradation of fatty acids into shorter chain homologues (for review, see [2]). Trapping experiments with glutathione peroxidase [3] or stannous chloride [4] suggested that fatty acid (hydro)peroxides serve as intermediates in α-oxidation. However, it was not until 1999 that an enzyme catalyzing oxygenation of fatty acids into 2-hydroperoxides was discovered [5]. This enzyme was originally detected in tobacco as a pathogen-induced oxygenase ("PIOX") showing homology to mammalian prostaglandin endoperoxide synthases-1 and -2 [6]. Studies of the catalytic function of the recombinant tobacco oxygenase and a homologous oxygenase from *Arabidopsis* using a set of fatty acids revealed conversion of each fatty acid into a 2(*R*)-hydroperoxy derivative, and accordingly, the new class of enzymes was named "α-dioxygenases" [5,7]. Members of this family of enzymes catalyze the initial step of the α-oxidation sequence (Fig. 1) and may additionally be involved in plant defensive reaction against bacteria and other pathogens.

Catalytic properties of α-dioxygenases

α-Dioxygenases from tobacco, Arabidopsis, cucumber, and rice

Incubation at 23 °C of linolenic acid with recombinant α-DOX1 from tobacco or *Arabidopsis* was accompanied by oxygen uptake and led to the formation of a major product identified as 8(*Z*),11(*Z*),14(*Z*)-heptadecatrienal [5]. When the reaction was conducted at 0 °C, formation of 8,11,14-heptadecatrienal was strongly suppressed and instead 2(*R*)-hydroperoxylinolenic acid appeared as the main product. In agreement with previous studies on the chemistry of 2-hydroperoxy carboxylic acids [8], 2(*R*)-hydroperoxylinolenic acid was chemically unstable and in aqueous buffer underwent a first order decay ($t_{1/2}$, ca. 30 min at 23 °C) into 8,11,14-heptadecatrienal. The stereochemistry of the α-dioxygenase reaction followed from

* Corresponding author.
E-mail address: Mats.Hamberg@mbb.ki.se (M. Hamberg).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.117

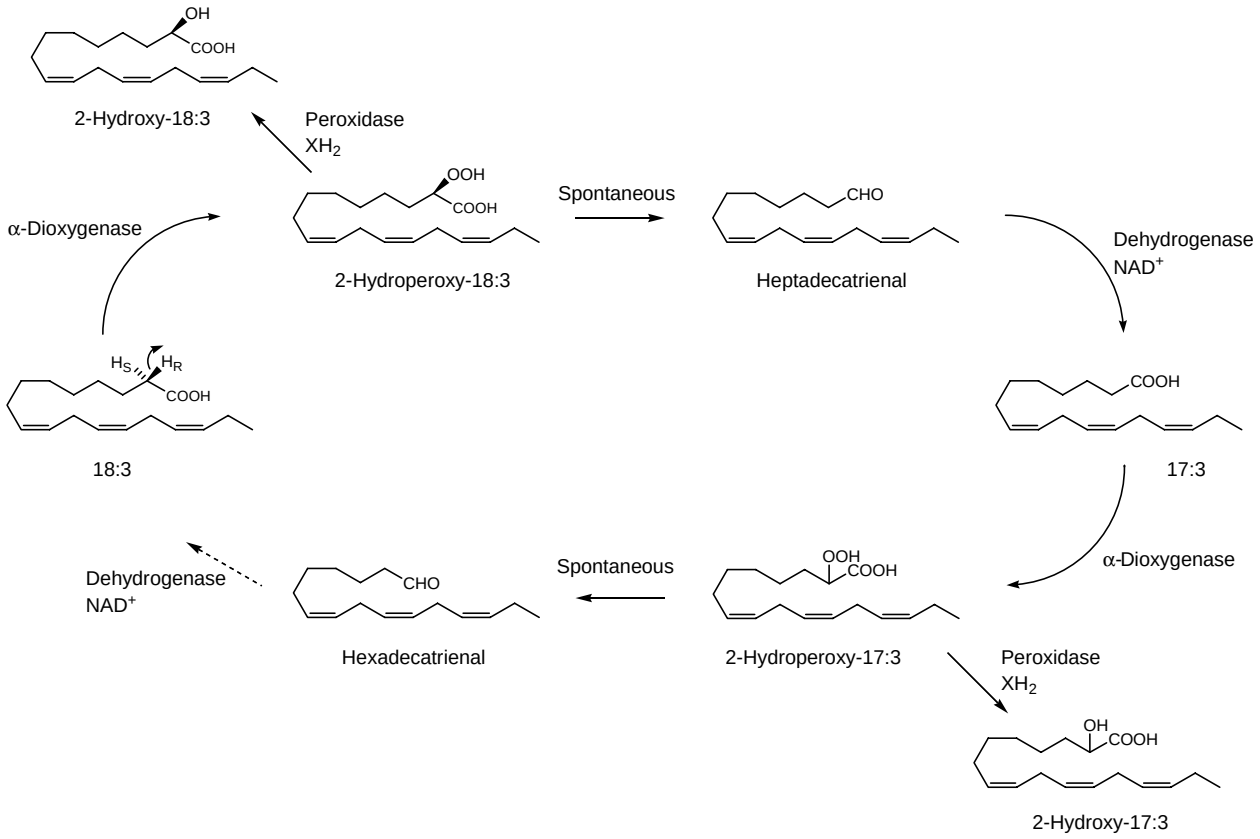

Fig. 1. α-Oxidation of linolenic acid (18:3) in plants. As shown, peroxidase activity will inhibit the oxidation spiral by reducing part of the 2-hydroperoxides into 2-hydroxides.

the absolute configuration established for the 2-hydroperoxy acid products, i.e., "*R*," and from isotope studies using linolenic acid stereospecifically deuterated in the C-2 position [9]. As seen in Fig. 1, the reaction involved stereospecific abstraction of the *pro-R* hydrogen from C-2 followed by insertion of molecular oxygen with retention of absolute configuration. α-DOX from cucumber [5] and rice [10] also produced unstable 2-hydroperoxy acids as determined using linolenic and palmitic acids, respectively, as the substrates.

The α-DOX1 enzymes from tobacco and *Arabidopsis* showed significant homology to prostaglandin H synthases-1 and -2, and like the prostaglandin synthases possessed heme-binding proximal and distal histidines [6]. The two enzymes possessed insignificant peroxidase activity [5,6], and lack of peroxidase activity was noted also for the related α-dioxygenases from cucumber [5] and rice [10]. A recent study of recombinant α-DOX1 from *Arabidopsis* showed that cyanide bound relatively weakly to the heme vicinity (K_d ca. 10 mM) and that carbon monoxide, azide, and imidazole showed even less binding [11]. It was concluded that α-DOX1 resembles a *b*-type cytochrome although with much more restricted access to the heme moiety. Interestingly, cyanide strongly inhibited the enzyme (K_i ca. 5 μM) and showed high-affinity binding to an unknown non-metal center distinct from the heme [11].

α-Dioxygenase from pea

Palmitic acid incubated at 0 °C with a fatty acid α-dioxygenase enzyme complex purified from germinating pea was converted into 2-hydroperoxypalmitic acid as the major product [12]. Gel filtration indicated a molecular mass of 230 kDa, and analysis of the SDS-denatured enzyme by SDS–PAGE revealed the presence of two subunits, i.e., a 50-kDa enzyme identical to a turgor-responsive NAD^+ aldehyde dehydrogenase from pea and a 70-kDa enzyme showing similarity to α-DOX1 from tobacco and *Arabidopsis*. Incubations in the presence of the peroxidase co-substrate guaiacol led to reduction of 2-hydroperoxypalmitic acid into 2-hydroxypalmitic acid demonstrating the presence of appreciable peroxidase activity. The α-dioxygenase complex purified from pea has the capacity of performing a complete cycle of α-oxidation. It is noteworthy that in the presence of a suitable peroxidase hydrogen donor, the 2-hydroperoxy fatty acids will be reduced into 2-hydroxides and thus escape from the cycle (Fig. 1).

α-Dioxygenase from Ulva pertusa

A preparation from the green alga *U. pertusa* has been reported to contain an enzymatic activity which catalyzes the formation of 2(*R*)-hydroperoxypalmitic acid from

palmitic acid [13], thus indicating that α-oxidation of fatty acids in marine algae proceeds by the same mechanism as that in higher plants. Medium- and long chain aldehydes such as 8,11,14-heptadecatrienal exhibit a distinct seaweed odor, and it can be speculated that the capacity of generating this type of compound is widely spread among marine algae.

α-Dioxygenase from Physcomitrella patens

The moss *P. patens* has been found to be a rich source of lipoxygenase activity [14]. In addition, we here report the presence of a prominent α-dioxygenase activity in this primitive plant. Thus, incubation of palmitic acid (100 μM) with a whole homogenate preparation of the moss afforded two hydroxy acids in a 10:1 ratio, i.e., 2-hydroxypalmitic acid and 2-hydroxypentadecanoic acid, the major of which was identified using a chemically synthesized specimen [15] as reference (Fig. 2, inset). Steric analysis of the (−)-menthoxycarbonyl derivative [5] of the enzymatically generated 2-hydroxypalmitic acid demonstrated that the absolute configuration at C-2 was "*R*." Although 2-hydroperoxy acids were not detected with this whole homogenate preparation, conversion of palmitic acid into the two 2-hydroxy acids mentioned is readily explained by the α-oxidation scheme shown in Fig. 1. In further experiments, fatty acids ranging in chain length from C_{12} to C_{20} were incubated with the enzyme preparation and the yield of 2-hydroxy acids was determined by GC–MS. Palmitic acid was clearly the preferred substrate in this series of compounds (Fig. 2). This fact and the finding that oleic acid was a very poor substrate for the enzyme gave biochemical evidence for the *Physcomitrella* α-dioxygenase being distinct from α-DOX1 of tobacco and *Arabidopsis*.

To further analyze the presence of α-dioxygenase in *P. patens*, an EST database was searched (http://moss.nibb.ac.jp) [16] and a gene was found with high sequence similarity to α-dioxygenases of higher plants. We completed the sequence of the putative transcript (pphb11c23), and the deduced amino acid sequence showed 49–53% identity to α-dioxygenases of higher plants, containing the two conserved heme-binding histidines. Sequence alignment of the putative *P. patens* α-DOX with those of higher plants is shown in Fig. 3A. The amino acid identity levels between α-dioxygenases of higher plants were higher (59–95%), placing the putative *P. patens* α-DOX as a distinct phylogenetic group (Fig. 3B). These observations are consistent with the fact that the last common ancestor of bryophytes and higher plants diverged 450 million years ago. Interestingly, exposure of *P. patens* to plant pathogens resulted in a rapid induction of α-dioxygenase transcript (Ponce de León et al., unpublished results).

Biological roles of α-dioxygenases

The recent identification of a number of α-dioxygenase-encoding genes in different plant species has opened a new avenue to investigate the role and relevance of this enzymatic activity. Studies with tobacco and *Arabidopsis* α-DOX1 revealed the participation of this enzyme in plant

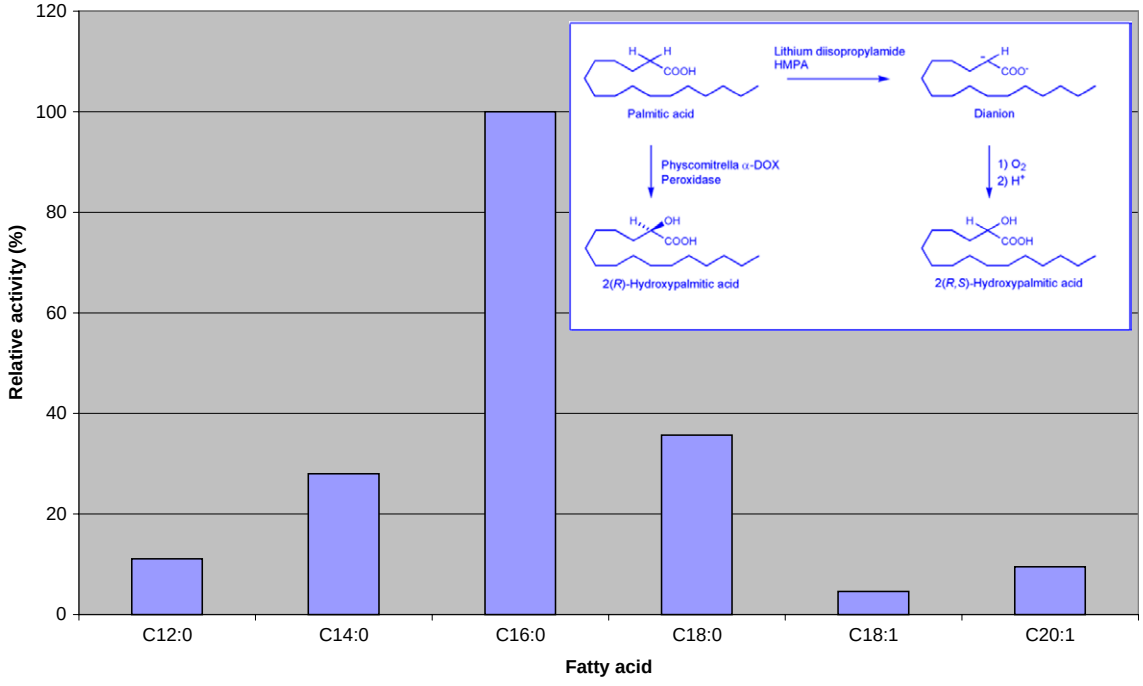

Fig. 2. Oxidation of fatty acids by *P. patens* α-dioxygenase. Whole homogenates of *P. patens* were incubated at 23 °C for 30 min with fatty acids C_{12}–C_{20} (300 μM) and the amounts of the corresponding 2-hydroxy fatty acids were determined by GC–MS. Inset: oxidation of 16:0 by *P. patens* α-dioxygenase and chemical synthesis of 2-hydroxypalmitic acid.

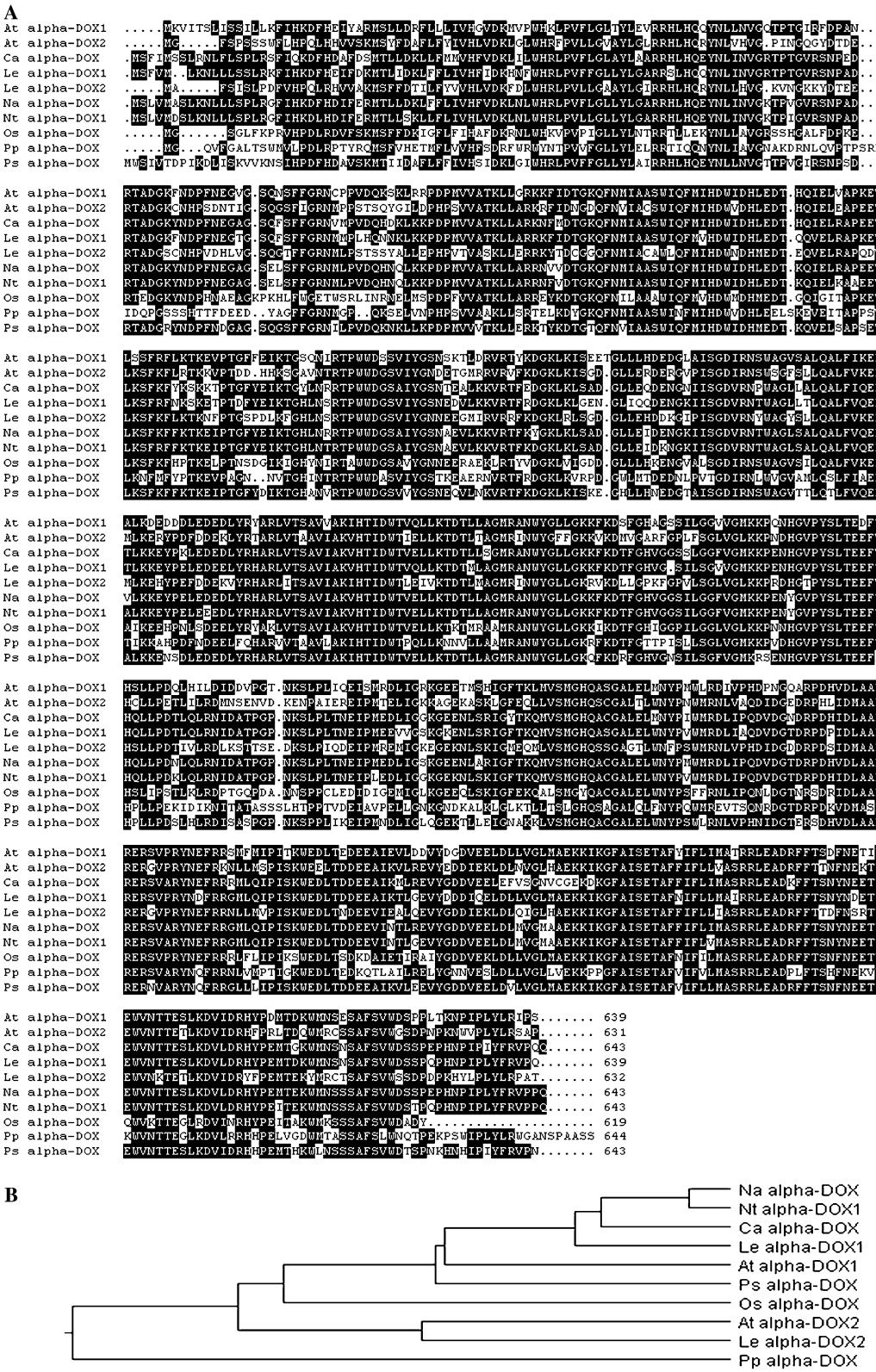

Fig. 3. Sequence alignment and phylogenetic tree of α-DOX proteins of plants. (A) Sequences shown correspond to the *Arabidopsis thaliana* α-DOX1 and α-DOX2 proteins GenBank Accession Nos. (At alpha-DOX1: AAL87742, At alpha-DOX2: AAG52078), *Capsicum annuum* α-DOX (Ca alpha-DOX: AAK85133), *Lycopersicon esculentum* α-DOX1 and α-DOX2 (Le alpha-DOX1: AAR05646, Le alpha-DOX2: CAH64542), *N. attenuata* α-DOX (Na alpha-DOX: AAG59584), *Nicotiana tabacum* α-DOX (Nt alpha-DOX: CAA07589), *Oryza sativa* α-DOX (Os alpha-DOX: AAF64042), *P. patens* α-DOX (Pp alpha-DOX: cDNA clone pphb11c23), and *Pisum sativum* α-DOX (Ps alpha-DOX: CAH05011). (B) The phylogram shown was constructed with the sequences given in (A).

defence against microbial infection [17,18]. In both plant species, gene expression was highly activated in leaves responding to pathogen inoculation, reaching higher accumulation of transcripts and protein when the infection resulted in a hypersensitive reaction (HR), a well-characterized plant defence response activated through the SA-dependent transduction pathway [19]. The HR involves programmed cell death at the point of pathogen invasion that results in part from an oxidative burst leading to the production of cytotoxic reactive oxygen species. Accordingly, studies on the cellular signals mediating the activation of α-*DOX1* in *Arabidopsis* [18] revealed that gene expression is induced by salicylic acid (SA), intracellular superoxide (O_2^-) or singlet oxygen and nitric oxide (NO), three signal molecules mediating cell death. Further characterization using wild type plants and transgenic lines altered in the production of the α-DOX1 protein supported the correlation between α-DOX1 induction and the HR response, and led to suggest that this enzymatic activity protects plant tissues from undergoing excessive necrosis, and that activation of α-*DOX1* is part of the defence mechanisms induced to protect cells from oxidative stress. In agreement with these results, biochemical studies using bacterially infected tobacco leaves showed that high levels of 2-hydroxylinolenic acid accumulated in HR-responding leaves and that this compound might have a role in controlling cell death [17]. In addition to plant defence against microbial infection, results with *Nicotiana attenuata* and tomato α-*DOX1* indicated that this enzymatic activity might play a role in the response of plants to insect attack and salt stress [20,21].

A second *Arabidopsis* α-dioxygenase-like sequence was previously reported [7]. Moreover, protein alignment to additional α-dioxygenase sequences led to suggest that this *Arabidopsis* protein and the closely related FEEBLY (FB) tomato sequence [22] might define a distinct type of isoform that was designated as α-DOX2. In support of this suggestion, results shown here revealed that expression of α-*DOX2* genes differs significantly from that described above for α-DOX1. Thus, in contrast to α-*DOX1*, analyses in tomato and *Arabidopsis* plants revealed that the α-*DOX2* gene was not induced in response to microbial infection but that expression was weakly activated by mechanical damage (not shown). Furthermore, the accumulation of α-*DOX2* transcripts was highly enhanced in leaves subjected to artificial senescence by leaf detachment. As shown in Fig. 4, a clear increase of α-*DOX2* transcripts was observed three days after leaf detachment. High levels of α-*DOX2* RNA were maintained for three or four additional days in tomato and *Arabidopsis* leaves, respectively, starting to decay at later stages after detachment. Also, a weak induction of α-*DOX1* was observed in tomato and *Arabidopsis* leaves at 5 and 7 days after detachment, respectively. In line with the results discussed above, the induction of α-dioxygenases in detached leaves might be part of the protecting mechanisms activated during senescence to control cell disruption, a critical process to allow appropriate remobilization and redistribution of nutrients to the growing parts of the plant.

Previous studies with tomato plants reported that the α-DOX2 protein plays a role in plant development. This assumption was based on data showing that the mutant *feebly* (*fb*), and a previously described mutant *divaricata* (*div*), were mutated at the α-*DOX2* locus. Both mutants showed a strong phenotypic alteration producing seedlings with high anthocyanin levels that developed into small fragile plants [22]. In accord with these results, data in Fig. 4C show that α-*DOX2* is highly expressed in seedlings of tomato wild type plants at 1 and 2 weeks after seed germination, reaching maximum levels of transcript accumulation in RNA samples from hypocotyls, whereas α-*DOX2* transcripts were nearly absent from similar tissues of *divaricata* mutants (data not shown). Confirmation that mutation of the α-*DOX2* gene was the cause of the *divaricata* phenotype came from sequence and complementation analyses. Comparison of the DNA sequences of the α-*DOX2* gene from

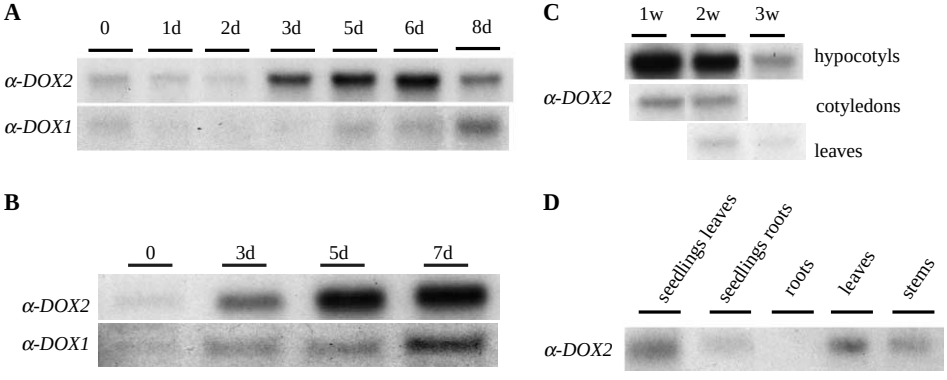

Fig. 4. Analysis of α-*DOX2* and α-*DOX1* gene expression in *A. thaliana* and *L. esculentum* plants during development and in response to leaf detachment. (A) RNA was extracted from detached leaves of mature *L. esculentum* var. Condine; (B) RNA was extracted from detached leaves of mature *A. thaliana* ecotype Columbia; (C) RNA was extracted from different plant organs of healthy *L. esculentum* seedlings at the indicated week (w) after germination; (D) RNA was extracted from different plant organs of healthy *A. thaliana* 8-day-old seedlings and mature 4-week-old plants. Gene expression was examined at the times indicated. Loading controls were analyzed by ethidium bromide staining. Blots were hybridized with riboprobes derived from an *A. thaliana* and *L. esculentum* α-*DOX1* and α-*DOX2*, respectively.

divaricata with its corresponding allele from wild type plants revealed a single nucleotide deletion in exon number 4 that produced a change in the open reading frame creating a stop codon at the amino acid number 433 of the predicted protein. Further confirmation was obtained by results showing the complementation of the *divaricata* phenotype by transformation with a *35S*:α-*DOX2* construct. As shown in Fig. 4D, expression analyses in *Arabidopsis* developing plants detected the presence of α-*DOX2* transcripts in leaves of seedlings and mature plants. However, in contrast to tomato, the characterization of an α-*DOX2* mutant from the SALK collection (SALK-066160, containing a T-DNA insertion in exon number 6 of the α-*DOX2* gene sequence), revealed that the absence of this enzymatic activity does not produce any apparent developmental alteration and, therefore, that significant differences distinguish tomato and *Arabidopsis* with respect to the functionality of the α-DOX2 gene product.

Acknowledgments

The *P. patens* EST was obtained from the RIKEN Biological Research Center. The financial support by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Project 2004-833), the Education and Science Ministry, Spain (Grant BIO2003-02338), DINACYT, Fondo Clemente Estable, Uruguay (Project 9008), and the European Union (Project QLK5-CT-2001-02445) is gratefully acknowledged.

References

[1] P.K. Stumpf, J. Biol. Chem. 223 (1956) 643–649.
[2] T. Galliard, in: P.K. Stumpf, E.E. Conn (Eds.), The Biochemistry of Plants, Vol. 4, Academic Press, London, 1980, pp. 85–116.
[3] W.E. Shine, P.K. Stumpf, Arch. Biochem. Biophys. 162 (1974) 147–157.
[4] W. Adam, W. Boland, J. Hartmann-Schreier, H.-U. Humpf, M. Lazarus, A. Saffert, C.R. Saha-Möller, P. Schreier, J. Am. Chem. Soc. 120 (1998) 11044–11048.
[5] M. Hamberg, A. Sanz, C. Castresana, J. Biol. Chem. 274 (1999) 24503–24513.
[6] A. Sanz, J.I. Moreno, C. Castresana, Plant Cell 10 (1998) 1523–1537.
[7] M. Hamberg, I. Ponce de León, A. Sanz, C. Castresana, Prostaglandins Other Lipid Mediat. 68–69 (2002) 363–374.
[8] W. Adam, O. Cueto, V. Ehrig, J. Org. Chem. 41 (1976) 370–371.
[9] M. Hamberg, A. Sanz, C. Castresana, in: Y. Ishimura et al. (Eds.), Oxygen and Life—Oxygenases, Oxidases and Lipid Mediators. Excerpta Medica International Congress Series, 1233, Elsevier, Amsterdam, 2002, pp. 307–317.
[10] T. Koeduka, K. Matsui, Y. Akakabe, T. Kajiwara, J. Biol. Chem. 277 (2002) 22648–22655.
[11] W. Liu, C.E. Rogge, B. Bambai, G. Palmer, A.-L. Tsai, R.J. Kulmacz, J. Biol. Chem. 279 (2004) 29805–29815.
[12] A. Saffert, J. Hartmann-Schreier, A. Schön, P. Schreier, Plant Physiol. 123 (2000) 1545–1551.
[13] Y. Akakabe, K. Matsui, T. Kajiwara, Tetrahedron Lett. 40 (1999) 1137–1140.
[14] T. Senger, T. Wichard, S. Kunze, C. Göbel, J. Lerchl, G. Pohnert, I. Feussner, J. Biol. Chem. 280 (2005) 7588–7596.
[15] D.A. Konen, L.S. Silbert, P.E. Pfeffer, J. Org. Chem. 40 (1975) 3253–3258.
[16] T. Nishiyama, T. Fujita, T. Shin-I, M. Seki, H. Nishide, I. Uchiyama, A. Kamiya, P. Carninci, Y. Hayashizaki, K. Shinozaki, Y. Kohara, M. Hasebe, Proc. Natl. Acad. Sci. USA 100 (2003) 8007–8012.
[17] M. Hamberg, A. Sanz, M.J. Rodriguez, A.P. Calvo, C. Castresana, J. Biol. Chem. 278 (2003) 51796–51805.
[18] I. Ponce de León, A. Sanz, M. Hamberg, C. Castresana, Plant J. 29 (2002) 61–72.
[19] J.L. Dangl, J.D.G. Jones, Nature 411 (2001) 826–833.
[20] D. Hermsmeier, U. Schittko, I.T. Baldwin, Plant Physiol. 125 (2001) 683–700.
[21] A. Tirajoh, T.S.T. Aung, A.B. McKay, A.L. Plant, J. Exp. Bot. 412 (2005) 713–723.
[22] E.A. Van der Biezen, B.F. Brandwagt, W. van Leeuwen, H.J. Nijkamp, J. Hille, Mol. Gen. Genet. 251 (1996) 267–280.