Tandem orientation of duplicated xanthine dehydrogenase genes from *Arabidopsis thaliana*: differential gene expression and enzyme activities

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SUMMARY

Xanthine dehydrogenase from the plant *Arabidopsis thaliana* was analyzed on molecular and biochemical level. While most other organisms appear to own only one gene for xanthine dehydrogenase *Arabidopsis thaliana* possesses two genes in tandem orientation spaced by 704 basepairs. The cDNAs as well as the proteins AtXDH1 and AtXDH2 share an overall identity of 93% and show high homologies to xanthine dehydrogenases from other organisms. Whereas *AtXDH2* mRNA is expressed constitutively alterations of *AtXDH1* transcript levels were observed at various stresses like drought, salinity, cold and natural senescence, but also after abscisic acid treatment. Transcript alteration did not necessarily result in changes of xanthine dehydrogenase activities. While salt-treatment had no effect on xanthine dehydrogenase activities, cold stress caused a decrease, but desiccation and senescence a strong increase of activities in leaves. Because *AtXDH1* presumably is the more important isoenzyme in *Arabidopsis thaliana* it was expressed in *Pichia pastoris*, purified and used for biochemical studies. *AtXDH1* protein is a homodimer of about 300 kDa consisting of two identical subunits of 150 kDa. Like xanthine dehydrogenases from other organisms *AtXDH1* uses hypoxanthine and xanthine as main substrates and is strongly inhibited by allopurinol. *AtXDH1* could be activated by purified molybdenum cofactor sulfurase ABA3 that converts inactive desulfo- into active sulfo-enzymes. Finally it was found that *AtXDH1* is a strict dehydrogenase and not an oxidase, but is able to produce superoxide radicals indicating that besides purine catabolism it might also be involved in response to various stresses that require reactive oxygen species.
INTRODUCTION

Xanthine oxidoreductase (XOR) is a ubiquitous metallo-flavo enzyme with a central role in purine catabolism where it catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. The enzyme from higher eukaryotes is active as a homodimer composed of two identical subunits of 150 kDa, each being subdivided into three domains: a N-terminal domain of 20 kDa for binding of two iron-sulfur clusters of the [2Fe-2S]-type, a 40 kDa domain harboring a FAD-binding site, and a C-terminal molybdenum cofactor (Moco)-binding domain of 85 kDa. XOR enzymes in mammals are present either as the predominantly existing xanthine dehydrogenase (XDH; EC 1.1.1.204) or as O$_2$-dependent xanthine oxidase (XO; EC 1.1.3.22). While XDH possesses high reactivity towards NAD$^+$ and low reactivity towards O$_2$ as electron acceptor, XO reacts in a strictly O$_2$-dependent manner with negligible reactivity towards NAD$^+$. Both forms can be interconverted reversibly by oxidation of cysteine residues (1) whereas the conversion of XDH into XO by limited proteolysis is irreversible (2). In contrast to mammalian XOR the avian enzyme is exclusively present in the dehydrogenase form (3). The ability of mammalian XO to produce superoxide and hydrogen peroxide by reducing molecular oxygen (4) led to the suggestion that it might play an important role in the pathogenesis of cellular injury (5, 6). Among all XOR enzymes studied so far the bovine enzyme from milk is the most exhaustively studied one and recently its crystal structure has been determined for both the XDH and the XO form (7).

Besides functional characterization of XDH/XO also the corresponding nucleotide and protein sequence information was published for organisms like humans (8), cow (9), rat (2), mouse (10), chicken (3), insects (e.g. 11, 12), fungi (13) and bacteria (14, 15). With the exception of the silkworm all organisms analyzed so far possess one XOR gene.

In plants the XDH form but not the oxidase form was purified from nodules of bean (16), from the green algae *Chlamydomonas reinhardtii* (17), from wheat leaves (18) and
leaves of legumes (19) as well as from pea seedlings (20). All plant XDH proteins were found to be homodimers with a molecular mass of approximately 300 kDa and showed highest substrate specificity for hypoxanthine and xanthine but were also able to convert purines, pterines and aldehydes at much lower rate. Beside purine degradation, plant XDH is supposed to play a role in important cellular processes: (i) plant-pathogen interactions between phytopathogenic fungi and legumes and cereals (21, 22), (ii) cell death associated with hypersensitive response (23, 24), and (iii) natural senescence (25). As all these processes require the formation of reactive oxygen species XDH was supposed to be able to produce superoxide anions and/or hydrogen peroxide (25). Supporting this hypothesis, XDH activity was found to be increased concomitant with superoxide dismutase and other oxygen related enzymes in senescent pea leaves (25). Although much effort was spent at purification and biochemical characterization of plant XDH neither cloning of the corresponding cDNAs nor molecular data are published so far.

In this work we describe the cloning of two XDH cDNAs from *Arabidopsis thaliana*, their tandem arrangement in the genome, their mRNA expression levels as well as the enzymatic activities at various stresses and treatments, and the recombinant expression of *AtXDH1* cDNA in the methylotrophic yeast *Pichia pastoris* with subsequent purification and characterization of the AtXDH1 protein.
EXPERIMENTAL PROCEDURES

Plant Material and Plant Growth.

*A. thaliana* Col-O, Ler and *aba3.2* seeds were grown in pots containing low-nutrient soil in an AR-36L *A. thaliana* growth chamber (Percival Scientific, Perry, USA) at 13 hr light/11 hr darkness, 21°C and 70% relative humidity for periods as given in the text.

Stress Treatment.

For drought stress experiments, soil was completely removed from the roots prior to incubation under normal conditions in the chamber for 4 hr or as given in the text (loss of fresh weight about 50%). Subsequently, roots and leaves were detached and used for RNA and activity analysis. ABA treatment in case of plants without drought treatment was performed by spraying plants with 50 µM (+/-)-*cis,trans*-ABA in water uniformly onto the leaves and lastet for 4 hr. In case of combined ABA and drought treatment plants were sprayed with ABA prior to removal of plants from the soil and 2 hr after removal. Treatment also lasted for 4 hr and control plants were sprayed with water instead of ABA solution. For NaCl treatment, 3 week old plants were transferred to hydroponic culture 2 days before treatment and then incubated in nutrient solution containing 200 mM NaCl for 6 hr and 20 hr. Cold stress treatment at 4°C was performed in a chamber with ambient temperature for 6 hr and 20 hr and freezing stress was applied by incubating plants in small chambers precooled to -4°C. Because longer freezing stress will result in freezing of the soil freezing temperature treatment lasted only 6 hr.

Preparation of RNA.

Total RNA was prepared either as described by (26) or by using the NucleoSpin RNA Plant kit (Macherey & Nagel, Dueren, Germany) according to the manufacturers instructions.

Relative quantitative reverse transcriptase-polymerase chain reaction (RT-PCR).
For each RT reaction 2 µg *A. thaliana* total RNA was reverse-transcribed with AMV-reverse transcriptase (Promega, Madison, USA) and oligo-d(T)\textsubscript{18}-BamHI primer according to standard procedures (27). RT-PCR was performed on a PCR-Express gradient cycler (Hybaid, Heidelberg, Germany) by using the SAWADY Taq-DNA-Polymerase (Peqlab, Erlangen, Germany). *AtXDH1* specific primers were AtXDH1+: 5’-CAC ATT TAC TGA GCT AGT A-3’ and AtXDH1-: 5’-GTT TCC CCT CTG ATG AGT TTC-3’; *AtXDH2* specific primers were AtXDH2+: 5’-TCT TCT CAA GGG TAA TCC A-3’ and AtXDH2-: 5’-TTC TCC CCT CTA TTA AAG TTT-3’. The following PCR program was used: 3 min at 94°C for initial denaturing of templates, 30 cycles including denaturing for 30 sec at 94°C, annealing for 1 min at 56°C and elongation for 1 min at 72°C, and a final elongation step for 6 min at 72°C. RT-PCR generated fragments were directly ligated to pGEM-T Easy (Promega, Madison, USA) and sequenced for ascertaining proper amplification.

**Cloning of *AtXDH2* cDNA.**

Two overlapping cDNA-subfragments were generated by PCR from reverse transcribed total RNA of *A. thaliana (Col-O)* and subsequently fused by PCR according to standard procedures (27). The obtained full length cDNA of *AtXDH2* was directly ligated to pGEM-T Easy (Promega, Madison, USA) and sequenced. Specific PCR primers for generation of the 5’ subfragment (2080 base pairs) were AtXDH2-ATG: 5’-GTT CAG TGA AGA TGG AGC AGA AC 3’ and AtXDH2-2622rev: 5’-GCG ACA AGC ACA CCA ATA-3’. Primers for the 3’ subfragment (2085 base pairs) were AtXDH2-2400for: 5’-TTA TTT GCT ACA GAC GTG-3’ and AtXDH2-3’: 5’-TGA TCC ATC TTT CTC CCC-3’.

**Generation of *AtXDH1* expressing *P. pastoris.*

The cDNA clone AV548322 coding for full length *A. thaliana* XDH1 was obtained from the Kazusa DNA Research Institute (Chiba, Japan). The yeast expression vector pPICZA with C-terminal 6x His-tag and *P. pastoris* strain KM71 mut\textsuperscript{i} were purchased from Invitrogen (Carlsbad, USA). Standard molecular cloning techniques were used for DNA
manipulation. The \textit{AtXDH1} cDNA was used as template for PCR to remove 5’-untranslated region and stop-codon and to generate a \textit{KpnI} site at the 5’ end and a \textit{ApaI} site at the 3’ end. Primers used for introducing restriction sites were: \textit{AtXDH1} \textit{KpnI} start: 5’-ATA TAT GGT ACC ATG GGT TCA CTG AAA AAG GAC GGC-3’ and \textit{AtXDH1} \textit{ApaI} stop: 5’-ATA TAT GGG CCC AAC ACT AAG ATT AGG GTA GAA ATC TGA-3’. The resulting PCR fragment containing the total coding region was cloned into pPICZA. \textit{P. pastoris} was transformed with pPICZA/AtXDH1 and pPICZA (control) by electroporation according to manual (EasySelect \textit{Pichia} expression Kit Version A, Invitrogen). The presence of \textit{AtXDH1} cDNA in zeocin-resistant colonies was confirmed by PCR on the \textit{P. pastoris} colonies.

\textbf{Expression and purification of AtXDH1.}

Several positive transformants were grown in 25 ml BMGY (1% yeast extract, 2% pepton, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.04‰ biotin, 1% glycerol, and 100 µg/ml zeocin) in a 250 ml baffled flask for 16 to 20 hours (OD$_{600}$ 2 to 3) at 30°C and 150 rpm. Cells were collected by centrifugation and resuspended in 10 ml BMMY (1% yeast extract, 2% pepton, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acids, 0.04‰ biotin, 0.3 mM sodium molybdate and 0.5% methanol) in a 100 ml baffled flask and cultured again at 30°C and 150 rpm. Cells were harvested after 0, 6, 10, 14, 18, 24, 36 hours of methanol induction by centrifugation, and resuspended in breaking buffer (50 mM sodium phosphate pH 7.4, 0.5 mM EDTA, 200 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and 5% glycerol). Cells were broken by vigorous vortexing with equal amounts of acid washed glass beads (425 - 600 µ, Sigma) before cell debris and glass beads were removed by centrifugation. In the resulting supernatant XDH activity was examined by activity staining after native PAGE. Strongest intensity was detected 10 hours after methanol induction with a gradual decrease until 36 hours of incubation. The clone showing highest XDH activity was selected for large scale expression culture. Cells were grown in 250 ml BMGY in a 1 L baffled flask for 20
hours, collected by centrifugation and resuspended in 50 ml BMMY in a 500 ml baffled flask. After cultivation for 10 hours in BMMY the cells were harvested by centrifugation and resuspended in breaking buffer. Depending on the quantity of cells, they were broken either by vigorous vortexing with an equal volume of glass beads at 4°C for a total of 30 min in bursts of 30 s alternating with cooling on ice or by three passages through a french press pressure cell with 14000 psi operating pressure. After centrifugation the supernatant was used for purification of the His-tagged AtXDH1 protein by affinity chromatography with Ni-nitrilotriacetic acid (Ni-NTA)-superflow matrix (Qiagen, Hilden, Germany) under native conditions at 4°C according to the manufacturers instructions. The sample was rebuffered to 50 mM Tris/HCl pH 8.0, 5 mM EDTA, 2.5 mM DTT. For further purification AtXDH1 was subjected to anion exchange chromatography using a Source™ 15Q column (Amersham Biosciences, Freiburg, Germany) equilibrated with 50 mM Tris/HCl pH 8.0, 5 mM EDTA, 2.5 mM DTT (buffer A). Protein samples were applied to the column and eluted with buffer A followed by a linear gradient of 0 to 1 M NaCl in buffer A. Final purification and size determination was achieved by chromatography on a Superdex® 200 HR10/30 size exclusion column (Amersham Biosciences, Freiburg, Germany) equilibrated with 50 mM Tris/HCl pH 8.0, 200 mM NaCl, 1 mM EDTA.

**Determination of protein concentrations.**

Concentrations of total soluble protein were determined by use of Roti Quant solution (Roth, Karlsruhe, Germany) according to (28).

**Wavescan of AtXDH1.**

Absorption spectroscopy was carried out using an Ultrospec 3000® spectrophotometer (Pharmacia, Freiburg, Germany).

**Sequence analysis.**
Sequence analysis was performed with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 310 cycle sequencer (PE Applied Biosystems, Warrington, UK) with a pop 6 polymer.

**Expression of ABA3 and AOα from A. thaliana.**

Recombinant molybdenum cofactor sulfurase ABA3 and aldehyde oxidase AOα from *A. thaliana* were expressed and purified as described earlier by (29) and (30).

**Enzyme assays.**

For preparation of plant crude extracts plant material was squeezed at 4°C in two volumes of extraction buffer (100 mM potassium phosphate, 2.5 mM EDTA, 5 mM DTT, pH 7.5), sonificated and centrifugated and supernatant was used for activity assays. XDH activity in plant crude extracts and of recombinant AtXDH1 was visualized according to (31), except that native electrophoresis in the absence of SDS was run with 7.5% polyacrylamide gels and staining solution contained 1 mM hypoxanthine as substrate, 1 mM MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide) and 0.1 mM PMS (phenazine methosulphate) in 250 mM Tris/HCl pH 8.5. For standard *in gel* XDH activity assays each lane was loaded either with 80 µg plant crude extract protein or with 1 µg recombinant AtXDH1. The *in vitro*-reconstitution of recombinant AtXDH1 by ABA3 was performed in a total volume of 0.4 ml in 50 mM Tris/HCl, pH 8.0. AtXDH1 (20 µg) was incubated with ABA3 (40 µg) in the presence of 1 mM L-cysteine for 1 h at 30°C, followed by native PAGE with 1/5 volume of the reaction mixture and activity staining with hypoxanthine as substrate. Spectrophotometric determination of XDH activity was measured at 340 nm in a 1 ml reaction mixture containing 1 mM of the respective substrate, 1 mM NAD⁺, 50 mM Tris/HCl pH 8.0, 1 mM EDTA and a suitable amount of recombinant AtXHD1. Reaction was started by addition of substrate. Inhibitors were preincubated with the enzyme for 5 min before starting the reaction. The xanthine-O₂ reductase activity was measured under the same conditions but without NAD⁺, and O₂-dependent production of uric acid was monitored at
295 nm. The production of superoxide radicals was monitored by following the reduction of cytochrome c at 550 nm. The specificity of $O_2^-$-dependent reduction of cytochrome c was estimated by incorporating an excess of bovine superoxide dismutase in the assay mixture according to (32, 33).

RESULTS

Cloning of AtXDH1 and AtXDH2 cDNAs and their tandem arrangement in the genome.

Sequence similarity searches at „The Arabidopsis Information Resource“ (TAIR, http://www.arabidopsis.org) using human and bovine full length XDH as query sequences detected two putative genes in the A. thaliana genome with higher similarity to XOR than to aldehyde oxidases (AO). Both genes were found in a tandem orientation on chromosome 4 with their reading frames pointing to the same direction (Fig. 1A). Based on the BAC clone AL079347/ATF11I11 as reference both putative XDH genes, annotated as T11I11.130 and T11I11.140 and designated by us as AtXDH1 and AtXDH2, respectively, are located within the center region of this clone with an interspace of only 704 bp. The predicted genes range from position 50304 (ATG) to 44057 (TGA) for T11I11.130/AtXDH1 and from position 56627 (ATG) to 51009 (TGA) for T11I11.140/AtXDH2, thereby spanning regions of 6248 and 5619 basepairs, respectively. For both predicted genes expressed sequence tags (ESTs) were found indicating that both genes actually are transcribed. Although these ESTs clearly showed that annotation of the putative coding sequences is not fully correct they confirmed the predicted transcription start- and stop-sites. Neither extended database searches nor genomic DNA hybridizations (data not shown) revealed more than these two XDH genes within the A. thaliana genome.
Complete sequencing of the *A. thaliana* EST AV548322 has shown that the full length open reading frame of *AtXDH1* is interrupted by 13 introns and contains 4083 basepairs encoding a protein of 1361 amino acids (Fig. 1B) with 47% identity to human and bovine XDH. A full length cDNA of *AtXDH2* with an open reading frame of 4059 base pairs was obtained by reverse transcriptase PCR using sequence information of the predicted *AtXDH2* gene. The *AtXDH2* gene also contains 13 introns highly conserved in exon/intron junctions compared to *AtXDH1*. The encoded AtXDH2 protein exhibits a length of 1353 amino acids with identities of 46% to human and bovine XDH. Both proteins, AtXDH1 and AtXDH2, reveal lower identities of 29 - 31% to AO proteins from *A. thaliana* but share overall identities of 93% to each other, indicating that they are similar to AO but functionally divergent. Full length cDNA and protein sequences were deposited at GenBank (AY171562 for AtXDH1; AY518202 for AtXDH2).

Comparative primary structure analysis of AtXDH1 and AtXDH2 and XOR proteins from other organisms revealed a three domain structure for both *A. thaliana* XDH monomers as is typical for XOR proteins. Like the chicken XDH (2) both *A. thaliana* XDH proteins contain a N-terminal domain including 8 strictly conserved cysteine residues for binding of two non-identical iron-sulfur clusters of the [2Fe-2S]-type spanning amino acid positions 19 to 173 in AtXDH1 and 11 to 164 in AtXDH2, respectively. In each protein, the [Fe-S] binding domain is followed by a FAD-binding domain (amino acids 260 to 440 in AtXDH1 and 252 to 432 in AtXDH2) whereas both domains are separated by hinge regions that are less conserved among all XOR proteins. FAD domains of both XDH proteins contain a FFLGYR-motif (amino acids 417 to 422 in AtXDH1 and 409 to 414 in AtXDH2) that is supposed to be responsible for binding the second substrate NAD⁺ via the invariant tyrosine (34, 2). The third and C-terminal domain includes the Moco- and substrate-binding sites as well as the dimerization motif (35). In AtXDH1 it spannes amino acid residues 612 to 1272 and in AtXDH2 604 to 1264, respectively, and is separated from the FAD/NAD domain by
another hinge region. Within the Moco domain of XOR proteins both a strictly conserved glutamate and an arginine residue are supposed to be essential for binding and proper positioning of purine substrates (36). AtXDH1 and AtXDH2 exhibit identical residues at the corresponding positions (Glu831 and Arg909 in AtXDH1, Glu832 and Arg901 in AtXDH2) indicating that the favored substrates of both proteins should be purines rather than aldehydes.

To find out at what evolutionary point XDH gene duplication might have occurred we analyzed the phylogenetic relationships of XDH proteins from various eukaryotic organisms (Fig. 2). Since AO is homologous to XDH but functionally divergent we have chosen three AO proteins from *A. thaliana* as an outgroup. The sequences used for this analysis show a splitting into three groups, among which plant sequences clearly form an own monophyletic subgroup besides animal and fungi XDH. Therein, *A. thaliana* XDH gene duplication appears to have happened long after the separation of dicots and monocots. Different from *A. thaliana*, none of the fully sequenced genomes of rice and *C. reinhardtii* were found to contain more than one XDH gene. Among the animals, vertebrates, insects, and nematodes group separately. Generally, the phylogenetic tree based on XDH protein similarities mirrors the species phylogeny and gives one more indication that AtXDH1 and AtXDH2 are in fact xanthine dehydrogenases rather than aldehyde oxidases.

**Differential transcription and enzyme activities of AtXDH1 and AtXDH2.**

Due to the high similarities between the mRNAs of *AtXDH1* and *AtXDH2* and nearly identical transcript sizes, one cannot distinguish both transcripts by use of mRNA hybridization. Therefore we performed relative quantitative reverse transcriptase PCR, using specific PCR primers designed for binding within the 3’-regions of each transcript and generating PCR-fragments of 693 basepairs for *AtXDH1* and 279 basepairs for *AtXDH2*, respectively. Proper amplification of the corresponding fragments was confirmed by sequencing.
As shown in Fig. 3A expression of \textit{AtXDH1} and \textit{AtXDH2} on mRNA level can be detected in roots, leaves, stem, flowers, and siliques, indicating that both mRNAs are ubiquitously expressed in \textit{A. thaliana}, although with varying amounts. Consistent with these findings also XDH activities were found in these organs. Unfortunately, discrimination of two XDH isoforms in non-denaturing polyacrylamide gels was impossible, either due to very similar physicochemical properties of both XDH proteins or due to the fact that only one isoform is actually translated. When analyzing the expression levels of \textit{AtXDH1} and \textit{AtXDH2} in plants of different age it turned out that mRNA levels of \textit{AtXDH1} increased in aging and senescent leaves while \textit{AtXDH2} transcript levels remained unaltered (Fig. 3B) thereby simultaneously serving as an internal standard. In the same plants, a strong increase of XDH activity could be observed in senescent leaves but not at any other stage of development (Fig. 3B).

When applying salt and cold stress to \textit{A. thaliana} plants again the mRNA levels of \textit{AtXDH1}, but not of \textit{AtXDH2}, were altered. While salinity (200 mM NaCl) caused a strong accumulation of \textit{AtXDH1} transcripts after 20 hr, persistent cold at 4°C as well as rapid freezing at -4°C appeared to cause a decrease of \textit{AtXDH1} transcript levels (Fig. 3C). Although the change in mRNA amounts is more apparent under salt stress than at cold and freezing stress conditions no change in XDH activity could be observed at salinity, neither in leaves (Fig. 3C) nor in roots (data not shown). However, cold stress and rapid freezing clearly resulted in decreased activities of XDH.

Because dehydration is another common stress that plants have to cope with we exposed \textit{A. thaliana} plants to drought stress that lasted for 4 hr and resulted in a loss of fresh weight of about 50%. During this stress period mRNA amounts of \textit{AtXDH1} increased strongly in rosette leaves but at the same time dramatically decreased in roots (Fig. 3D) while again the levels of \textit{AtXDH2} mRNA basically remained unchanged. To find out whether this accumulation of \textit{AtXDH1} transcripts in leaves is due to the applied drought stress itself or due
to associated stress-induced abscisic acid (ABA) synthesis we additionally analyzed mRNA amounts in wildtype plants that were treated with exogenously applied ABA. This analysis was repeated with \textit{aba3} mutants that were exposed to the same drought stress, either with or without pretreatment of ABA. Due to a mutation in the Moco sulfurase gene \textit{aba3}, mutants have lost the ability to activate XDH and AO by sulfuration, thereby rendered unable to respond to stresses that require AO-dependent ABA synthesis (37, 29, 38). Both the wildtype and the \textit{aba3} mutants accumulated \textit{AtXDH1} transcripts upon ABA-treatment but no increase was observed in \textit{aba3} mutants at drought stress without pretreatment of ABA (Fig. 3E). These data strongly suggest that transcript accumulation in \textit{A. thaliana} leaves at drought stress is a consequence of stress-induced ABA synthesis and thereby solely indirectly related to drought.

Concomitant to \textit{AtXDH1} transcript alteration XDH activity at drought stress was markedly increased in leaves and decreased in roots 4 hr and 20 hr after starting stress treatment (Fig. 3D). ABA treatment without drought stress also resulted in slightly increased XDH activities in leaves of wildtype plants (Fig. 3E), supporting that expression and activity of XDH are directly regulated by ABA rather than by drought stress itself.

\textbf{Heterologous expression and purification of AtXHD1.}

The phylogenetic analyses (Fig. 2) indicate that AtXDH1 and AtXDH2 are in fact xanthine dehydrogenases rather than aldehyde oxidases, but unequivocal evidence comes only from the biochemical characterization of purified enzyme. First attempts to produce recombinant AtXDH1 in \textit{E. coli} yielded only negligible amounts of recombinant protein, above all lacking cofactors and therefore being inactive. For this reason a eukaryotic system, the methylotrophic yeast \textit{P. pastoris}, was chosen for heterologous expression of AtXHD1. The recombinant protein was purified by affinity chromatography followed by anion exchange chromatography. After this purification procedures the protein displayed one major band of 150 kDa in a Coomassie-stained SDS gel, well corresponding to the calculated
molecular mass of 150.2 kDa for the deduced 6xHis-AtXDH1 monomer. These data were confirmed by immunoblot analysis with anti-His antibody where the appropriate band was detected (Fig. 4A).

XOR and AO form a class of Mo-enzymes that are activated by sulfuration of their active center (39). This step is catalyzed by a highly specific enzyme named Moco sulfurase. Since we have recently cloned and purified the *A. thaliana* Moco sulfurase ABA3 (29) we wanted to know whether it could activate purified AtXDH1. After coincubation of AtXDH1 with the Moco sulfurase ABA3 an increase of AtXDH1 activity was observed (Fig. 4B), indicating that the recombinant protein as obtained from *P. pastoris* is not fully active. A second band appearing in this experiment above the typical AtXDH1 band might indicate the formation of an AtXDH1-ABA3 complex. When comparing gel mobility of purified recombinant AtXDH1 to native XDH from *A. thaliana* leaf crude extracts it was found that both bands appearing after native PAGE and subsequent activity staining showed identical mobilities (Fig. 4B). By use of gelfiltration chromatography the molecular mass of recombinant AtXDH1 was found to range between 270 and 300 kDa, indicating that AtXDH1 in its native form is a homodimer (data not shown).

The absorption spectrum of AtXDH1 (Fig. 4C) is characterized by a maximum at 454 - 458 nm and a shoulder at about 540 - 590 nm, thereby corresponding to spectra of other molybdenum containing hydroxylases with typical peaks at 450 nm due to bound flavin chromophore, and a shoulder at 550 nm related to absorption of iron-sulfur centers. The absorption ratio of 2.8 at these two wavelengths is close to the ratio of 3 described for other XDH proteins revealing a FAD to FeS ratio of 1:4 (40, 41). Additionally, AtXDH1 shows strong absorption between 310 and 330 nm which might be related to enedithiolene- and sulfo-molybdenum charge transfer bond.
Substrate specificity of AtXDH1.

XOR enzymes catalyze the oxidation of hypoxanthine to xanthine and of xanthine to urate with concomitant reduction of either NAD$^+$ or molecular oxygen (42). When using hypoxanthine as substrate recombinant AtXDH1 reacted well with NAD$^+$. However, molecular oxygen as electron acceptor yielded a maximum substrate hydroxylation of about 2.5% compared to NAD$^+$ (Table 1) indicating that AtXDH1 occurs just in the dehydrogenase form. Based on the reaction with molecular oxygen, superoxide formation by AtXDH1 was measured by reduction of cytochrome c in the presence and absence of superoxide dismutase (32, 33). It was found that up to 22% of the electrons from xanthine were transferred to molecular oxygen to form superoxide radicals during catalysis.

In addition to hypoxanthine and xanthine, AtXDH1 catalyzes the hydroxylation of purine and various aldehydes (Table 2). Compared to hypoxanthine oxidation, hydroxylation rates of aldehydes ranged from 12.5% for heptaldehyde up to 31.3% for aromatic aldehydes like indole-3-carboxaldehyde under steady state conditions. AtXHD1 activity was found to be totally inhibited by allopurinol which is converted to alloxanthine and not released from the enzyme, as well as by $p$-hydroxymercuribenzoate, an inhibitor of SH-groups, and potassium cyanide that removes the terminal sulfur ligand from the Moco of Mo-hydroxylases like XDH and AO. Comparing the substrate specificities of AtXDH1 to recombinant A. thaliana AO$\alpha$ demonstrated that hypoxanthine and xanthine are converted only by AtXDH1 (Fig. 5). Although AtXDH1 also converts several aldehydes conversion of these aldehydes by AO$\alpha$ is much more efficient. The pH optimum of AtXDH1 was found to range between 8.0 and 8.5 with NAD$^+$ as oxidizing substrate.
DISCUSSION

The present study has demonstrated that *A. thaliana* possesses duplicated XOR genes that are arranged in tandem orientation in the genome. Both proteins represent xanthine oxidizing hydroxylases rather than AOs which do not require reduction of NAD⁺ for substrate conversion. Proving this assumption, recombinant AtXDH1 was shown to convert hypoxanthine and xanthine much more efficiently than aldehydes, although oxidation of aromatic indole-3-carboxaldehyde, abscisic aldehyde and naphthaldehyde was significant (Tab. 2 and Fig. 5). Recombinant AOα in turn was found to be unable to convert hypoxanthine and xanthine but oxidizes aldehydes much more efficiently than AtXDH1 (Fig. 5). Furthermore, AtXDH1 activity was completely inhibited by treatment with the typical XDH inhibitor allopurinol that is converted to alloxanthine, which remains tightly attached to the substrate binding pocket thereby preventing further substrate turn-over (43).

XORs from mammals are present mainly in the dehydrogenase form but can readily be converted to the oxidase form (44). While XDH is the predominant enzymatic form in normal tissues and is involved in purine catabolism XO dominates in milk and in tissues subjected to injury where the production of reactive oxygen species is required. Both XDH and XO produce superoxide anions when O₂ is used as electron acceptor. Although slower in comparison to XO, XDH produces more superoxide per mol O₂ but the formation of superoxide is nearly completely inhibited by the presence of NAD⁺. Therefore, the physiological significance of XDH-dependent superoxide generation in mammals is questionable due to excess amounts of NAD⁺ in cells under normal conditions (for review (6)). However, depletion of the NAD⁺ available to XDH relative to O₂, is supposed to be sufficient for XDH-catalyzed production of oxygen radicals (45). Interestingly, chicken XDH is not converted to XO and produces amounts of superoxide (40 - 44%; (46)) very similar to bovine milk XDH (35 – 42%; (47)) when using xanthine and O₂ as substrates. Like chicken
XDH, AtXDH1 appears to be present only in the dehydrogenase form indicated by the lack of two cysteine residues responsible for reversible conversion of rat XDH to XO (Cys535 and Cys992; (48)) and the inefficient use of O$_2$ as electron acceptor. Nevertheless, AtXDH1 transferred about 22% of the electrons from xanthine to O$_2$ to produce superoxide radicals. Although the total amount of superoxide radicals produced by recombinant AtXDH1 appears to be too low for physiological significance the following points have to be highlighted: $AtXDH1$ transcript amounts increase dramatically at senescence accompanied by an even more obvious increase of XDH activity indicating that alteration of XDH activity in A. thaliana can be ascribed to alteration of AtXDH1 amounts and/or activity. Thus, under *in vivo* conditions higher amounts of native AtXDH1 protein that are activated far beyond normal levels are likely to produce also more superoxide. Whether or not superoxide production by AtXDH1 (and AtXDH2?) actually is of physiological importance remains to be investigated. But it is without doubt that plant XDH is involved in processes that require the formation of reactive oxygen species like pathogen defense (21, 22), cell death associated with hypersensitive reaction (23, 24) and natural senescence (25), thereby most likely fulfilling a function beyond purine degradation.

XDH gene duplication in A. thaliana, a situation that otherwise is known only from the silkworm Bombyx mori (12), forms the basis for differential gene regulation. Initially, expression studies on mRNA level have shown that both $AtXDH1$ and $AtXDH2$ are ubiquitously expressed in all plant parts and distribution of XOR activity corresponds well with the mRNA distribution. However, in leaves of different age it became obvious that aging and natural senescence caused an increase of $AtXDH1$ transcripts only, but not of $AtXDH2$ transcripts. Concomitant to mRNA levels, senescent leaves showed strongly increased XDH activities. These results are in accordance to Pastori and del Rio (29) who have observed a 7- to 8-fold increase of XDH activities in leaves of senescent pea plants concomitant to increasing activities of superoxide dismutase. Since oxidative processes during senescence
require the generation of reactive oxygen species like superoxide radicals it might well be that
the function of plant XDH at senescence is the production of superoxide radicals rather than
the degradation of purines, although the latter point should not be neglected due to the
requirement of purine rescue for carbon and nitrogen remobilization during aging of plants.
As found for AtXDH1, other degradative enzymes like RNases (49), proteinases (50, 51, 52)
and lipases (53) are known to increase during the process of senescence. Interestingly, leaves
of aging plants did not display altered XDH activities although senescent leaves, showing
nearly the same increase of AtXDH1 transcripts, did so. This can be explained by the fact that
XDH like AO requires a posttranslational activation of the holoenzyme. According to
changing environmental conditions the Moco sulfurase ABA3 is controlling the activities of
XDH and AO (37, 29, 38) by changing the ratio of sulfurated/active to non-sulfurated/inactive
enzymes, relatively independent from the total amount of holoprotein. It is most likely that the
Moco sulfurase in aging leaves does not activate XDH beyond regular levels but is doing so at
the stage of senescence.

Like in plants of different developmental stages also alteration of transcripts at various
stresses is not necessarily associated with alterations in XDH activities. But it appears that a
decrease of XDH activity is regulated by transcript down-regulation like in cold stressed
plants and desiccated roots. On the other hand, increasing activities appear to result from two
successive events, i.e. the accumulation of AtXDH1 transcripts and, presumably, subsequent
sulfuration of XDH protein by its Moco sulfurase. As shown for AtXDH1, transcripts of
ABA3 accumulate in drought stressed leaves (29, 38) but not in roots (Bittner et al.,
unpublished data), and ABA3 expression in leaves is stimulated by ABA treatment (38). In
case of AtXDH1 leaf-specific transcript accumulation at drought stress was found to be
directly correlated to enhanced ABA levels rather than to the drought stress itself (Fig. 3E).
This might indicate a common regulation for AtXDH1 and its activating enzyme ABA3
during processes that require ABA response. Similar to the situation at senescence the
relevance of enhanced XDH activity at desiccation is not absolutely clear. It remains to be shown whether leaf-specific purine degradation is required for maintaining cell viability or whether also under drought stress conditions superoxide is produced in adaptation to this stress. Remarkably, at all conditions tested the amount of \( \text{At} \text{XDH2} \) transcripts remained more or less unaltered while \( \text{AtXDH1} \) displayed strong changes. Hence, one can conclude that altered XDH activities might be a consequence of increasing or decreasing amounts of \( \text{AtXDH1} \) mRNA but not of \( \text{AtXDH2} \) mRNA. Because final activation of XDH holoenzyme is carried out by ABA3 and therefore is not directly correlated to the amount of preexisting protein it is so far unclear whether both XDH genes are actually translated and active or whether there is only one XDH protein in \( A. \text{thaliana} \).

Up to now we have not been able to detect and distinguish two separate XDH activity bands in native PAGE supporting the possibility of only one active XDH isoenzyme. On the other hand, physicochemical properties of \( \text{AtXDH1} \) and \( \text{AtXDH2} \) might be nearly identical due to their high degree of homology and therefore might lead to identical migration in native PAGE making a discrimination impossible. Nevertheless, we favour \( \text{AtXDH1} \) either to be the only active XDH enzyme in \( A. \text{thaliana} \) or to be the one of greater physiological importance based on several observations: (i) at all stresses and treatments tested only \( \text{AtXDH1} \) reacted significantly on transcript level while levels of \( \text{AtXDH2} \) remained unaltered, (ii) concomitant to the alterations of \( \text{AtXDH1} \) on transcript level also XDH activities changed the same way (except at salt stress), (iii) recombinant \( \text{AtXDH1} \) and XDH from \( A. \text{thaliana} \) crude extracts displayed identical migration properties in native PAGE, and (iv) \( \text{AtXDH1} \) was found to be able to produce superoxide, thereby likely being the protein reacting at senescence. In case of translated and active \( \text{AtXDH2} \) we propose a more general and constitutive function during purine degradation but not at stress adaptation.
REFERENCES


FOOTNOTES

The abbreviations used are: ABA, abscisic acid; AO, aldehyde oxidase; EST, expressed sequence tag; Moco, molybdenum cofactor; PAGE, polyacrylamide gel electrophoresis; XDH, xanthine dehydrogenase; XO, xanthine oxidase; XOR, xanthine oxidoreductase

ACKNOWLEDGMENTS

We thank the Kazusa DNA Research Institute (Chiba, Japan) for providing the EST AV548322 containing the *AtXDH1* full length cDNA, Tomokazu Koshiba (Tokyo, Japan) for kindly providing the AOα-expressing *P. pastoris* strain, and Jan Zeevaart (East Lansing, MI) for donating abscisic aldehyde. The authors also are grateful to Joern Petersen (Braunschweig, Germany) for help with phylogeny, to Günter Schwarz for critically reading of the manuscript, and to Saskia Helmsing for technical assistance.
FIGURE LEGENDS

Fig. 1: Genomic and cDNA structure of AtXDH1 and AtXDH2. (A) Tandem orientation of AtXDH1 and AtXDH2 genes on chromosome 4, represented by the BAC clone ATF1I11. Large arrows indicate the orientation of genes. (B) Exon-intron structure of AtXDH1 and AtXDH2 genes. The overall sizes of the respective open reading frames (orf) are indicated and the relative sizes of exons (boxes) and introns (peaks) are shown; their lengths are indicated as numbers of base pairs (bp).

Fig. 2: Phylogenetic neighbor joining tree of XDH proteins. Full length sequences of XDH proteins and A. thaliana AOs were aligned using ClustalX and phylogeny of XDH proteins was constructed by use of the neighbor joining method using PAUP 4.0. Numbers in the phylogenetic tree indicate 100 bootstrap replicates. A. thaliana AO1, AO2 and AO3 were used as an outgroup. The accession numbers of the XDH sequences are as follows: AtXDH1 (AY171562), AtXDH2 (AY518202), Oryza sativa (cDNA: AK065099), Chlamydomonas reinhardtii (not available), Emericella nidulans (CAA58034), Neurospora crassa (EAA27223), Homo sapiens (P47989), Rattus norvegicus (P22985), Mus musculus (CAA44705), Bos taurus (CAA58497), Gallus gallus (P47990), Drosophila melanogaster (S07245), Calliphora vicina (JQ0407), Bombyx mori XDH1 (BAA21640), Bombyx mori XDH2 (BAB47183), Caenorhabditis elegans (NP_502747). Accession numbers for the AOs from A. thaliana are AtAO1 (BAA28624), AtAO2 (BAA28625) and AtAO3 (BAA82672).

Fig. 3: Relative AtXDH1 and AtXDH2 mRNA expression and XDH activity at different conditions. (A) Relative mRNA expression (upper image) and enzymatic activity (lower
image) of *A. thaliana* XDHs in different tissues, (**B**) in leaves of different age (seedlings: 6 days; young: 2 weeks; adult: 3.5 weeks; aging: 6 weeks; senescent: 8 weeks), (**C**) at salinity, cold and freezing stress, (**D**) at drought stress treatment, and (**E**) at ABA treatment in wild types and *aba3.2* mutants. After RT-PCR, 2% agarose gels were loaded with 10 µl of the respective PCR reaction (M = 100 base pair ladder, upper band = 1000 base pairs; **C** = control; **D** = 4 hr drought stressed). For XDH activity measurements, each lane on 7.5% native polyacrylamide gels was loaded with 80 µg of *A. thaliana* crude extract protein, and subsequently stained in the presence of hypoxanthine as substrate. Each figure represents one of at least 3 independent experiments that basically gave same results.

**Fig. 4:** Purification of AtXDH1 after expression in *P. pastoris*. (**A**) SDS PAGE analysis of AtXDH1 after different purification steps. Coomassie Brilliant Blue staining shows AtXDH1 as obtained from *P. pastoris* crude protein extract (lane A; 15 µg protein), after Ni-NTA purification (lane B; 15 µg protein), and after anion exchange chromatography (lane C; 5 µg protein). Lane D reveals immunoblot analysis of Ni-NTA purified AtXDH1 detected by use of anti-His antibody. (**B**) Activation of AtXDH1 by ABA3 and comparison of mobilities of AtXDH1 and XDH from leaf crude extracts on native PAGE. For XDH activation assay, 20 µg of AtXDH1 either were used as control (left lane) or were coincubated with 40 µg ABA3 in the presence of 1 mM L-cysteine for 1 h at 30°C before subjecting 1/5 volume of the reaction mixture to native PAGE (middle lane). Right lane was loaded with 80 µg of *A. thaliana* leaf crude extract and activity staining was performed with hypoxanthine as substrate. (**C**) UV-visible absorption spectrum of AtXDH1, recorded in 50 mM Tris/HCl pH 8.0, containing 1 mM EDTA.
Fig. 5: Substrate preferences of recombinant AtXDH1 and AOα. After native PAGE, activity bands were developed separately with strips from two lanes with equal concentrations (1 mM) of the following substrates: heptaldehyde (heptald.), 1-naphthaldhyde (naphthald.), indole-3-carboxaldehyde (indolecarb.), hypoxanthine (hypoxanth.), and xanthine. Each lane contained 10 µg of recombinant AtXDH1 (XDH) or AOα (AO).
Table 1: Use of molecular oxygen as electron acceptor and production of superoxide radicals by AtXDH1. Activity was assayed by monitoring the production of urate at 295 nm, superoxide-dependent reduction of cytochrome c was measured at 550 nm.

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>Formation of urate (mol/min)</th>
<th>Reduction of cytochrome c (mol/min)</th>
<th>Flux (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD$^+$</td>
<td>35.541</td>
<td></td>
<td></td>
</tr>
<tr>
<td>O$_2$</td>
<td>0.930</td>
<td>0.415</td>
<td>22</td>
</tr>
</tbody>
</table>
Table 2: Substrate specificity of AtXDH1. Activity was measured as NAD$^+$ reduction at 340 nm. AtXDH1 and the respective inhibitor were coincubated 5 min prior to starting the reaction with hypoxanthine. Substrates were used in concentrations of 1 mM except for allopurinol and hydroxymercuribenzoate (each 0.1 mM) and potassiumcyanide (50 mM).

<table>
<thead>
<tr>
<th>Substrate/Inhibitor</th>
<th>Formation of NADH (mol/min)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypoxanthine</td>
<td>43.07 ± 1.57</td>
<td>100</td>
</tr>
<tr>
<td>Xanthine</td>
<td>40.78 ± 0.75</td>
<td>94.7</td>
</tr>
<tr>
<td>Purine</td>
<td>4.44 ± 0.15</td>
<td>10.3</td>
</tr>
<tr>
<td>Indole-3-carboxaldehyde</td>
<td>13.48 ± 0.87</td>
<td>31.3</td>
</tr>
<tr>
<td>Abscisic aldehyde</td>
<td>12.45 ± 1.47</td>
<td>28.9</td>
</tr>
<tr>
<td>Heptaldehyde</td>
<td>5.38 ± 1.66</td>
<td>12.5</td>
</tr>
<tr>
<td>1-Naphthaldehyde</td>
<td>11.83 ± 0.4</td>
<td>27.5</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxymercuribenzoate</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Potassiumcyanide</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 1

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Tandem orientation of duplicated xanthine dehydrogenase genes from Arabidopsis thaliana: differential gene expression and enzyme activities
Christine Hesberg, Robert Hänsch, Ralf R. Mendel and Florian Bittner

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