Evidence for the existence of rhodanese (thiosulfate:cyanide sulfurtransferase) in plants: preliminary characterization of two rhodanese cDNAs from *Arabidopsis thaliana*

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Abstract The existence of rhodanese (thiosulfate:cyanide sulfurtransferase; EC 2.8.1.1) in plants has been highly controversial. We have isolated and characterized for the first time in plants two cDNAs encoding rhodanese isoforms in *Arabidopsis thaliana*, *AtRDH1* and *AtRDH2*. Both cDNAs contained a full-length open reading frame, the expression of which increased the rhodanese activity of transgenic yeast. AtRDH1 protein was mitochondrial, while AtRDH2 was cytosolic. *AtRDH1* and *AtRDH2* genes originated from the duplication of a large genomic region in chromosome 1 which took place before the appearance of the *Arabidopsis* genus. Our results confirm the existence of rhodanese in plants.

Key words: Rhodanese; Sequence analysis; Thiosulfate:cyanide sulfurtransferase; Bioenergetics; Green fluorescent protein targeting; *Arabidopsis thaliana*

1. Introduction

Rhodanese (thiosulfate:cyanide sulfurtransferase; EC 2.8.1.1) catalyzes the reaction thiosulfate+cyanide → thiocyanate+sulfite. In bovine, this enzyme is a single polypeptide of 293 residues and 33 kDa, composed of two globular domains of the same size separated by a connecting loop [1]. The two domains have a highly homologous tertiary structure, but show little sequence homology. A conserved cysteine is involved in the binding of the sulfane moiety of thiosulfate at the active site [1]. The rhodanese is located in mitochondria, where it forms stable complexes through disulfur bonds with membrane-bound enzymes, and catalyzes the formation of iron–sulfur centers [2]. Phosphorylation inactivates the rhodanese activity, and may convert the enzyme into a protein sulfurase, which would remove the sulfur from iron–sulfur centers [3]. Rhodanese would regulate the respiration rate, through the control of the status of the iron–sulfur centers of enzymes of the respiratory chain [2,3]. The rhodanese activity itself would be regulated by a protein kinase/phosphatase, and would be the terminal step of hormonal or neurotransmitter signaling pathways acting on ATP production or oxygen consumption.

Rhodanese in plants is poorly understood. The enzyme has been proposed to have a role in cyanide detoxification, but no correlation between cyanide accumulation by plants and rhodanese activity has been observed [4]. A rhodanese purified from tapioca leaves showed properties similar to that of bovine rhodanese [5], but rhodanese activity in plants is so low that the very existence of a rhodanese enzyme has been controversial [6,7]. In order to ascertain the existence of rhodanese in plants, we searched an expressed sequence tag (EST) database for cDNAs encoding rhodanese-like proteins from *Arabidopsis thaliana*. Here we describe, for the first time in plants, the isolation and preliminary characterization of two rhodanese cDNAs from *A. thaliana*.

2. Materials and methods

2.1. Miscellaneous techniques

Molecular cloning, PCR amplification, yeast media, cultures and heat shock transformation were performed according to standard procedures [8]. *A. thaliana* var. *Columbia* was grown vertically for 3 weeks on solid MS medium at 24°C and 16 h light per day, then transferred to fresh MS medium for 2 days before use.

2.2. Isolation of rhodanese-like cDNAs in A. thaliana

The *A. thaliana* subset of the dbEST database was searched for ESTs encoding putative homologues of bovine rhodanese [9] using the various BLAST algorithms. The ESTs identified were separated into families based on their homologies using the GELMERGE algorithm of the GCG package (Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI, USA). ESTs encoding putative rhodanese-like proteins were obtained from the Arabidopsis Biological Resource Center (http://aims.cps.msu.edu/aims/), and sequenced on both strands using the Thermo Sequenase kit (Amersham Pharmacia Biotech) and a DSQ2000 sequencer (Shimazu, Japan), following the instructions of the manufacturers.

2.3. Expression of rhodanese isoforms in yeast

The yeast–bacteria shuttle expression vector pYPGE15, which contains a polylinker placed between the yeast phosphoglycerate kinase (PGK) gene promoter and the CYC1 terminator, was used to constitutively overexpress rhodanese proteins in yeast. A *Bgl*II–*Xba*I fragment of EST plasmid 141K15T7, enclosing the complete coding sequence of AtRDH1, was introduced downstream the PGK promoter of pYPGE15. A *Sal*I site was engineered upstream of the AtRDH2 ORF by polymerase chain reaction, using synthetic oligonucleotide primers (Rho2TP: TCACTTTGGTCGACTCATGGCTTC; M13-20: GTAAAACGACGGCCAGT)) and EST plasmid TAP0319 as a template. The resulting fragment was digested by *Sal*I and *Kpn*I and introduced downstream of the PGK promoter of pYPGE15. The resulting expression vectors were introduced into *Saccharomyces cerevisiae* strain CC371-4C (*matα leu2 ura3 met3*).

2.4. Determination of rhodanese activity in yeast and plants

Transformed yeast were cultured in adequate medium until they reached an absorbance at 600 nm of 1.0, then were harvested by centrifugation, rinsed in water and resuspended in ice-cold extraction

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buffer (100 mM potassium phosphate pH 7.2, 1 mM EDTA, 10 mM $Na_2S_2O_3$, 10% (v/v) glycerol). Cells were disrupted by sonication by five pulses of 20 s, 100 W, on melting ice. A cell-free crude protein extract was obtained after centrifugation for 10 min at 10 000 × *g*. Plant tissues were ground into liquid nitrogen, and the resulting powder was homogenized into ice-cold extraction buffer. A cell-free crude protein extract was obtained after centrifugation for 10 min at 10 000 × *g*. Rhodanese activity was measured on fresh crude protein extracts by measuring thiocyanate formation by the method of Westley [10]. Results were corrected for spontaneous thiocyanate formation. One activity unit was defined as the synthesis of 1 μmol of thiocyanate per minute. Proteins were assayed using a protein assay kit (Bio-Rad) with bovine serum albumin as a standard.

2.5. Construction of rhodanese–GFP chimeric proteins

The expression vector sGFP(S65T) [11], which contains a plant-improved GFP coding sequence controlled by the CaMV 35S promoter and the nopaline synthase gene terminator, was used to produce rhodanese targeting peptide–GFP fusion proteins.

An *Nco*I site was engineered in the *AtRDH1* cDNA sequence by PCR, using synthetic oligonucleotide primers (M13-reverse (forward): GGAAACAGCTATGACCATG; RhodTP (reverse): GCTCATCCGCCATGGACCATGAAGCATCC) and EST plasmid 141K15T7 as a template. The amplified DNA fragment was digested by *Sal*I and *Nco*I and inserted into the expression vector, resulting in the expression of a chimeric protein containing the 108 N-terminal amino acids of the AtRDH1 protein fused upstream of the full-length GFP protein.

An *Nco*I and a *Sal*I site were engineered in the *AtRDH2* cDNA sequences by PCR, using synthetic oligonucleotide primers (Rho2TP (forward): TCACTTTGGTCGACTCATGGCTTC; RhodTP (reverse): GCTCATCCGCCATGGACCATGAAGCATCC) and EST plasmid TAP0319 as a template. The amplified DNA fragment was digested by *Sal*I and *Nco*I and inserted into the expression vector, resulting in the expression of a chimeric protein containing the 45 N-terminal amino acids of the AtRDH2 protein fused upstream of the full-length GFP protein.

2.6. Transient expression of GFP–protein fusion by particle bombardment

Particle gun bombardment was carried out as previously described [12] using a Helios Gene-Gun System (Bio-Rad Biosystem) and following the protocol provided by the supplier. Leaves were observed with a fluorescent microscope (BX50-FLA, Olympus) using Chroma's dual band filters, which provide excitation at 475–490 and 545–565 nm and emission at 510–530 and 585–620 nm.

3. Results and discussion

3.1. Identification and functional characterization of rhodanese cDNAs in A. thaliana

The presence of a detectable rhodanese activity in *A. thaliana* (Table 1) led us to search for rhodanese-encoding cDNAs by sequence analysis of the *A. thaliana* subset of the dbEST database, using animal rhodanese sequences as probes. Among the 45 752 ESTs of *A. thaliana* present in dbEST (release 102999), eight could be assigned to encode a putative rhodanese, and they could be separated into two families.

Table 1
Rhodanese activity in soluble protein extracts of *A. thaliana* and transformed yeast expressing *AtRDH1* and *AtRDH2*

Organism		Rhodanese activity (mU/mg protein)
A. thaliana	Shoot	23.1 ± 0.2
	Roots	129.4 ± 2.8
Yeast	pYPGE15	37.5 ± 11.9 (100%)
	AtRDH1	84.0 ± 1.8 (224%)
	AtRDH2	75.9 ± 0.4 (202%)

Rhodanese activity was determined as described by Westley [10]. Data are mean ± S.D.

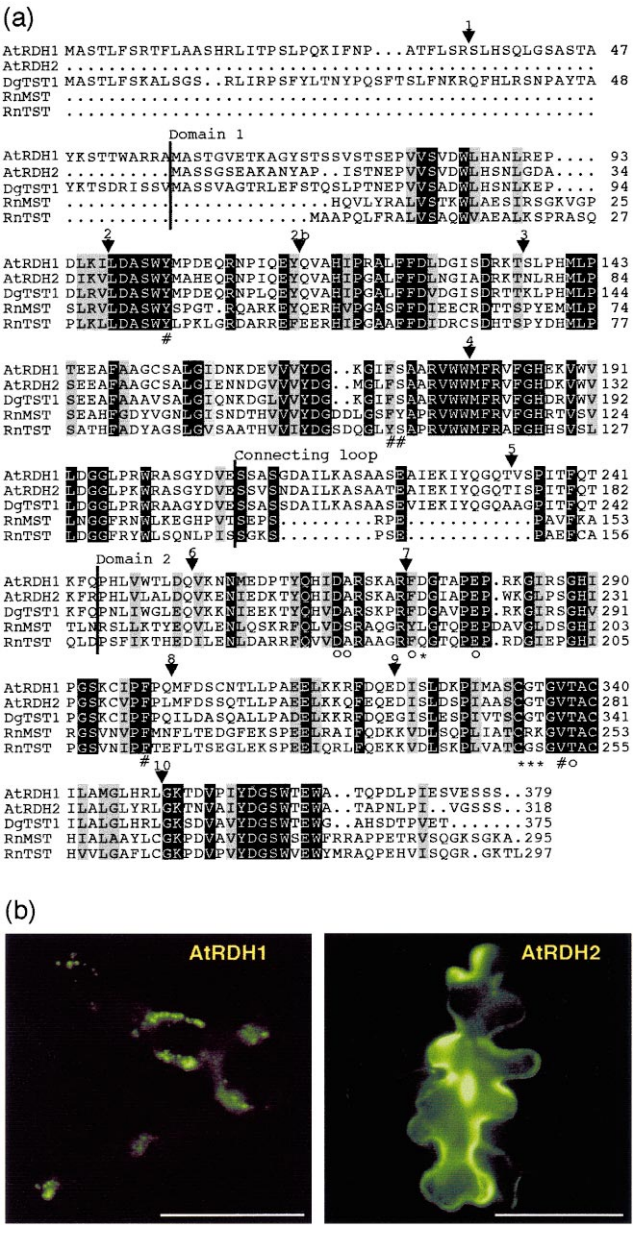

Fig. 1. Isolation of *AtRDH1* and *AtRDH2* cDNAs encoding two rhodanese genes from *A. thaliana*. a: Alignment of plant rhodanese and rat rhodanese and MST proteins. Full-length protein sequences were aligned using Clustal X. Conserved identical (black) or similar (gray) residues are shaded. Boundaries of domains 1 and 2 are shown. The arrows show the conserved intron positions in *AtRDH1* and *AtRDH2* sequences. The extra intron in *AtRDH2* is indicated (2b). *: residues forming the active site in mammal rhodanese. #, o: hydrophobic and hydrophilic residues surrounding the active site in mammal rhodanese. RnMST: rat MST. RnTST: rat rhodanese. b: Subcellular localization of GFP fused to the AtRDH1 and AtRDH2 N-terminal sequence. AtRDH1 108 N-terminal and AtRDH2 45 N-terminal amino acid–GFP fusion proteins were expressed in 4-week-old *A. thaliana* leaves by particle bombardment. Expression was monitored for 20 h after bombardment. Scale bar: 100 μm.

Representative ESTs were sequenced and named *AtRDH1* and *AtRDH2* for *Arabidopsis thaliana* rhodanese homologue 1 and 2 respectively (accession numbers AJ011045 and AJ010500, respectively).

AtRDH1 contained a full-length open reading frame (ORF)

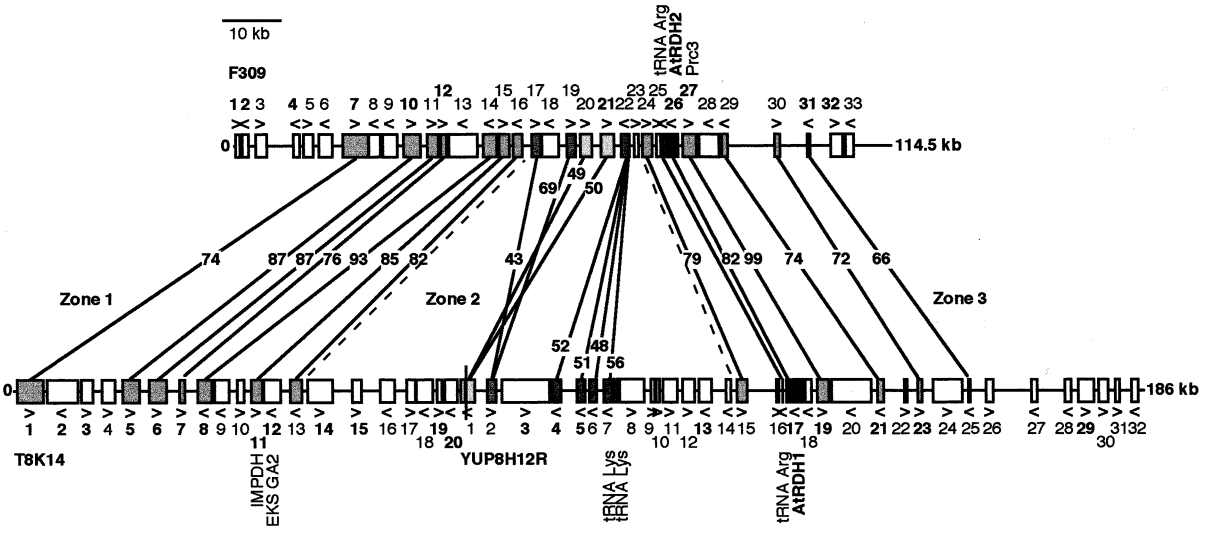

Fig. 2. Structure of *AtRDH1* and *AtRDH2* neighboring genomic regions in *A. thaliana* chromosome 1. The names of the studied BACs are indicated in bold characters. Putative protein-encoding regions are boxed and numbered. Putative tRNA genes are indicated, but not numbered. Gene orientation is shown by the arrowhead. Genes with corresponding ESTs or mRNA in the databases have their numbers written in bold face. Genes whose function has been characterized are indicated. Homologous putative genes present in both genomic regions are shaded and connected, with the amino acid identity percentage indicated. Zones 1, 2 and 3 are delimited by dotted lines. IMPDH: inositol monophosphate dehydrogenase. Prc3: proteasome subunit Prc3. EKS GA2: ent-kaurene synthase GA2. AtRDH: rhodanese. Only the first 32 putative genes (of 46) present in BAC YUP8H12R are shown.

encoding a 379 amino acids long protein of 41.9 kDa. *AtRDH2* contained a full-length ORF encoding a 318 amino acids long protein of 34.7 kDa. AtRDH1 and AtRDH2 were 81% identical to each other, and respectively 76% and 71% identical to a putative mitochondria-targeted rhodanese-like protein of *Datisca glomerata*, DgTST, whose actual function had not been investigated [13]. Most of the essential amino acids defined in animal rhodanese were conserved in the plant proteins (Fig. 1a), whose predicted tertiary structure could be superimposed on that of bovine rhodanese (not shown). The expression of *AtRDH1* and *AtRDH2* cDNAs in transgenic yeast resulted in a two-fold increase of their rhodanese activity (Table 1). Thus, we conclude that *AtRDH1* and *AtRDH2* encode two functional rhodanese isoforms in *A. thaliana*.

In animals, cytosolic mercaptopyruvate:cyanide sulfurtransferase (EC 2.8.1.2; MST), which catalyzes the reaction mercaptopyruvate+cyanide → pyruvate+thiocyanate, is closely related to mitochondrial rhodanese [14]. Both enzymes catalyze the MST and rhodanese reactions, and they are actually defined by their preferential use of mercaptopyruvate or thiosulfate, respectively [14,15]. The sequence of the active site of plant rhodanese (CGTG) is closer to that of animal MST (CGSG) than to that of animal rhodanese (CRKG) (Fig. 1a). In rat rhodanese, the substitution of CRKG by C<u>G</u>KG increases the MST activity of the mutated protein [14]. Therefore, plant rhodanese may also have MST activity, in addition to their rhodanese activity. Kinetic characterization of purified recombinant AtRDH1 and AtRDH2 proteins would address this question.

The AtRDH1 protein had a 57 amino acids long N-terminal extension compared to AtRDH2 and animal sequences (Fig. 1a), which had the features of a mitochondria-targeting peptide. The expression of a chimeric protein consisting of the 108 N-terminal amino acids of AtRDH1 fused to the N-terminus of the green fluorescent protein (GFP) in *A. thaliana* leaves showed a pattern identical to that obtained with spinach mitochondrial cysteine synthase-like CS-C N-terminus–GFP fusion protein [12] (Fig. 1b). The expression of a chimeric protein consisting of the 45 N-terminal amino acids of AtRDH2 fused to the GFP showed a pattern identical to that of the cytosolic sGFP(S65T) protein. Together with the computer prediction for protein targeting (PSORT program, http://www.genome.ad.jp/SIT/SIT.html), these results suggest strongly that AtRDH1 would be targeted to the mitochondria, while AtRDH2 would be located in the cytosol. In animals, rhodanese is mitochondrial while MST is cytosolic [14]. The presence of a cyanide sulfurtransferase activity in both subcellular compartments could be a general feature of higher organisms. The 22 N-terminal amino acids in bovine rhodanese are sufficient for mitochondrial import [16], and are not removed during enzyme maturation [9]. In contrast, the presence of an N-terminal extension in mitochondrial AtRDH1 and putative mitochondrial DgTST1 (Fig. 1a), and its absence in cytosolic AtRDH2, suggests strongly that in plants, the mitochondrial import of rhodanese could involve the cleavage of a targeting peptide.

3.2. Gene sequence analysis

AtRDH1 was identical to gene *YUP8H12R.17* (accession number AC002986), while *AtRDH2* was identical to gene *F309.26* (accession number AC006341), both located in chromosome 1. *AtRDH1* and *AtRDH2* genes contained respectively 10 and 11 well conserved introns (Fig. 1a). We investigated whether *AtRDH1* and *AtRDH2* could have appeared after a genomic duplication or a single gene duplication event. The complete genetic surroundings of both *AtRDH1* and *AtRDH2*, spanning respectively 186 kb (BAC sequences T8K14 (accession number AC007202) and part of YUP8H12R) and 114.5 kb (BAC sequence F309) were compared (Fig. 2).

The *AtRDH1* surroundings contain 52 predicted genes, of which 20 have a homologue in *AtRDH2* surroundings. The

deduced proteins of these common genes show 70 ± 17% average sequence identity with their counterparts. Thus we conclude that the two rhodanese genes arose from the duplication of a complete genomic region containing numerous genes. Three zones can be defined in *AtRDH1* and *AtRDH2* surroundings. The first zone shows seven common genes, disposed in the same order, with the same orientation and spanning the same space in both genomic regions. Their deduced proteins share 84 ± 7% identity with their counterpart. The second zone is extensively reorganized, exhibiting the events of two duplications, one quadruplication and a number of gene insertions and deletions. The third zone shows six common ORFs, disposed in the same order, with the same orientation, and spanning roughly the same space in both genomic regions. This zone includes the rhodanese gene, bordered by an identical $tRNA^{Arg}$ gene. The deduced proteins of these six common genes share 79 ± 12% identity. Therefore, the two genomic regions were not subjected to an extensive reorganization after the duplication event, except in the second zone. The size of the genomic duplication, which could extend upstream and downstream of the regions investigated in this study, and the relative positions of the two homologous regions in chromosome 1 are unknown. The completion of the *A. thaliana* genome sequencing project will address these questions.

The deduced proteins of the 13 genes contained in the first and third zones share an average 81 ± 9% sequence identity. Using the calibrated protein clock published by Feng et al. [17], we estimate that the duplication in chromosome 1 could have happened 130–450 million years ago. This duplication would therefore long predate the appearance of the *Arabidopsis* genus, estimated to have occurred 14.5–20.4 million years ago [18]. A similar genome structure could exist in other plant genera and families.

3.3. Conclusion

To the best of our knowledge this is the first time that rhodanese-encoding cDNAs from plants are characterized. This report confirms the existence of rhodanese in plants, and gives a final answer to an older debate [6,7]. The availability of plants rhodanese cDNAs will make it possible to better characterize the biochemical characteristics and the physiological function of this enzyme, which remains poorly understood even in animals.

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