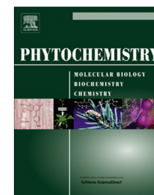
Phytochemistry 94 (2013) 45–52

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem



Abolishing activity against ascorbate in a cytosolic ascorbate peroxidase from switchgrass

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ARTICLE INFO

Article history:
Received 25 January 2013
Received in revised form 8 May 2013
Available online 26 June 2013

Keywords:
Ascorbate
Peroxidase
Panicum virgatum
Poaceae
Mutant
ABTS

ABSTRACT

Switchgrass (*Panicum virgatum* L.) is being developed as a bioenergy species. Recently an early version of its genome has been released permitting a route to the cloning and analysis of key proteins. Ascorbate peroxidases (APx) are an important part of the antioxidant defense system of plant cells and present a well studied model to understand structure–function relationships. Analysis of the genome indicates that switchgrass encodes several cytosolic ascorbate peroxidases with apparent varying levels of tissue expression. A major cytosolic ascorbate peroxidase was thus selected for further studies. This gene was cloned and expressed in *Escherichia coli* cells to obtain purified active protein. Full heme incorporation of the enzyme was achieved utilizing slow growth and supplementing the media with 5-aminolevulinic acid. The enzyme was observed to be monomeric in solution via size exclusion chromatography. Activity toward ascorbate was observed that was non-Michaelis–Menten in nature. A site-directed mutant, R172S, was made in an attempt to differentiate activity against ascorbate versus other substrates. The R172S protein exhibited negligible ascorbate peroxidase activity, but showed near wild type activity toward other aromatic substrates.

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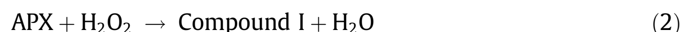
1. Introduction

Cytosolic ascorbate peroxidase (APx) catalyzes the reduction of H_2O_2 utilizing small organic substrates as electron donors with the physiological substrate being ascorbate (AsA) as shown below (Eq. (1)).

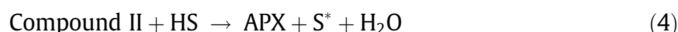
$$H_2O_2 \overset{APX}{\rightarrow} 2H_2O + 2MDAsA \quad (1)$$

In plants, the monodehydroascorbate radical (MDAsA) is converted back to AsA by MDAsA reductase or in the absence of reductase, the 2 MDAsAs can disproportionate to AsA and dehydroascorbic acid (DAsA) (Chen et al., 2003; Shigeoka et al., 2002). The catalytic mechanism of ascorbate oxidation by APx has been well studied and is generally represented as shown in Eqs. (2)–(4), where APx first reacts with H_2O_2 and becomes Compound I, the fully oxidized form of APx. Compound I is converted back to APx via two one-electron transfers from an electron donating substrate (HS).



$$APX + H_2O_2 \rightarrow \text{Compound I} + H_2O \quad (2)$$

$$\text{Compound I} + HS \rightarrow \text{Compound II} + S^* \quad (3)$$



$$\text{Compound II} + HS \rightarrow APX + S^* + H_2O \quad (4)$$

There are many excellent reviews dealing with APx (Dunford, 1999; Raven, 2003; Raven et al., 2004), and only a brief review is provided here. Ascorbate-dependent peroxidases from spinach and pea were first described in 1979 (Groden and Beck, 1979; Kelly and Latzko, 1979) and are key proteins protecting cellular health in plants by scavenging peroxides generated during photosynthesis and cellular metabolism (Alvarez and Lamb, 1997; Bowler et al., 1992; Ishikawa and Shigeoka, 2008; Noctor and Foyer, 1998). They have been investigated at the structural and functional level because unlike cytochrome *c* peroxidase (CcP), one of the best known and characterized peroxidases, they have the more typical Compound I intermediate with a porphyrin-based radical and they utilize small molecule substrates as electron donors (Raven, 2003). Ascorbate-dependent peroxidases are essentially defined by having high specificity to ascorbate, which has a clear physiological role as an electron source for detoxifying cells of H_2O_2. However many of these enzymes also have significant activity toward other aromatic electron donors, which usually do not have clear physiological roles. For the most part, these ascorbate-dependent peroxidases have higher activity toward ascorbate than other substrates.

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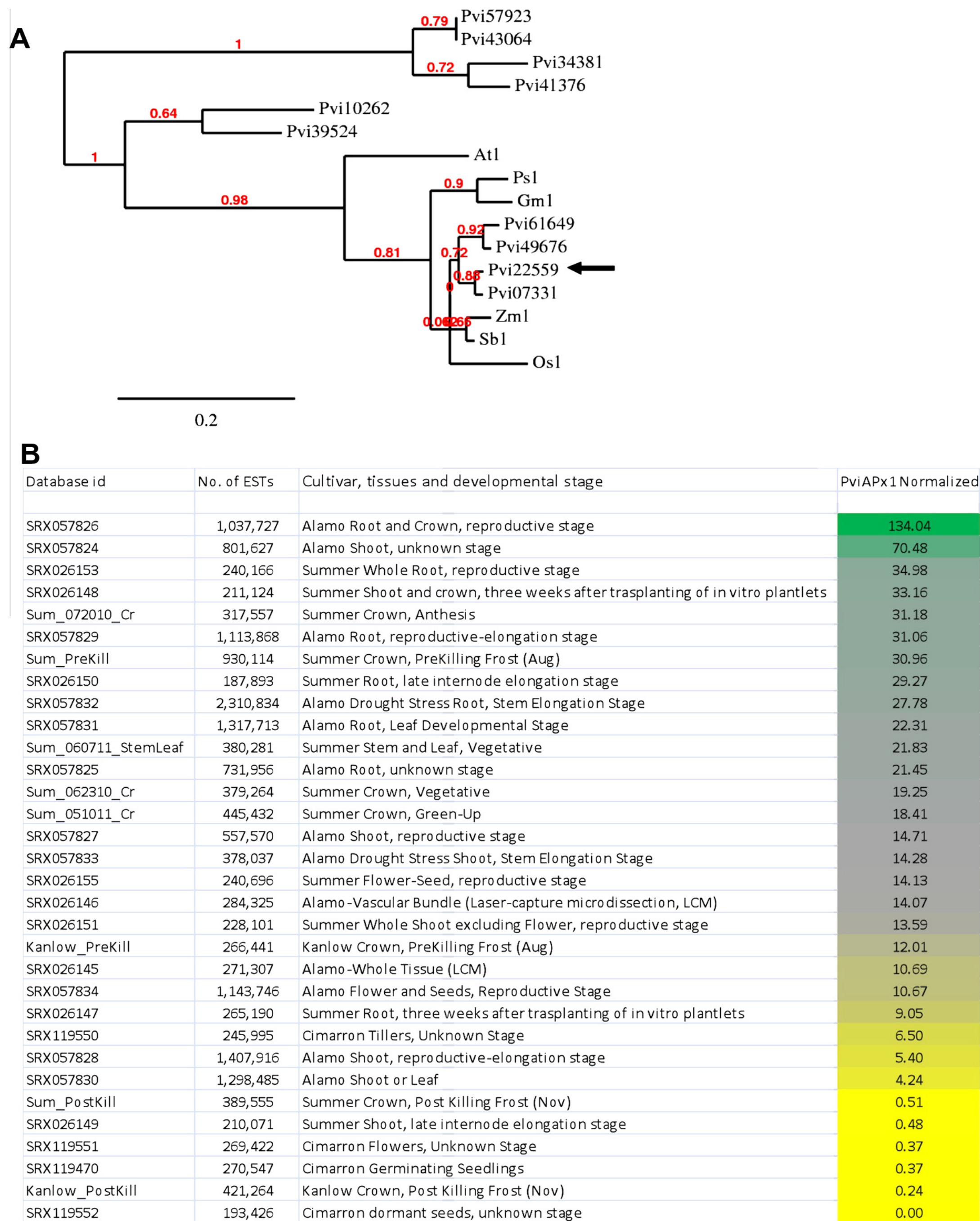
http://dx.doi.org/10.1016/j.phytochem.2013.05.016



Database id	No. of ESTs	Cultivar, tissues and developmental stage	PviAPx1 Normalized
SRX057826	1,037,727	Alamo Root and Crown, reproductive stage	134.04
SRX057824	801,627	Alamo Shoot, unknown stage	70.48
SRX026153	240,166	Summer Whole Root, reproductive stage	34.98
SRX026148	211,124	Summer Shoot and crown, three weeks after trasplanting of in vitro plantlets	33.16
Sum_072010_Cr	317,557	Summer Crown, Anthesis	31.18
SRX057829	1,113,868	Alamo Root, reproductive-elongation stage	31.06
Sum_PreKill	930,114	Summer Crown, PreKilling Frost (Aug)	30.96
SRX026150	187,893	Summer Root, late internode elongation stage	29.27
SRX057832	2,310,834	Alamo Drought Stress Root, Stem Elongation Stage	27.78
SRX057831	1,317,713	Alamo Root, Leaf Developmental Stage	22.31
Sum_060711_StemLeaf	380,281	Summer Stem and Leaf, Vegetative	21.83
SRX057825	731,956	Alamo Root, unknown stage	21.45
Sum_062310_Cr	379,264	Summer Crown, Vegetative	19.25
Sum_051011_Cr	445,432	Summer Crown, Green-Up	18.41
SRX057827	557,570	Alamo Shoot, reproductive stage	14.71
SRX057833	378,037	Alamo Drought Stress Shoot, Stem Elongation Stage	14.28
SRX026155	240,696	Summer Flower-Seed, reproductive stage	14.13
SRX026146	284,325	Alamo-Vascular Bundle (Laser-capture microdissection, LCM)	14.07
SRX026151	228,101	Summer Whole Shoot excluding Flower, reproductive stage	13.59
Kanlow_PreKill	266,441	Kanlow Crown, PreKilling Frost (Aug)	12.01
SRX026145	271,307	Alamo-Whole Tissue (LCM)	10.69
SRX057834	1,143,746	Alamo Flower and Seeds, Reproductive Stage	10.67
SRX026147	265,190	Summer Root, three weeks after trasplanting of in vitro plantlets	9.05
SRX119550	245,995	Cimarron Tillers, Unknown Stage	6.50
SRX057828	1,407,916	Alamo Shoot, reproductive-elongation stage	5.40
SRX057830	1,298,485	Alamo Shoot or Leaf	4.24
Sum_PostKill	389,555	Summer Crown, Post Killing Frost (Nov)	0.51
SRX026149	210,071	Summer Shoot, late internode elongation stage	0.48
SRX119551	269,422	Cimarron Flowers, Unknown Stage	0.37
SRX119470	270,547	Cimarron Germinating Seedlings	0.37
Kanlow_PostKill	421,264	Kanlow Crown, Post Killing Frost (Nov)	0.24
SRX119552	193,426	Cimarron dormant seeds, unknown stage	0.00

Fig. 1. Phylogenetic relationships of PviAPx1 (A) (Pvi22559; arrow). Other switchgrass Apx sequences are identified by Pvi. The full database entry will read, for example as Pavirv00022559 m etc. Other cytosolic APx sequences used for this analysis are: At1 = *Arabidopsis thaliana*; Ps1 = *Pisum sativum*; Gm1 = *Glycine max*; Zm1 = *Zea mays*; Sb1 = *Sorghum bicolor*; Os1 = *Oryza sativa*.Relative expression of PviAPx1 in switchgrass tissues (B).

Crystal structures of APxs have been used to identify two different substrate binding sites, one for ascorbate (PDB ID: 1OAF) (Sharp et al., 2003) and one for aromatic substrates (PDB IDs: 1VOH, complexed with salicyhydroxamic acid and 2VCS, complexed with isoniazid)(Metcalfe et al., 2008; Sharp et al., 2004). This would strongly indicate that the substrate specificity for the other aromatic compounds comes from a different binding site than that of ascorbate.

In 1995, the first crystal structure of APx was determined for recombinant pea cytosolic APx (PDB ID: 1APX) (Patterson and Poulos, 1995). This structure showed a dimeric enzyme held together non-covalently by ionic interactions. The structure was remarkably similar to that of CcP, especially in the heme active site where the same hydrogen bonds exist between key conserved amino acids that are known to be essential for function. One striking difference was the discovery of a cation (K^+) binding site that has been observed to influence the ligation of the distal histidine to the heme iron (Cheek et al., 1999). In 2003, another set of structures were determined for recombinant soybean cytosolic APx that included a structure of the APx-ascorbate complex (PDB ID: 1OAF) (Sharp et al., 2003). This structure positively identified the γ-edge of the heme as the primary location of ascorbate binding and paved the way to better understanding the electron transfer mechanism for the enzyme. Presently, there are almost 30 APx structures in the protein structural database (http://www.rscb.org) that are predominantly mutant structures for cytosolic APx.

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