Characterization of a Plant, Tyrosine-Specific Phosphatase of the Aspartyl Class†

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ABSTRACT: The *Arabidopsis thaliana* homologue of the Eyes Absent genes (*AtEYA*) encodes a protein corresponding to the C-terminal conserved domain of the animal Eyes Absent proteins. We show here that AtEYA is a tyrosine-specific phosphatase that hydrolyzes its substrates in a metal-dependent reaction analogous to the phosphoserine phosphatases of the haloacid dehalogenase (HAD) family. The animal Eyes Absent proteins are a novel family of dual-function enzymes: they are transcription factors as well as phosphatases. They also represent a new mechanistic class of tyrosine phosphatases (PTPs) that do not have the Cys-containing signature motif. In contrast, AtEYA is only a tyrosine phosphatase and has no transactivation domain. Using the reaction mechanism of other HAD family enzymes as a model, we have conducted mutational analyses on AtEYA to query the roles of conserved residues. This analysis confirms the importance of the putative nucleophile, the general acid, and the metal-binding residues. Additionally, an inhibitory profile that is diagnostic of this new class of protein tyrosine phosphatases is described. The results of these studies on AtEYA reveal that while the animal and plant Eyes Absent proteins catalyze the same dephosphorylation reaction, the details of their specificity and active site environment, as well as their biological roles, are distinct.

Protein function in multicellular organisms is often modulated by reversible phosphorylation, with approximately 30% of all cellular proteins being phosphorylated (*1*). The phosphorylation state of a protein can influence function, stability, and localization. The main sites of phosphorylation in eukaryotic proteins are serine, threonine, and tyrosine residues. Dephosphorylation is carried out by phosphatases, which are classified on the basis of their substrate specificity as Ser/Thr phosphatases, dual-specificity phosphatases, or tyrosine phosphatases. The Ser/Thr phosphatases share a common three-dimensional fold and an acid−base catalytic mechanism. The classical tyrosine phosphatases and dual-specificity phosphatases are structurally and mechanistically distinct from the Ser/Thr phosphatases. They include a Cys-containing "signature" motif and hydrolyze phosphotyrosines via a thiol phosphate intermediate (reviewed in refs *2* and *3*). An additional class of tyrosine phosphatases that use an aspartate as a nucleophile in a metal-dependent reaction, exemplified by the Eyes Absent (EYA)[1] proteins (*4*−*6*), has also been recently described (reviewed in ref *7*).

While tyrosine phosphorylation in higher plants was considered rare until relatively recently, it is now believed that plant proteins are phosphorylated on tyrosine residues at levels comparable to those seen in animals (*8*). Tyrosine phosphorylation levels have been shown to vary as a response to stress signals and mitogens, with movement (*9*, *10*), and with developmental stage (*8*). A recently reported virtual screening of the *Arabidopsis thaliana* proteome (*11*) suggests that about 4% of the over 900 putative protein kinases are tyrosine-specific kinases, contradicting the previously held view that tyrosine phosphorylation in plants, as in yeast, was catalyzed by a few dual-specificity kinases. Interestingly, of the 112 phosphatases identified in the *Arabidopsis* genome, only one was a protein tyrosine phosphatase (*12*). In addition, there are 17 dual-specificity phosphatases and one low molecular weight phosphatase. The tyrosine-specific phosphatase, AtPTP1, has been cloned and characterized (*13*, *14*). One other plant phosphatase PTPKIS1 has very weak activity toward phosphotyrosine peptides but no activity toward phosphotyrosine (*15*). This enzyme was not definitively classified as either a tyrosine-specific or a dual-specificity phosphatase.

Here we report the biochemical characterization of the Eyes Absent homologue in *A. thaliana*, which is a tyrosine-specific phosphatase lacking the diagnostic Cys-containing signature of the classical PTPases and belongs to the Asp-based class of PTPs (classification as in ref *7*). This 307-residue protein (NCBI protein gi no. 23506145) shares 39% sequence similarity (23% identity) with the conserved C-terminal domain of the animal Eyes Absent proteins. The animal Eyes Absent proteins are involved in cell-fate determination in both vertebrates and invertebrates. In *Drosophila* eye development, eyes absent (eya) is part of a genetic cascade that also includes the genes twin of eyeless (toy), eyeless (ey), eyes absent (eya), sine oculis (*so*), and *dachshund* (*dac*) (reviewed in ref *16*). In various combinations, these genes have also been implicated in leg, gonad, kidney, muscle, ear, and brain development. Animal Eyes

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[1] Abbreviations: EYA, Eyes Absent; PTP, protein tyrosine phosphatase; HAD, haloacid dehalogenase; ED, Eya domain; TAIR, The Arabidopsis Information Resource; GST, glutathione *S*-transferase; Ni-NTA, nickel nitrilotriacetic acid; pNPP, *p*-nitrophenyl phosphate; pY, phosphotyrosine; pS, phosphoserine; pT, phosphothreonine.

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Absent proteins range in size from 510 to 760 amino acids, containing a highly conserved 271−274-residue C-terminal domain [Eya domain (ED)] that participates in protein−protein interactions (*17*). We, and others, have recently shown that the ED domain has protein tyrosine phosphatase activity (*4*−*6*). In addition, the poorly conserved N-terminal domain has a transactivation function (*18*), although the Eyes Absent proteins have no known direct DNA-binding activity.

Eyes Absent homologues have been identified in both monocots and dicots: rice (OsEYA1), *A. thaliana* (AtEYA), and alfalfa (MsEYA) (*19*). These plant Eyes Absent homologues consist of the ED domain and a short (15−23 amino acid) N-terminal peptide. In rice OsEYA1 is expressed in the embryo, caryopsis, and shoot apex and is thought to inhibit proliferation of the primordia of lateral organs and thus function in pattern formation (*19*). In this report we describe the biochemical characterization of the phosphatase activity and specificity of AtEYA. This protein is the first representative of the metal-dependent, Asp-based class of PTPs in plants and only the second tyrosine-specific plant phosphatase to be reported.

EXPERIMENTAL PROCEDURES

Recombinant Proteins. The AtEYA cDNA obtained from TAIR (The Arabidosis Information Resource) was used as a PCR template to insert the AtEYA coding sequence into the *Nde*I and *Bam*HI sites of expression vector pET15b and the *Bam*HI and *Xho*I sites of pGEX4T. All mutations were introduced using standard PCR-based protocols. All of the resulting fusion proteins [glutathione *S*-transferase (GST) for pGEX4T and $(His)_6$ for pET15b] were overexpressed in BL21(DE3). Bacterial cultures were grown at 37 °C. At mid-log ($A_{595} = 0.6-0.8$) protein overexpression was induced at 15 °C with 0.3 mM IPTG. Cells overexpressing GST fusion proteins [AtEYAΔ15, AtEYA(D27N), AtEYA(T29A), AtEYA(D25E), AtEYA(D25C), AtEYA(T197A), AtEYA(G252A), AtEYA(D253N), AtEYA(E257Q)] were lysed by sonication in 20 mM Tris, pH 8, 150 mM NaCl, and 1% Triton X-100. GST-tagged AtEYA proteins were present in the soluble fraction. The cell lysates were loaded on glutathione−agarose beads and washed extensively with lysis buffer. The proteins were then released by thrombin treatment. Except in the case of AtEYA(D27N), the proteins were then purified by size exclusion chromatography (Superdex-75). The resulting proteins were homogeneous as estimated by overloaded SDS−PAGE gels stained with both Coomassie and silver. AtEYA(D27N) was first purified by ion-exchange chromatography (Mono-Q), using a NaCl gradient of 0−300 mM NaCl at pH 8, and then by size exclusion chromatography on Superdex-75.

Cells expressing His-tagged proteins [AtEYA, AtEYA(D25N), AtEYA(D27A)] were lysed by sonication in 20 mM Tris, pH 8, 500 mM NaCl, 5 mM imidazole, and 1% Triton X-100. The proteins were present in the soluble fraction. The cell lysate was loaded on a Ni-NTA column and washed with lysis buffer. The proteins were released by thrombin treatment. AtEYA(D25N) and AtEYA(D27A) were then purified to homogeneity by size exclusion chromatography (Superdex-75). AtEYA was first purified by ion-exchange chromatography on a Mono-Q column and eluted using a NaCl gradient of 0−300 mM NaCl at pH 8. AtEYA eluted from the Mono-Q column near the end of the gradient. It was concentrated and further purified to homogeneity by size exclusion chromatography on Superdex-75.

Arabidopsis MPK4 was expressed in *Escherichia coli* as a maltose-binding protein fusion. It was purified by affinity chromatography on amylose resin and eluted with maltose.

PTP1B, used as a control in some experiments, was purchased as a GST fusion protein from Upstate Biotechnologies Inc.

Phosphatase Assays. For the *p*-nitrophenyl phosphate (pNPP) assay, reaction mixtures (60 μL) in 20 mM MES (pH 5.5), various concentrations of pNPP, and cations were preequilibrated at 30 °C for 10 min. The assay was initiated by the addition of 0.3 μg of enzyme in 20 mM Tris (pH 8.0) and 150 mM NaCl. The reaction mixture was incubated for 20 min. Reactions were quenched with 100 μL of 0.5 M EDTA, pH 10.0. Release of *p*-nitrophenol (pNP) was monitored by measuring A_{410} and extrapolating the values to a *p*-nitrophenol standard curve. The data were fit directly into the Michaelis−Menten equation using SIGMAPLOT (SPSS Science, Chicago, IL). In initial experiments we plotted the production of pNP as a function of time when various enzyme concentrations were used. These experiments indicated that using 0.3 μg of AtEYA or AtEYAΔ15 yielded a linear response for at least 30 min.

In other assays, reaction mixtures (60 μL) with different concentrations of substrate (50−1500 μM phosphopeptides, 0.1−5 mM phosphoamino acids, or dNTPs), 20 mM MES, pH 5.5, and 2 mM $MgCl_2$ were preheated at 30 °C for 10 min and reactions started by adding enzyme. Reactions were quenched after 20 min with 50 μL of malachite green reagent (Promega). Phosphate released was determined by measuring A_{650} and extrapolating the values to a phosphate standard curve. The conditions used for all experiments were in the linear range in terms of enzyme concentration and time.

AtMPK4 Dephosphorylation Assay. AtMPK4 was incubated with 5 mM ATP in kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35) at 30 °C for 2 h. The reaction mixture was then loaded on a centri-spin-10 column to separate free ATP. For the dephosphorylation assay, phosphorylated AtMPK4, various amounts of AtEYA or AtEYA(D25N) as indicated in Figure 3, and various additives were mixed in phosphatase buffer (20 mM MES, pH 5.5, 2 mM $MgCl_2$). The mixture was incubated for 2 h at 30 °C. An aliquot of this reaction was analyzed by SDS−PAGE and visualized by western blotting using anti-phosphotyrosine antibody.

Myelin Basic Protein Dephosphorylation Assay. The phosphatase activity of AtEYA toward MyBP substrates was measured using the Tyr phosphatase and Ser/Thr phosphatase assay kits from New England Biolabs. Briefly, 0.2 mM MyBP was tyrosine phosphorylated with either Abl kinase (for tyrosine phosphorylation) or cAMP-dependent protein kinase A [for Ser and Thr phosphorylation (*20*)] in 50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM DTT, 0.01% Brij 35, 1 mM ATP, and 0.074 mCi of [γ-^{32}P]-triphosphate. The phosphorylated proteins were purified as per the manufacturer's protocol. pY/S/T-MyBP (50 μM) was then treated with varying amounts of AtEYA in 20 mM MES, pH 5.5, and 2 mM $MgCl_2$ for the times indicated in Figure 3. The samples were then electrophoresed on 15% SDS−PAGE gels and visualized by autoradiography.

Table 1: Effect of Cations on AtEYA-Catalyzed pNPP Hydrolysis

	AtEYA		AtEYAΔ15	
metal	K_{metal} (mM)	k_{cat} (s^{-1})	K_{metal} (mM)	k_{cat} (s^{-1})
Ni^{2+}	0.04 (±0.005)	3827 (±121)	0.011 (±0.001)	2185 (±45)
Mg^{2+}	0.104 (±0.01)	3747 (±131)	0.101 (±0.01)	2375 (±53)
Co^{2+}	0.123 (±0.03)	3744 (±221)	0.014 (±0.001)	2081 (±34)
Mn^{2+}	0.03 (±0.006)	2275 (±91)	0.011 (±0.001)	1464 (±33)
Zn^{2+}	0.02 (±0.005)	2093 (±140)	0.008 (±0.001)	1150 (±38)
Ca^{2+}	no detectable activity			
Co^{3+}	no detectable activity			

RESULTS

AtEYA Is a Metal-Dependent Phosphatase. AtEYA is able to hydrolyze the standard substrate *p*-nitrophenyl phosphate (pNPP) in the presence of Mg^{2+} with standard Michaelis−Menten kinetics, yielding $K_m = 0.73$ mM and $k_{cat} = 3630$ s^{-1} at pH 5.5 (similar to that previously reported in ref *4*). The catalysis is strictly metal-dependent, being completely inhibited by 2 mM EDTA. The reaction is favored to varying degrees by Mg^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+} (Table 1). Zn^{2+} binds with the greatest affinity while having the lowest k_{cat}, while Ni^{2+} yields the greatest catalytic rate, closely followed by Mg^{2+} and Co^{2+}. Interestingly, AtEYA has no activity in the presence of Ca^{2+}. Ca^{2+} has a larger ionic radius (1.14 Å when octahedrally coordinated) than the other divalent ions tested (Ni^{2+}, 0.83 Å; Mn^{2+}, 0.81 Å; Mg^{2+}, 0.86 Å; Zn^{2+}, 0.88 Å; Co^{2+}, 0.79 Å), and it is possible that Ca^{2+} binding induces an active site geometry that does not support catalysis. Trivalent cations such as Co^{3+} inhibit the phosphatase reaction. These results suggest that both the chemical nature of the metal ion and its correct coordination in the active site are essential for catalysis. The metals that support catalysis all prefer octahedral coordination geometry. Divalent metal-dependent catalytic activity and lack of activity in the presence of Ca^{2+} are properties that AtEYA shares with the phosposerine phosphatases.

The metal dependence of the phosphatase reaction catalyzed by AtEYA differs in one notable respect from that of the animal Eyes Absent proteins (*4*). While AtEYA has activity in the presence of Zn^{2+}, Eya3(ED) (the ED domain of mouse Eya3 corresponding to residues 238−510) has no activity in the presence of Zn^{2+} (*4*). These differences could reflect different active site geometries.

The phosphatase activity of AtEYA is comparable to that of the only other characterized plant tyrosine phosphatase AtPTP1 (*14*) and orders of magnitude higher than that of PTPKIS1, which was reported to have V_{max} of 8.8 ± 1.7 nM min^{-1} μg^{-1} using pNPP as a substrate and 0.5 ± 0.5 pM min^{-1} μg^{-1} using pY peptides as substrates (*15*).

The phosphatase activity reported here is unlikely to arise from trace contaminants, since we used recombinant AtEYA produced in *E. coli* and purified to homogeneity (as estimated by overloaded, silver-stained gels; Figure 4b). While Ser, Thr, and Tyr phosphorylation is much less common in prokaryotes than in eukaryotes, there have been reports of Ser/Thr phosphatases in *E. coli* [PrpA and PrpB (*21*)] that are expressed under conditions of stress and PTPs that are secreted by pathogenic bacteria. Our *E. coli* cell lysates not expressing AtEYA showed no phosphatase activity using pY, pS, or pT as substrates. pNPP was not used as a substrate in these control experiments since other phosphatases present in *E. coli*, such as alkaline phosphatase, can hydrolyze pNPP. Furthermore, phosphatase activity (using pNPP as a substrate) is lost when catalytic residues in AtEYA are mutationally altered (described below and in Figure 1). Taken together, these observations support the conclusion that the reported phosphatase activity arises from AtEYA and not an incidentally copurified bacterial protein.

Mutational Analysis of AtEYA Supports the Assignment of Active Site Residues. The Eyes Absent proteins share a sequence signature (Figure 1), consisting of three conserved motifs, with the haloacid dehalogenase (HAD) family of enzymes (*4*, *22*). These are an N-terminal hhhhDxDxT/S (motif I), a central hhhT (motif II) motif, and a C-terminal $Kx_{(n)}$hhhhGDxxxD/E (motif III), where h refers to hydrophobic residues. We have inferred the roles played by the conserved side chains in these motifs by analogy with well-studied HAD enzymes, such as phosphoserine phosphatase (*23*), Ca-ATPase (*24*), and MDP-1 (*25*), and conducted mutational analyses to probe mechanistic similarities between AtEYA and other HAD enzymes.

HAD family enzymes use the first aspartate residue in motif I as a nucleophile. Conservative mutations of the nucleophilic Asp 25 in AtEYA to either Asn (*4*) or Glu inactivated the enzyme. The fact that a glutamate cannot substitute for the nucleophilic Asp suggests strict geometric constraints at the active site, in agreement with the results of similar mutagenesis on other HAD family enzymes (*23*, *25*). As might be expected, replacement of Asp 25 with Cys (the nucleophilic residue for the classical PTPs) resulted in an inactive enzyme. The second aspartate in the motif, which is not completely conserved among the HAD enzymes, is proposed to act as a general acid. Changing Asp 27 in AtEYA to an Ala [or an Asn (*4*)] significantly reduced activity. There is precedent for HAD family enzymes retaining some low level of activity when residues at this position are altered; specifically an Asp to Asn mutation at the second aspartate in motif I of MDP1 retains 8% activity relative to wild type (*25*). The hydroxylic residue in motif I is thought to stabilize the nucleophilic Asp side chain, and when this was replaced by an Ala in AtEYA (T29A), catalytic activity was reduced 50-fold. The Thr/Ser in motif II and the Lys side chain in motif III are both expected to participate in substrate binding. Mutation of Thr 197 to an Ala reduced catalytic activity 55-fold. The acidic side chains in motif III as well as the nucleophilic Asp are believed to participate in metal binding. Conservative replacement of the motif III residues in AtEYA (D253N and E257Q) significantly inactivated the enzyme. The Gly in motif III is believed to play a structural role, and when we replaced it with a β-carbon-containing residue (G255A), catalytic activity was significantly reduced. All of these mutationally altered proteins were purified to homogeneity, including a final gel filtration step. The proteins eluted as sharp peaks off the column (Superdex-75) with the same retention time as the wild-type protein (data not shown). This suggests that there was no global unfolding of the protein that might account for loss of activity.

Mutational analyses of the putative active site residues of AtEYA support the proposal that AtEYA shares a catalytic mechanism with the metal-dependent aspartyl phosphatases. The results of altering active site residues in AtEYA and mouse Eya3 (*4*) are in general similar. One noteworthy

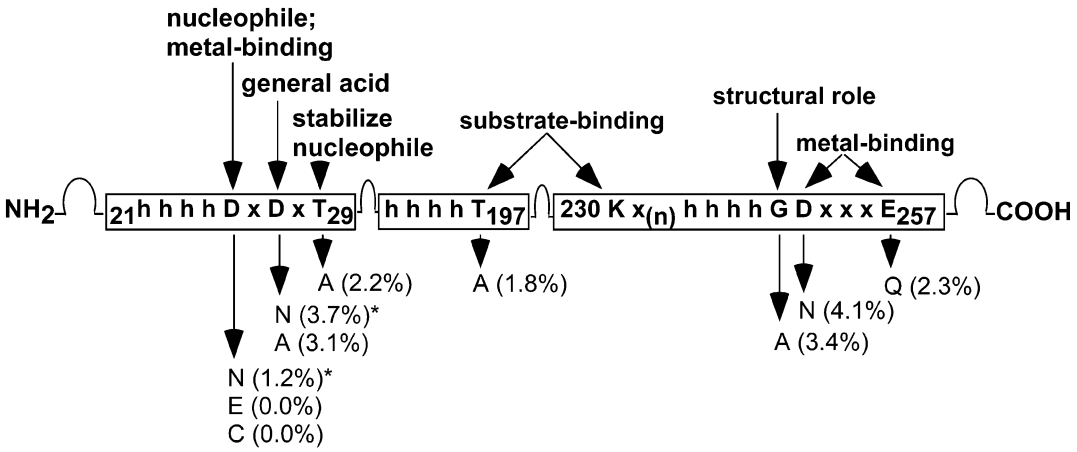

FIGURE 1: The Eyes Absent proteins belong to the haloacid dehalogenase class of enzymes, which are defined by the three motifs shown here. The roles of the conserved residues, inferred by comparison with other HAD family enzymes, are indicated above the sequence. The effect of altering the conserved residues is indicated below the sequence as a percentage of pNPP dephosphorylation activity (at pH 5.5 in the presence of 2 mM $MgCl_2$) relative to the activity of wild-type AtEYA. Values highlighted by an asterisk were previously reported in ref *4*.

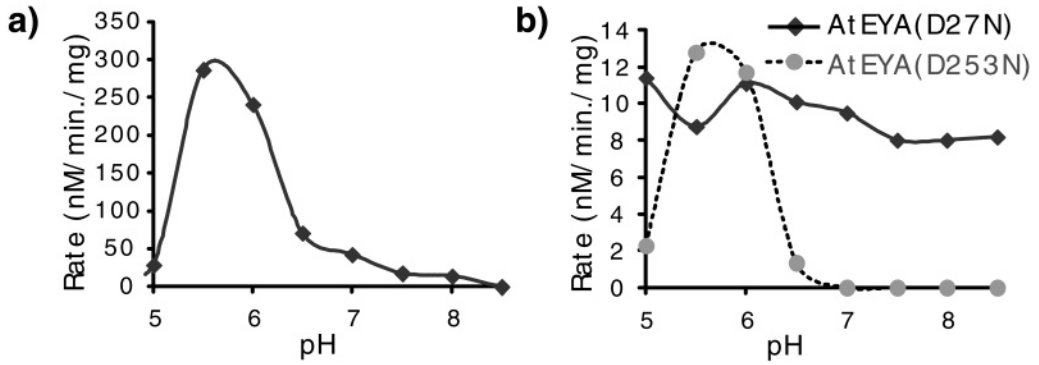

FIGURE 2: pH dependence of the dephosphorylation of phosphotyrosine catalyzed by AtEYA. (a) Phosphotyrosine dephosphorylation by AtEYA. (b) Replacing the putative general acid Asp 27 in AtEYA with an Asn results in significant reduction of catalytic activity (3.7% relative to wild type), and the reaction is insensitive to pH. As a control we used another inactivating mutation, D253N, of a putative metal-binding residue. AtEYA D253N has 4.1% activity relative to wild type, but the reaction mirrors the wild-type enzyme in terms of pH dependence. These reactions were conducted in the presence of 2 mM $MgCl_2$.

difference is that when the metal-binding residue Asp 474 in motif III of Eya3 was replaced by an Asn, it retained 30% activity, while the corresponding mutation in AtEYA resulted in almost complete inactivation.

pH Dependence. The phosphatase activity of AtEYA was assayed in the pH range 5−8.5 using both pNPP (data not shown) and pY (Figure 2) as substrates. In both cases, the pH dependence of the catalytic activity was described by a bell-shaped curve with a clear optimum at pH 5.5. These results are consistent with a general acid being involved in catalysis. The pH optimum of the animal Eyes Absent proteins is 6.0 (*4*), suggesting that the local environment at the active site is different enough to affect the pK_a of catalytic residues.

Asp 27 of AtEYA is predicted to act as a general acid in the phosphatase reaction. This assignment is supported by the observation that the pH dependence of the reaction is completely abrogated in the D27N mutant (Figure 2b). As a control, we assayed the pH dependence of catalysis for AtEYA (D253N), a mutation in motif III of a potentially metal-coordinating side chain. AtEYA (D253N) has 4.1% activity relative to wild type and retains the pH dependence seen with the wild-type enzyme.

AtEYA Acts Specifically on Phosphotyrosine and Phosphotyrosine-Containing Peptides. To establish the substrate specificity of AtEYA, we tested several phosphorylated amino acids using a commercial malachite green assay that measures released phosphate (Table 2). AtEYA showed strong activity toward phosphotyrosine and not toward phosphoserine or phosphothreonine.

Table 2: Substrate Specificity of AtEYA[a]

substrate	K_m (mM)	k_{cat} (s^{-1})
phosphotyrosine	2.0 (±0.2)	144 (±11)
phosphothreonine	no detectable activity	
phosphoserine	no detectable activity	
dNTPs	no detectable activity	
RRLIEDAEpYAARG[b]	0.27 (±0.06)[g]	259 (±21)[g]
TRDIpYETDYYRK[c]	0.15 (±0.04)	37 (±3.5)
DADEpYLIPQQG[d]	0.06 (±0.01)	31 (±0.98)
ENDpYINASL[e]	0.22 (±0.06)	33 (±3)
RRApTVA[f]	no detectable activity	
RRApSVA[f]	no detectable activity	

[a] All experiments were conducted at pH 5.5 in the presence of 2 mM $MgCl_2$. [b] Tyrosine 419 phosphorylation site in Src. [c] Phosphorylation site in the insulin receptor precursor, residues 1142−1153. [d] Autophosphorylation site in the EGF receptor precursor, residues 1014−1024. [e] Phosphorylation site in T-cell phosphatase TC-PTP, residues 65−73. [f] Standard peptides for the characterization of S/T phosphatases. [g] Reference *4*.

We further tested the ability of AtEYA to dephosphorylate pY-containing peptides (Table 2). Since we do not know

Table 3: Effect of Inhibitors on pNPP Hydrolysis by AtEYA[a]

inhibitor	concn	% activity AtEYA	% activity PTP1B
sodium orthovanadate	0.05 mM	77.3	6.4
	0.1 mM	74.1	2.1
	0.5 mM	58.5	0
	1 mM	52.6	0
	10 mM	2.3	0
H_2O_2	0.1 mM	100	0
	10 mM	79	0
sodium tungstate	0.01 mM	92.8	
	0.1 mM	78.2	
	1 mM	50.1	
	10 mM	6	
AlF_3	0.1 mM	1.5	
EDTA	20 mM	0	
NaF	50 mM	0	
okadaic acid	50 nM	96.7	
tyrosine	0.1 mM	89	
	1 mM	62	
	10 mM	32	
threonine	5 mM	98	
serine	5 mM	96	

[a] All reactions were conducted at pH 5.5 in the presence of 2 mM $MgCl_2$.

the sequence-context preferences of the Eyes Absent proteins, we chose to use peptides that have been useful in other attempts at characterizing novel phosphatases (*26*−*29*). AtEYA displayed strong affinity toward these substrates and catalyzed the reactions with kinetics comparable to those reported for other PTPs when tested with noncognate substrates (*29*). The differences in the catalytic parameters toward the various peptides tested suggest that the context of the pY residue affects AtEYA binding and catalysis. Interestingly, the relative affinities toward the peptides tested do not mirror those previously reported for Eya3(ED) (*4*), indicating that the two enzymes may have different specificities. Furthermore, of the peptides tested, AtEYA showed a clearly better catalytic rate with RRLIEDAEpYAARG (*4*), approximately 8-fold greater than the rates for the other peptides tested here. In contrast, the previously reported (*4*) rates for dephosphorylation of the same peptides by Eya3-(ED) did not display such a clear preference for a single peptide.

Inhibitors of AtEYA Phosphatase Activity Reflect Its Mechanism and Substrate Specificity. To probe the active site of AtEYA, a series of mechanism-based modulators of its catalytic activity were tested (Table 3). The phosphate mimics, tungstate and sodium orthovanadate, inhibited the enzyme in a dose-dependent fashion. Tyrosine behaved as a product inhibitor, while serine and threonine did not affect activity. The Ser/Thr phosphatase inhibitor okadaic acid did not alter AtEYA's catalytic behavior, while NaF (which inhibits magnesium-requiring enzymes) inhibited AtEYA. As might be expected, the metal chelator EDTA inhibited the enzyme. AlF_3^-, which forms transition state analogues with aspartyl enzymes, inhibited AtEYA.

Inhibition by vanadate, mediated by covalent interaction with the nucleophilic cysteine residue, is one of the defining features of the classical protein tyrosine phosphatases. Since Eyes Absent does not have a nucleophilic Cys, such a mechanism cannot explain the inhibition that we observe with vanadate. The dose dependence of vanadate-mediated inhibition of AtEYA and the fact that even 10 mM vanadate does not completely inhibit the enzyme are consistent with product inhibition. As a comparison we conducted parallel experiments with the classical tyrosine phosphatase PTP1B. It was completely inhibited with 0.5 mM vanadate.

Another Cys-specific mechanism of inhibition of the classical PTPs is mediated by peroxide. H_2O_2 oxidizes the active site cysteine side chain to a sulfenic acid or the recently described sulfenylamide (*30*). In our hands, 0.1 mM H_2O_2 was sufficient to completely inhibit PTP1B. In contrast, AtEYA, which does not have a catalytically important Cys residue, retains full activity in the presence of 0.1 mM H_2O_2. Even at peroxide concentrations as high as 10 mM, which might be expected to have structural effects by oxidation of some of the nine Cys residues in AtEYA, significant (79%) catalytic activity is retained.

The inhibitory profile derived from the studies described here is fully consistent with a metal-dependent reaction not involving a Cys residue. Furthermore, this panel of inhibitors could serve as a diagnostic for other metal-dependent tyrosine phosphatases as they are identified. This inhibitory profile is distinct from that commonly used to define the classical PTPs: inhibition by low concentrations of vanadate and peroxide and insensitivity to metal chelators and okadaic acid.

AtEYA Can Dephosphorylate Phosphotyrosine-Containing Proteins. MAPKs are the best characterized family of tyrosine-phosphorylated proteins in higher plants (*31*, *32*). To determine whether AtEYA can dephosphorylate proteins, we chose to assay its activity using AtMPK4, an *A. thaliana* MAPK, as a substrate. AtMPK4 was overexpressed in bacteria as a maltose-binding protein (MBP) fusion. Purified MBP-AtMPK4 was found to be autophosphorylated on Tyr as recognized by anti-phosphotyrosine antibody. This observation is consistent with previous reports of autophosphorylation of GST-AtMPK4 on a Tyr residue (*33*, *34*). Incubation with ATP increased the level of AtMPK4 Tyr phosphorylation. AtEYA was able to dephosphorylate this Tyr-phosphorylated AtMPK4 in a dose-dependent fashion (Figure 3a). The activity of AtEYA is comparable to that reported in a similar experiment conducted using AtDsPTP1 (*35*). The activity was metal-dependent as evidenced by the lack of dephosphorylation in the presence of EDTA. The catalytically inactive mutant D25N was unable to dephosphorylate AtMPK4. In agreement with the results reported in Table 3, vanadate at high concentrations also inhibited AtEYA activity, while H_2O_2 was unable to inhibit the dephosphorylation reaction. As a comparison we also show that the ED domain of mouse Eya3 is able to dephosphorylate AtMPK4 (Figure 3b).

As a further examination of the substrate specificity of AtEYA we monitored its effect on either Tyr-phosphorylated or Ser/Thr-phosphorylated myelin basic protein (MyBP) (Figure 3c). AtEYA was able to efficiently dephosphorylate Tyr-phosphorylated MyBP while having no visible effect on its Ser/Thr-phosphorylated forms, as visualized on autoradiograms (Figure 3c). These experiments were also conducted in solution, yielding a specific activity of 389 nM min^{-1} mg^{-1} of AtEYA with Tyr-phosphorylated myelin basic protein as a substrate, as compared to 35 nM min^{-1} mg^{-1} of AtEYA with Ser/Thr-phosphorylated MyBP substrate. Thus,

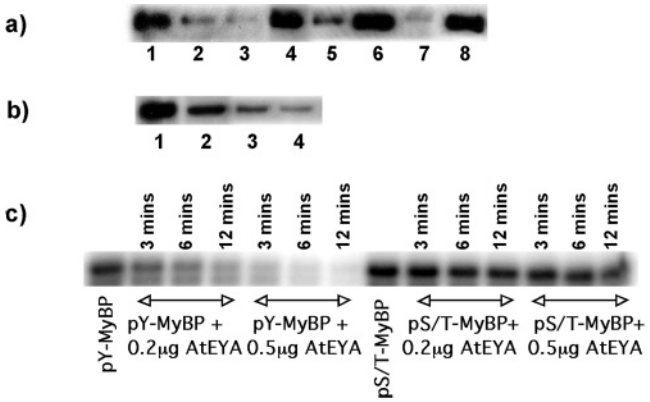

FIGURE 3: AtEYA is able to dephosphorylate AtMPK4, a tyrosine-phosphorylated *Arabidopsis* protein that participates in the MAPK pathway. The effects of treating phosphorylated AtMPK4 with Eyes Absent under different conditions are shown here. The gels were visualized by blotting with anti-pY antibody, and all reactions were conducted at pH 5.5 in the presence of 2 mM $MgCl_2$. (a) Treatment of 2 μg of phosphorylated AtMPK4 (lane 1) with 1 μg of AtEYA (lane 2), 2 μg of AtEYA (lane 3), 4 μg of AtEYA(D25N) (lane 4), 4 μg of AtEYA + 0.1 mM sodium orthovanadate (lane 5), 4 μg of AtEYA + 10 mM sodium orthovanadate (lane 6), 4 μg of AtEYA + 0.1 mM H_2O_2 (lane 7), and 4 μg of AtEYA + 10 mM EDTA (lane 8). (b) Treatment of phosphorylated AtMPK4 (lane 1) with 2 μg of mEya3(ED) (lane 2), 4 μg of Eya3(ED) (lane 3), and 8 μg of Eya3(ED) (lane 4). (c) Treatment of 15 μg of tyrosine-phosphorylated (left half of gel) or Ser/Thr-phosphorylated (right half of gel) myelin basic protein (MyBP) with varying amounts of AtEYA shows that AtEYA preferentially dephosphorylates tyrosine-phosphorylated substrate. Phosphorylation of MyBP was conducted using radiolabeled ATP, and the gel was visualized by autoradiography.

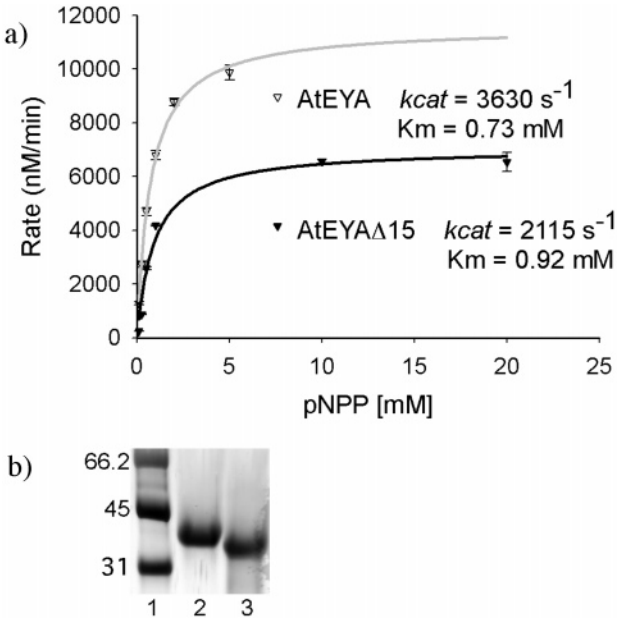

FIGURE 4: Comparison of the phosphatase activities of AtEYA and AtEYAΔ15 toward the standard substrate *p*-nitrophenyl phosphate at pH 5.5 in the presence of 2 mM $MgCl_2$. (a) The reactions follow Michaelis−Menten kinetics. Full-length AtEYA yields $k_{cat} = 3630$ s^{-1} and $K_m = 0.73$ mM (similar to that previously reported in ref *4*). AtEYAΔ15, which corresponds to the conserved ED domain of the animal Eyes Absents, is also able to dephosphorylate pNPP with $k_{cat} = 2115$ s^{-1} and $K_m = 0.92$ mM. (b) Silver-stained SDS−PAGE gel of recombinant AtEYA and AtEYAΔ15 used in these experiments. Lanes: 1, molecular mass markers [sizes of markers (in kDa) are shown to the left]; 2, AtEYA; 3, AtEYAΔ15.

all of our evidence suggests that AtEYA has a clear preference for phosphotyrosine-containing substrates.

The N-Terminal Peptide in AtEYA Is Necessary for Optimum Catalytic Activity. Most cytoplasmic PTPases have, in addition to the catalytic domain, various N- and C-terminal extensions that are believed to have targeting or regulatory functions (*36*). The animal Eyes Absents have a large N-terminal domain that has been shown to have transactivation potential (*18*). The plant Eyes Absent proteins have a short N-terminal region of 15−23 residues upstream of the conserved ED domain. The sequence in this region is not conserved and shows no obvious sequence trends. To determine whether the N-terminal peptide of AtEYA had any effect on either the activity or specificity of AtEYA, we compared the catalytic behavior of full-length AtEYA with AtEYAΔ15 (residues 16−307), a construct designed to correspond to the minimal ED domain conserved between the animal and plant Eyes Absent proteins. AtEYAΔ15 retained catalytic activity, but to a lesser degree (58% activity using pNPP as a substrate), when compared to the full-length enzyme (Figure 4). AtEYAΔ15 had similar affinity for substrates (pNPP, phosphotyrosine, and several phosphotyrosine-containing peptides) and displayed essentially similar divalent metal preferences as the full-length protein (Table 1). The only notable difference was that AtEYAΔ15 displayed an over 8-fold increase in affinity for Co^{2+} ($K_m =$ 0.014 mM) compared to the wild-type enzyme ($K_m = 0.123$ mM). We also examined whether the N-terminal 15 amino acids contributed in any way to substrate specificity by comparing the catalytic parameters for AtEYAΔ15 and AtEYA using several tyrosine-phosphorylated peptides. While AtEYAΔ15 consistently exhibited lower k_{cat} values, both full-length AtEYA and AtEYAΔ15 displayed similar sequence-context preferences. These results suggest that while the N-terminal 15 amino acids are necessary for optimum catalytic activity, they do not appear to directly participate in the reaction.

DISCUSSION

Here we have demonstrated that the *Arabidopsis* homologue of the animal Eyes Absent proteins is a metal-dependent, tyrosine-specific phosphatase. It is distinct from the animal Eyes Absent proteins in both its biological context of action and in the details of its biochemistry. It is also distinct from the classical PTPs in its mechanism of action and its sequence signatures. Furthermore, AtEYA becomes only the second protein tyrosine-specific phosphatase to be described in plants, adding a new dimension to the relatively scant understanding of the regulation of tyrosine phosphorylation in plants.

The animal Eyes Absent proteins have a transactivation function encoded by their poorly conserved N-terminal domain. Hence they are classified as transcription factors, possibly targeted to DNA by interaction with the Six/So family of DNA-binding proteins. The animal Eyes Absents thus represent, to our knowledge, the first example of transcription factors also having tyrosine phosphatase activity. The plant homologues of Eyes Absent differ in their biochemical function in that they lack the N-terminal transactivation domain, suggesting that the biological role(s) of this protein in the plant and animal kingdoms may be different. Another notable difference is that while the animal

Eyes Absents are known to be part of a genetic cascade (the retinal determination pathway) that includes the genes dachshund and Six/so, we could not find homologues for these other retinal determination genes in the *Arabidopsis* genome sequence. Thus the evidence suggests that plant Eyes Absent, unlike its animal homologues, is a simple protein tyrosine phosphatase that participates in regulating cellular tyrosine phosphorylation levels.

On the basis of our biochemical characterization it is apparent that the active sites of AtEYA and mouse Eya3 differ in subtle but noteworthy respects. The difference in the optimum pH for the catalytic reaction suggests that ionizable groups in the active site are in slightly different environments in the two enzymes. The fact that, unlike AtEYA, Eya3 has no activity in the presence of Zn^{2+} also suggests that their active site geometries could be distinctive. Furthermore, of the peptide substrates tested AtEYA shows a clear preference for RRLIEDAEpYAARG. This is in contrast to similar experiments previously conducted on the animal Eyes Absent proteins (*4*) where the k_{cat} values for various phosphotyrosine-containing peptide substrates varied over a more limited range (4-fold). Thus it appears as though AtEYA is more discriminating with regard to the sequence context of the phosphotyrosine residue that it dephosphorylates.

Li et al. have reported comparable phosphatase activity toward pY-, pT-, and pS-containing peptides for mouse Eya3 (*6*). However, we (*4*) and Tootle et al. (*5*) have previously reported that the conserved ED domain of the animal Eyes Absent proteins was unable to dephosphorylate pS/T-containing substrates. It is possible that the N-terminal domain of the animal Eyes Absent proteins alters the substrate specificity of the catalytic ED domain, accounting for these differences. The plant Eyes Absent proteins lack the additional N-terminal domain seen in the animal Eyes Absents, and thus the results reported here reflect the intrinsic specificity of full-length AtEYA. It remains possible that specificity will be modulated by interactions with other as yet unidentified protein partners, as in the case of PP1 (reviewed in ref *37*).

Accumulating data now suggest that tyrosine phosphorylation and dephosphorylation serve as important a function in plants as they do in animals (*10*, *38*, *39*). The task of tyrosine dephosphorylation has been assigned to the approximately 20 genes in the *Arabidopsis* genome that include dual-specificity phosphatases and the one tyrosine-specific phosphatase AtPTP1. AtEYA adds to this repertoire, although the role of AtEYA-mediated dephosphorylation in plant biology remains to be determined. A survey of the *Arabidopsis* genome, using tools available on the Arabidopsis Information Resource (TAIR) website, reveals that there are only 68 HAD motif containing proteins. Most of these (*39*) are ATPases. In addition, there are enzymes involved in metabolism (3-amino-5-hydroxybenoic acid synthesis, FAD synthesis), several phosphatases (histidinol phosphatase, phosphoglycolate phosphatase, phosphoserine phosphatase), an aldolase, and an alcohol phosphatidyl transferase. There are also six functionally unclassified genes, including AtEYA, which may well code for proteins with tyrosine phosphatase activity as seen with AtEYA. At this time we do not have a sufficient understanding of the sequence features (beyond the three motifs that describe the large HAD family of enzymes) to catalog other non-Eyes Absent proteins as Asp-based, metal-dependent protein tyrosine phosphatases. However, the identification of AtEYA as a tyrosine-specific phosphatase makes us aware of the possibility that plants (and animals) may encode more PTPs than might be apparent by scanning the genome for the Cys-containing signature motifs of the classical PTPs.

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