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The Plant S1-Like Nuclease Family Has Evolved a Highly Diverse Range of Catalytic Capabilities

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Plant S1-like nucleases, often referred to as nuclease I enzymes, are the main class of enzymes involved in nucleic acid degradation during plant programmed cell death. The catalytically active site of these enzymes shows a significant similarity to the well-described P1 nuclease from *Penicillium citrinum*. Previously published studies reported that plant S1-like nucleases possess catalytic activities similar to their fungal orthologs, i.e. they hydrolyze single-stranded DNA and RNA, and less efficiently double-stranded DNA, in the presence of zinc ions. Here we describe a comprehensive study of the nucleolytic activities of all Arabidopsis S1-like paralogs. Our results revealed that different members of this family are characterized by a surprisingly large variety of catalytic properties. We found that, in addition to Zn^{2+}-dependent enzymes, this family also comprises nucleases activated by Ca^{2+} and Mn^{2+}, which implies that the apparently well-known S1 nuclease active site in plant nucleases is able to cooperate with different activatory ions. Moreover, particular members of this class differ in their optimum pH value and substrate specificity. These results shed new light on the widely accepted classification of plant nucleases which is based on the assumption that the catalytic requirements of plant nucleases reflect their phylogenetic origin. Our results imply the need to redefine the understanding of the term 'nuclease I'. Analysis of the phylogenetic relationships between S1-like enzymes shows that plant representatives of this family evolve toward an increase in catalytic diversity. The importance of this process for the biological functions of plant S1-type enzymes is discussed.

Keywords: *Arabidopsis thaliana* • Gene family evolution • Metallonucleases • Nuclease I • Programmed cell death • S1-like nucleases.

Abbreviations: CAN, calcium-dependent nuclease; dsDNA, double-stranded DNA; ECFP, enhanced cyan fluorescent protein; GFP, green fluorescent protein; NBS, mononucleotide-binding site; PCD, programmed cell death; PEG, polyethylene glycol; ssDNA, single-stranded DNA.

Introduction

Many plant orthologs of bacterial and fungal genes have evolved as a multigene family whose members have been adapted to different biological processes that are strictly specific for the development of multicellular species (Copley et al. 2002). One such example concerns fungal genes encoding extracellular S1-type nucleases and their plant homologs that utilize nuclease activity during different types of programmed cell death (PCD).

Researchers have long observed the complex patterns of nucleolytic activities specific for individual plant tissues and developmental stages. These activities are also characterized by a variety of catalytic properties, regarding mainly their divalent metal ion requirements and pH optima. One of the primary research studies resulted in the identification of the enzymes commonly named nuclease I enzymes (Johnson and Laskowski 1968). Several biochemical characteristics defined these proteins as Zn^{2+}-dependent enzymes, having acidic pH optima, and molecular mass ranges between 30 and 45 kDa. These nucleases efficiently degrade RNA and denatured DNA, but are much less active toward native, double-stranded DNA (dsDNA). One of the first known members of this family was the mung bean nuclease which is now widely used in modern biotechnology as a commercially available enzyme (Johnson and Laskowski 1970).

Further studies resulted in the identification of a number of genes encoding plant nucleases. The determination of their primary amino acid sequences revealed that the vast majority of these genes are the orthologs of well-defined fungal S1

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and P1 nucleases from *Aspergillus oryzae* and *Penicillium citrinum*, respectively (Desai and Shankar 2003). The fungal P1 nuclease is an extracellular glycoprotein that at acidic pH exhibits high endonuclease cleavage specificity for single-stranded DNA (ssDNA), RNA and dsDNA with mismatches. X-ray crystallography revealed that this nuclease contains three zinc ions bound in the pocket forming the trinuclear active site. These ions are coordinated by five histidines, three aspartates and one tryptophan. Two zinc ligands, Zn1 and Zn3, activate the water molecule that acts as a nucleophile making an in-line attack on the phosphate group center, resulting in the cleavage of the P–O3′ bond. The third zinc ligand (Zn2) activates the phosphate and stabilizes the leaving O3′-oxyanion. Beside the residues that coordinate the Zn^{2+} ions, the reaction mechanism of P1 nuclease also involves one arginine (Arg48) that stabilizes the pentacovalent transition state (Romier et al. 1998, Liao et al. 2010).

In every plant S1-like nuclease studied so far, despite the varied levels of sequence similarity to their fungal orthologs, all nine amino acid residues responsible for interaction with metal ions are conserved in the same position as in P1 nuclease. This observation prompted researchers to examine plant S1-type nuclease activity in conditions characteristic for fungal P1 and S1 nucleases. Indeed, Zn^{2+}-dependent activation has been well documented in a number of these enzymes. In addition to the mung bean nuclease, zinc ions at low pH induce the catalytic activity of another well-defined plant S1-like nuclease, ZEN1 from *Zinnia elegans*, that is responsible for nuclear degradation during tracheary element differentiation (Ito and Fukuda 2002). The activity of another well characterized member of the S1-type class, Arabidopsis bifunctional nuclease BFN1 (Perez-Amador et al. 2000), is also stimulated by Zn^{2+}, but was reported at neutral pH. Similar catalytic properties have also been demonstrated for another Arabidopsis member of this class, the ENDO2 (Ko et al. 2012). The Zn^{2+}-dependent activity of three members of S1-type nucleases from tomato, hop and *Arabis brassica* at pH 6.2 has also been reported (Podzimek et al. 2011).

However, studies of endogenous nucleolytic enzymes have shown that, in addition to Zn^{2+}-dependent nucleases, strong and complex activities of Ca^{2+}-dependent nucleases are observed in plant tissues (Ito and Fukuda 2002, Lesniewicz et al. 2010). So far, only one calcium-dependent nuclease family, named CAN, possessing an active site similar to staphylococcal nucleases, has been relatively well documented in plants. In contrast to S1-type enzymes, the CAN nucleases are active only at neutral pH and show high sensitivity to zinc inhibition (Lesniewicz et al. 2012).

The study of endogenous and recombinant nuclease activities presented above consolidated the widespread belief that all plant nucleases can be classified into two main groups. The first of them, also called nuclease-I-type, includes all Zn^{2+}-dependent acidic and neutral enzymes, while the second comprises Ca^{2+}-dependent neutral nucleases. Since some authors have occasionally reported the identification of nucleolytic activity stimulated by ions other than Zn^{2+} and Ca^{2+}, they have also distinguished other subgroups, such as Mg^{2+}-dependent or Mn^{2+}-dependent nucleases (Sugiyama et al. 2000).

The classification of plant nucleases based on their requirements is important for reasons that go beyond the study of details of their catalytic mechanism. Since the physiological state of plant cells is highly variable depending on tissue specificity, developmental stage or cell compartments, determination of particular nuclease catalytic requirements is essential for an understanding of its biological function. The pH of the nucleus and cytoplasm of young and mature plant cells is characterized by neutral pH (around 7.5) and very low concentrations of Ca^{2+} and Zn^{2+} ions, while the apoplast and vacuolar lumen have a pH of around 5.5 (Martin et al. 1982). Moreover, since the vacuole is implicated in the storage of heavy metals ions, the concentration of vacuolar Zn^{2+} in mature cells exceeds its cytosolic content. Similarly, the concentration of Ca^{2+} in apoplasts is considerably higher than in the cytosol of normal cells. However, the cellular environment undergoes dramatic changes in response to developmental events or external stresses. During the final stage of different types of PCD, tonoplast rupture releases the vacuolar content, resulting in cytosolic acidification and an increase in metal ion concentration (Hara-Nishimura and Hatsugai 2011). Likewise, some biotic and abiotic stresses trigger a Ca^{2+} influx from apoplasts, temporarily increasing the cytosolic concentration of calcium (Tena et al. 2011). According to the common belief, the activation of particular nucleases is strictly connected with such events. Direct evidence showing relationships between the best characterized plant S1-like nucleases, ZEN1 (Ito and Fukuda 2002) and BFN1 (Farage-Barhom et al. 2008, Farage-Barhom et al. 2011), with two types of PCD, xylogenesis and senescence, respectively, apparently confirms this hypothesis. Based on this principle, it is often assumed that Ca^{2+}/Mg^{2+}-dependent neutral nucleases are targeted to the nucleus before the Zn^{2+}-dependent acidic nucleases, that are activated by vacuolar membrane disruption (Sugiyama et al. 2000).

Although the general requirements of plant S1-like nucleases seem to be well characterized, the following data have prompted us to examine this problem more closely. Our previous research on endogenous nuclease activities has shown the high diversity of detected Ca^{2+}-dependent nucleases in plant tissues (Lesniewicz et al. 2010), although only two Ca^{2+}-dependent CAN nucleases are encoded by the Arabidopsis genome (Lesniewicz et al. 2012). This suggests that plant genomes probably encode additional Ca^{2+}-activated nucleases belonging to other nuclease families. Moreover, we noticed that Triques and her colleagues (2007), who considered the application of plant S1-like nucleases for the TILLING method, were able to detect zinc-dependent DNase activity only for two of the five members of the S1 family, encoded by the Arabidopsis genome. These results suggest that the catalytic requirements of the individual members of this family may differ from one another. This assumption seems to be strengthened by the

finding that one of the plant S1-like nucleases, the CEL1 endonuclease isolated from celery, was reported as a Zn^{2+}- and Mg^{2+}-dependent enzyme (Yang et al. 2000).

On the basis of the available data, it is rather difficult to outline the general conclusions concerning the catalytic requirements of plant S1-like nucleases. The results reported in the literature usually refer to single members of this family isolated from different species. However, it should be noted that in known plant genomes the S1-like nuclease genes are present in multigene families, encoding proteins differing to varying degrees. Moreover, DNase detection assays performed in different laboratories may vary with the parameters, affecting the results obtained. Therefore, to define the catalytic requirements of plant S1-like nucleases, we studied the activity of five Arabidopsis members of this family. We decided that a comprehensive study of all Arabidopsis S1-like paralogs would provide more information than a comparison of orthologs derived from different species. The two different in-gel nuclease activity assays performed using denaturing and native gel electrophoresis enabled us to determine the pH and ion requirements of each nuclease as well as their preferences toward ss/dsDNA and RNA substrates. Surprisingly, these studies revealed an amazing diversity in the activity of the members of this family. In fact, only two of the five Arabidopsis S1-like nucleases revealed canonical properties of the nuclease I family. We have found that in addition to Zn^{2+}-dependent nucleases, the activity of other members of this family is determined by Ca^{2+} and Mn^{2+} ions or by simultaneous use of Ca^{2+} and Zn^{2+} ions. In our opinion, these results put the widely accepted classification principles of plant nucleases in a new light. Moreover, our findings open up interesting perspectives for further research into the reaction mechanism catalyzed by the active site of plant S1-type nucleases.

Results

The five Arabidopsis orthologs of fungal P1 nuclease possess conserved amino acid residues involved in divalent metal ion binding

The *Arabidopsis thaliana* genome contains five predicted genes encoding proteins similar to the well-defined fungal S1 and P1 nucleases from *A. oryzae* and *P. citrinum*, respectively. For four of these genes the mRNA sequences are available in public databases. As for the fifth gene, AT4G21585, mRNA transcripts are not annotated in databases, its encoded amino acid sequence was predicted from the gene sequence. In this paper, to describe our results we adopt the nomenclature proposed by Triques et al. (2007) according to which the Arabidopsis S1-like nucleases have been named as follows: ENDO1 (also known as BFN1, AT1G11190), ENDO2 (AT1G68290), ENDO3 (AT4G21590), ENDO4 (AT4G21585) and ENDO5 (AT4G21600).

Alignment of the P1 protein with its plant orthologs revealed a varying level of similarity within amino acid sequences of different functional domains (**Fig. 1**). All nine amino acid residues that are involved in P1 nucleases in the binding of three zinc ions are fully conserved among Arabidopsis S1-like nucleases. The most significant difference in the active site seems to be an arginine/lysine substitution at position 48 of ENDO2, ENDO3 and ENDO4, and at position 49 of ENDO5. However, it should be noted that S1 nuclease from *A. oryzae* that is highly homologous to P1 also possesses lysine at this position. A much lower degree of similarity is found in the regions responsible for nucleic acid recognition. Fungal P1 nuclease possesses two mononucleotide-binding sites, NBS1 and NBS2. The first site is located close to the active site and consists of two hydrophobic residues, Phe61 and Val132, forming a nucleotide base-binding pocket, and asparagines (Asp63) located at the pocket bottom. In the second binding site, the next nucleotide base is sandwiched between two tyrosines (Tyr144/Tyr155) and interacts with Asp146 at the bottom of the pocket (Romier et al. 1998). As shown in **Fig. 1**, the first nucleotide-binding site is partially conserved only in ENDO3, while the other plant nucleases share very limited sequence similarity to NBS1 in the corresponding region. The sequence corresponding to the second mononucleotide-binding sites does not exhibit any similarity to fungal NBS2.

To analyze how the above-described differences in the primary sequence influence the protein structure, we constructed three-dimensional models of all Arabidopsis S1-like nucleases based on the well-defined structure of P1 nuclease. All Arabidopsis S1-like nucleases, despite their overall low sequence similarity to the P1 enzyme, appear to have essentially the same folding (**Supplementary Fig. S1**) as the P1 protein. Moreover, as shown in **Fig. 2**, the orientations of all predicted zinc-binding residues at the active sites of all Arabidopsis S1-like nucleases are basically similar to those observed in P1. The only significant difference concerns the ENDO2 protein, whose Asp45 and Lys48 side chains display different orientations compared with the corresponding residues of other Arabidopsis and fungal S1-type nucleases. Since the Asp45 residue in P1 nuclease plays a crucial role in coordination of the Zn2 ligand and the hydroxide ion acting as the attacking nucleophile, and as Lys48 is important for stabilization of the reaction transition state, the differences in their orientation in ENDO2 nuclease suggest that the catalytic mechanism of this enzyme may differ from those of other S1-type nucleases.

The members of the Arabidopsis S1-like family are characterized by different pH optima and activating divalent ion requirement

The main aim of our study was to determine the catalytic requirements of Arabidopsis S1-like nucleases. For this purpose, the cDNA sequences of all members of this family were amplified from Arabidopsis seedlings and cloned into the plant expression vectors. The transient expression of recombinant proteins was next performed in Arabidopsis leaf protoplasts. We first determined the catalytic activities of overexpressed proteins using an in-gel nuclease assay conducted under

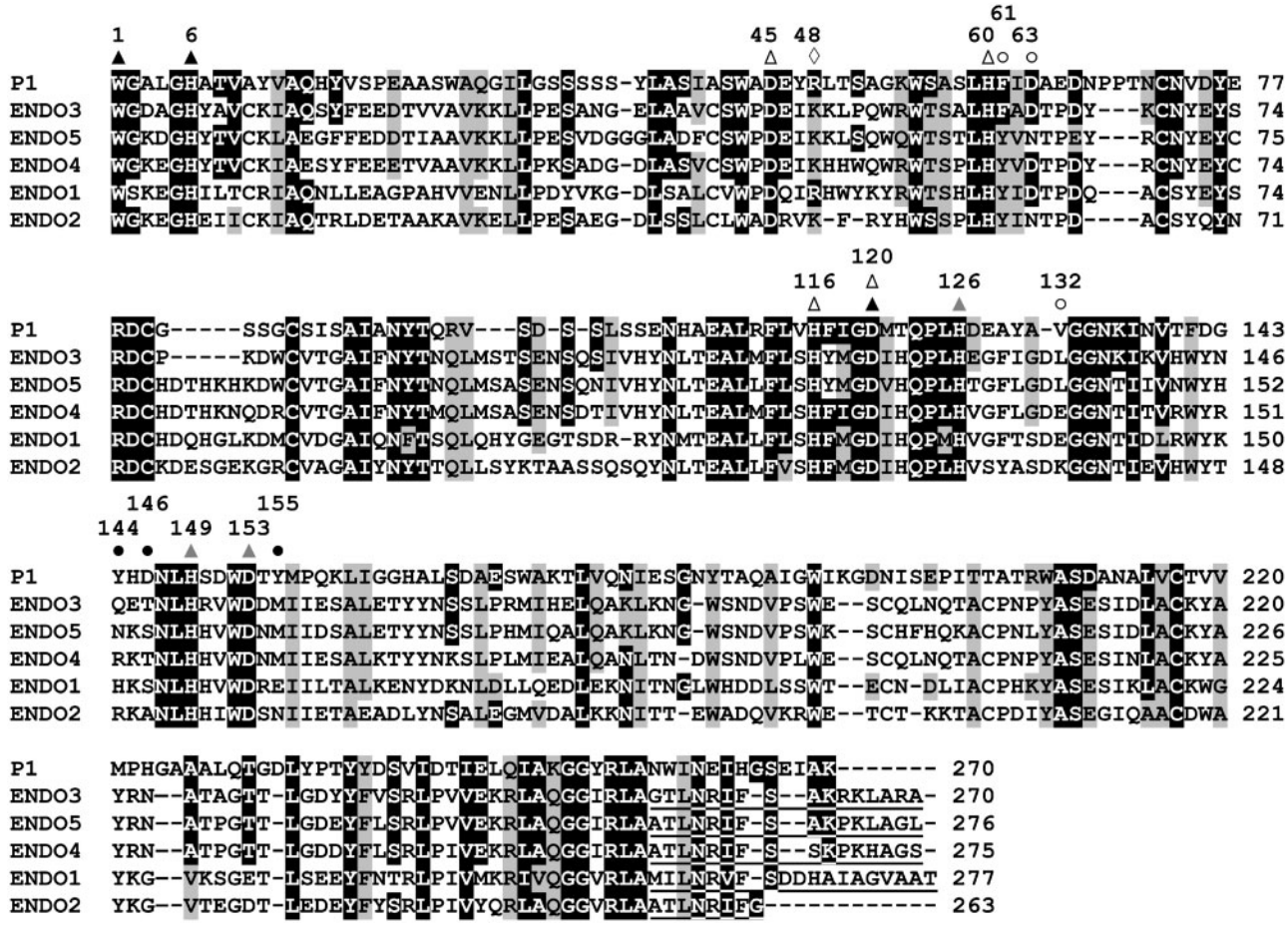

Fig. 1 Alignment of amino acid sequences of Arabidopsis S1-like nucleases with P1 nuclease from *P. citrinum*. Black and gray boxes indicate the amino acid positions of Arabidopsis enzymes that are identical and similar, respectively, to the corresponding residues of P1 nuclease. The active site residues that coordinate Zn1, Zn2 and Zn3 ligands in P1 nuclease are indicated by white, gray and black triangles, respectively. The active site arginine and corresponding lysine residue are marked by a white diamond. The white and black dots indicate P1 nuclease residues forming the first and second mononucleotide-binding sites, respectively. Numbers above the depicted residues refer to the P1 amino acid sequence. The N-terminal signal sequences that are removed from the mature proteins are not shown. The C-terminal regions removed in the deletion constructs are indicated by underlining.

denaturing conditions. In our study, we analyzed the influence on nuclease activity of three divalent metal ions, i.e. Zn^{2+}, Ca^{2+} and Mn^{2+}. According to published research, Zn^{2+} and Ca^{2+} are the most common metal ion cofactors of plant nucleases. Much less is known about the role of Mn^{2+} on nuclease activity, but our preliminary results suggested the great importance of this ion for some members of the S1-like family. Moreover, in our initial study we tested the potential role of Mg^{2+} and Fe^{2+}, but these ions seem not to affect Arabidopsis S1-like nuclease activity. In addition to the ions, pH is the second important factor determining nuclease activity. Since according to published data, as well as our findings, the majority of plant nucleases achieve the highest activity at acidic or neutral pH (Lesniewicz et al. 2010), we analyzed S1-like nuclease activity at two distinct pH values, pH 5.5 and pH 8.0. Moreover, since the available studies reported divergent opinions regarding S1-like nuclease specificity towards their substrates, we analyzed their nucleolytic activities using ssDNA and dsDNA, as well as RNA.

Identification of the metal cofactor responsible for enzyme activation is possible only under conditions that allow the removal of residual ions bound by a protein when it is overexpressed in a cell. Therefore, the protein extracts obtained from transformed protoplasts were heat treated in the presence of 5 mM EDTA before loading into the gel. As shown in **Fig. 3A–D**, the samples treated under such conditions did not reveal any nuclease activities after incubation in reaction buffer lacking any divalent ions. In turn, application of metal ions revealed an unexpectedly diverse picture of strong activities for four of the five members of the Arabidopsis S1-like family. As shown in **Fig. 3M**, ENDO3 is the only nuclease, of all members of this family, that demonstrates activity characteristic of fungal S1-type nucleases and mung bean plant nuclease, i.e. it digests ssDNA under acidic pH and in the presence of Zn^{2+}. The same

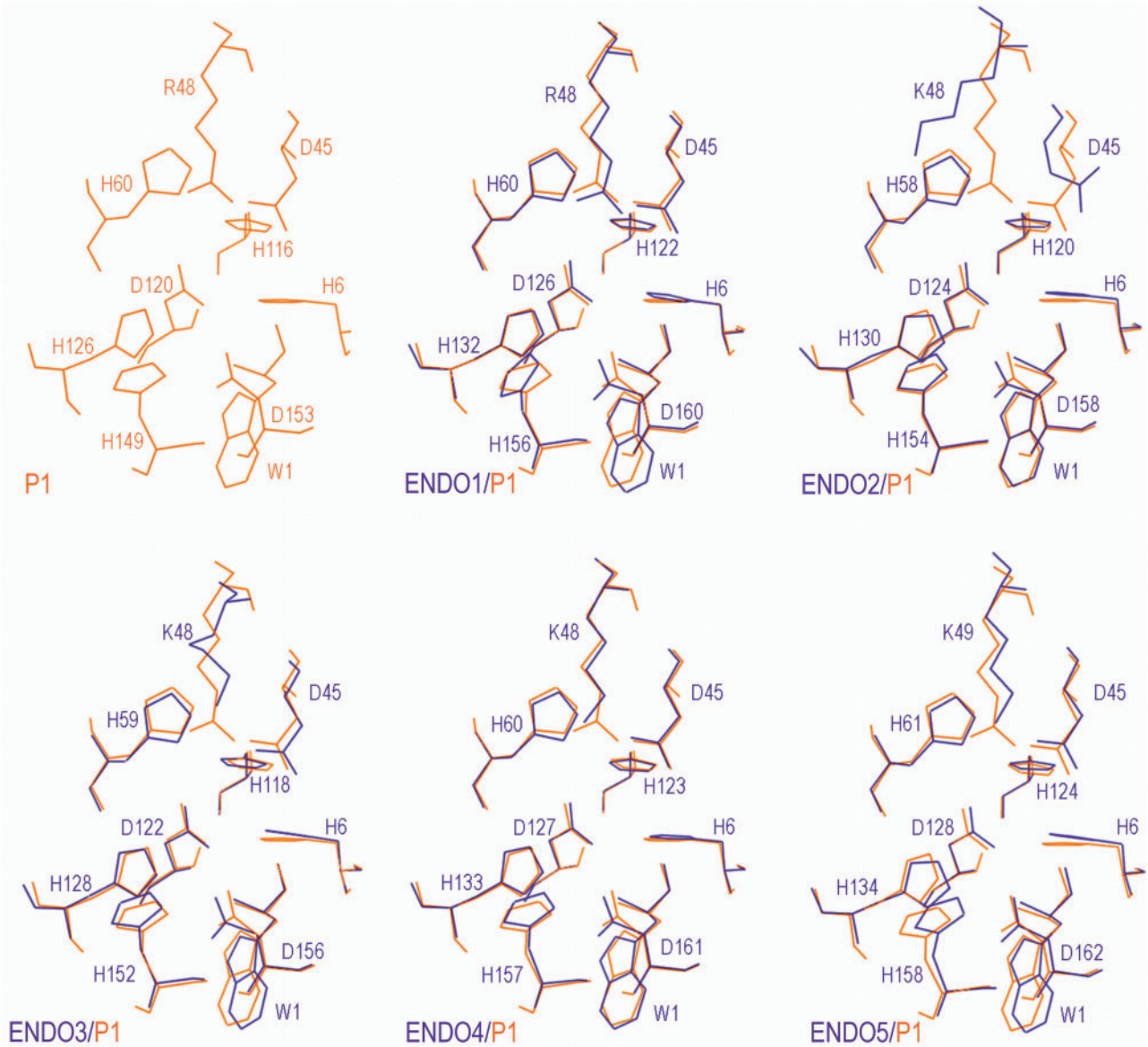

Fig. 2 The orientation of residues located in S1 nucleases active sites. The 3D models of Arabidopsis members of the S1 family were built on the basis of the P1 nuclease crystal structure. Nine Zn^{2+}-binding residues and the arginine residue involved in stabilization of the reaction transition state of P1 nuclease active sites are in orange. The corresponding residues in Arabidopsis nucleases are shown in blue. The residues of Arabidopsis enzymes are aligned with the corresponding amino acids of P1 nuclease for comparison.

nuclease achieved a much lower activity in the gel washed in buffer containing both Zn^{2+} and Ca^{2+} ions (**Fig. 3R**), which is evidence for the inhibition of ENDO3 by calcium ions.

In contrast to ENDO3, two other nucleases, ENDO1 and ENDO4, revealed optimal activity at neutral pH. Both of these enzymes are activated by Mn^{2+} and Ca^{2+} ions, although ENDO1 exhibits a stronger preference for calcium ions (**Fig. 3F, J**). Both of these enzymes are completely inactive in calcium buffer supplemented with Zn^{2+} (**Fig. 3S**). This finding confirms the common observation that plant Ca^{2+}-dependent nuclease activity can be inhibited by zinc ions (Sugiyama et al. 2000). Together with the observations that Zn^{2+}-dependent ENDO3 nuclease undergoes inhibition in the presence of Ca^{2+}, these results show that calcium and zinc ions exert the opposite effect on the activities of some plant S1-like nucleases.

Taking into account the above observations, ENDO2 nuclease revealed particularly interesting features. In contrast to the nucleases presented above, ENDO2 achieved strong DNase activity under two completely different conditions. As shown in **Fig. 3F** and **J**, in neutral pH this nuclease is strongly activated by Mn^{2+} and less intensively by Ca^{2+}. However, in low pH, the same protein can be activated by simultaneously applied Zn^{2+} and Ca^{2+} (**Fig. 3R**). The Zn^{2+}-dependent activity of ENDO2 in acidic pH has been reported recently (Ko et al. 2012), but results

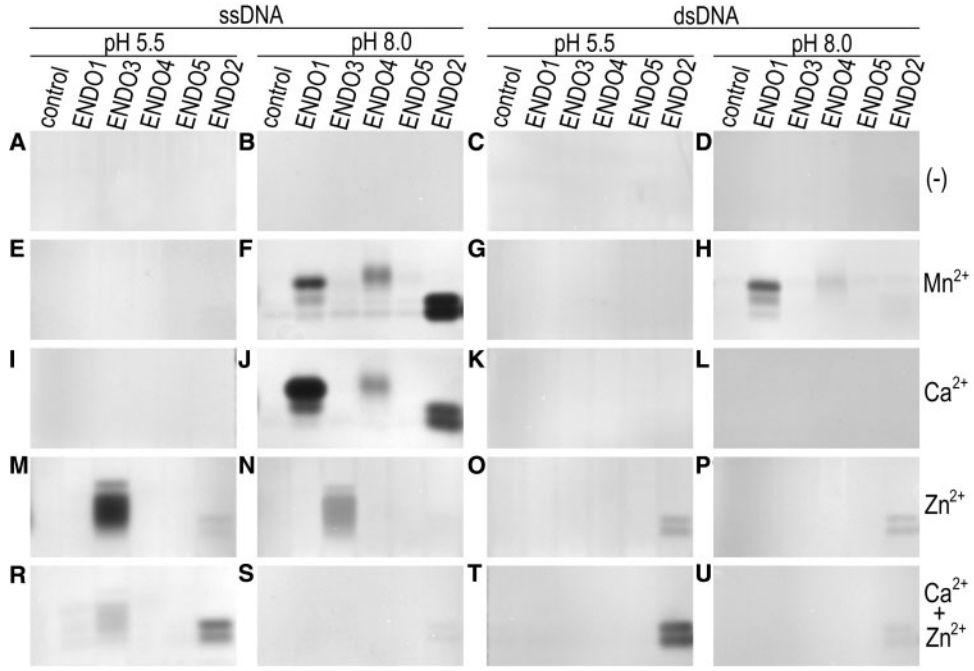

Fig. 3 DNase activities of Arabidopsis members of the S1-like family identified by in-gel nuclease assay performed under denaturing conditions. Different gels have been arranged according to the applied ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Ca^{2+} + Zn^{2+}), pH value (pH 5.5 and pH 8.0) and DNA substrate (ssDNA or dsDNA) as described on the right side and above the gels. All gels contained the same set of overexpressed nucleases, as depicted above the upper gels. The protein extract from protoplasts transformed with empty vectors (pSAT6A) was used as a negative control (contr.). The individual gels show the following substrate/ion/pH requirements of Arabidopsis S1-like nucleases: (A) ssDNA/−/pH 5.5, (B) ssDNA/−/pH 8.0, (C) dsDNA/−/pH 5.5, (D) dsDNA/−/pH 8.0, (E) ssDNA/Mn^{2+}/pH 5.5, (F) ssDNA/Mn^{2+}/pH 8.0, (G) dsDNA/Mn^{2+}/pH 5.5, (H) dsDNA/Mn^{2+}/pH 8.0, (I) ssDNA/Ca^{2+}/pH 5.5, (J) ssDNA/Ca^{2+}/pH 8.0, (K) dsDNA/Ca^{2+}/pH 5.5, (L) dsDNA/Ca^{2+}/pH 8.0, (M) ssDNA/Zn^{2+}/pH 5.5, (N) ssDNA/Zn^{2+}/pH 8.0, (O) dsDNA/Zn^{2+}/pH 5.5, (P) dsDNA/Zn^{2+}/pH 8.0, (R) ssDNA/Ca^{2+}+Zn^{2+}/pH 5.5, (S) ssDNA/Ca^{2+}+Zn^{2+}/pH 8.0, (T) dsDNA/Ca^{2+}+Zn^{2+}/pH 5.5, (U) dsDNA/ Ca^{2+} + Zn^{2+}/pH 8.0.

presented here show that Zn^{2+}-dependent activity of this protein can be strongly enhanced by the Ca^{2+}, despite the fact that both of these ions exert an antagonistic effect on other plant S1-like nucleases.

Most of the reports concerning plant S1-like nucleases express the opinion that members of this family preferentially digest ssDNA over dsDNA. As presented in **Fig. 3H** and **T**, under the employed conditions only two nucleases, ENDO1 and ENDO2, revealed the capacity to digest dsDNA. It is worth noting that, although ENDO1 nuclease digests ssDNA in the presence of both Ca^{2+} and Mn^{2+} ions, its ability to digest dsDNA can be stimulated only by Mn^{2+} ions. Similarly, ENDO2 nuclease that can degrade ssDNA in two different conditions exerts its nuclease activity towards dsDNA only in acidic buffer as a Ca^{2+}/Zn^{2+}-dependent enzyme (**Fig. 3T**).

In-gel nuclease assay performed in native conditions confirms the catalytic requirements of Arabidopsis S1-like enzymes

In-gel nuclease assay conducted under denaturing conditions is one of the main techniques used by researchers to analyze nuclease activities. The main advantages of this assay derive from its ability to analyze protein activity after its resolution in an SDS–polyacrylamide gel. As a result, an active enzyme is well separated from its cofactors as well as from other cellular proteins, and is located at the position corresponding to its molecular weight. Analysis of the molecular mass of the enzymes resolved by this technique may lead to the identification of potential post-translational modifications affecting the protein's electrophoretic mobility. Authors studying ENDO1 and ENDO2 nucleases have reported that the electrophoretic mobility of these proteins is decreased due to their glycosylation (Ko et al. 2012). The nuclease assay demonstrated here also confirmed the observation that the apparent molecular weights of Arabidopsis S1-like nucleases (range 30–40 kDa) are higher than those estimated from amino acid sequence analysis of matured proteins (range 29–32 kDa). In addition, the denaturing in-gel assay allowed us to determine that the activity of some S1-like nucleases, such as ENDO1 and ENDO2 (**Fig. 3J**) appears in more then one band, suggesting that these proteins can be active in a few post-translationally modified forms.

However, an in-gel nuclease assay conducted under denaturing conditions also has some important disadvantages. First of all, the denaturation treatment can permanently inhibit the activity of a protein that fails to renature after SDS–gel

electrophoresis. Also, any enzymes that are active only upon binding of the unknown cofactor, absent in the reaction buffer, or are active in a complex with other protein molecules will be undetectable by this assay. We assumed that some of those reasons could be responsible for our failure to identify the activity of ENDO5 nuclease. Moreover, one must reckon with the possibility that the denaturation–renaturation process may cause incorrect protein folding, resulting in its non-specific enzyme activity. For these reasons, we decided that our results obtained using denaturing in-gel assay should be supplemented by an additional in-gel nuclease assay, conducted under native conditions. For this purpose, we applied the following modifications to a conventional in-gel nuclease assay. First, we excluded all denaturing agents, such as SDS detergent and high temperature treatment of protein samples. Since the proteins that were not treated with SDS can migrate through the native gel only by their own intrinsic charges, we ran the electrophoresis in a slightly alkaline medium (pH 8.8), in which the protein acquires a net negative charge. Moreover, the polyacrylamide gel was supplemented with glycerol acting as a protein stabilizer and glycine facilitating native electrophoretic separation.

Adjusting the native electrophoresis procedure to the requirements of the in-gel nuclease assay, we tested various approaches to provide the ions for the examined nucleases. Since proteins migrating into native gel can maintain bonded ion(s), we assumed that different ions may be supplied to the protein sample prior to electrophoresis. These attempts have resulted in the observation that migration of native nucleases is strongly affected by the presence of Mn^{2+} ions in the sample buffer. As shown in **Supplementary Fig. S2**, addition of Mn^{2+} to the sample buffer resulted in much sharper and stronger bands representing the nuclease activity of four of the five S1 enzymes. It should be added that a similar application of other ions, such as Zn^{2+}, Ca^{2+} and Mg^{2+}, did not exert a similar influence on nuclease migration (data not shown).

Application of a native in-gel nuclease assay allowed us to identify the catalytic activities of all Arabidopsis S1-like nucleases. To screen the overall activities of these nucleases, we applied the same combination of divalent ions and pH values as in denaturing in-gel assays. As presented in **Fig. 4**, the electrophoretic mobility of native nucleases varies greaty, showing that they differ considerably in their net charge and/or structural conformation, despite the similarity of their primary structure.

Ions incorporated by particular nucleases during their overexpression were removed before electrophoresis using EDTA, as described in the Materials and Methods. However, since the protein designed for native electrophoresis could not be denatured, the limited effectiveness of this process should be considered. As shown in **Fig. 4A–D**, gels containing samples treated with EDTA prior to electrophoresis and washed in the absence of any divalent ions revealed the residual activity of ENDO2, ENDO3, ENDO4 and ENDO5 nucleases. We assume, however, that pronounced enhancement of nuclease activity in the presence of ions added to the reaction buffer demonstrates their activating role.

In principle, the native gels presented in **Fig. 4** confirmed the catalytic requirements of particular plant S1-like nucleases as identified using denaturing in-gel nuclease assay, namely the Ca^{2+}-dependent activity of ENDO1 and ENDO4 in neutral buffer (**Fig. 4J**) and the Zn^{2+}-dependent activity of ENDO3 under acidic conditions (**Fig. 4M**). Native in-gel assay also confirmed the ability of ENDO2 to digest ssDNA both as the Mn^{2+}-dependent neutral nuclease (**Fig. 4F**) and as the Ca^{2+}/Zn^{2+}-dependent acidic enzyme (**Fig. 4R**). Gels simultaneously treated with zinc and calcium ions again revealed the inhibitory effect of Zn^{2+} on ENDO1 and ENDO4 activity in neutral buffer (**Fig. 4S**) and the influence of inhibition of Ca^{2+} on ENDO3 nuclease (**Fig. 4R**). Moreover, the native in-gel assay revealed some additional nuclease activities that were undetectable under denaturing conditions. First of all, as shown in **Fig. 4M** and **N**, we were able to detect an activity of ENDO5 nuclease which proved to be the Zn^{2+}-dependent enzyme active in the neutral buffer and less efficiently in the acidic buffer.

Experiments performed with native gels supplemented with dsDNA confirmed our previous results showing that only the ENDO1 and ENDO2 nucleases have the capacity to digest dsDNA. Again, as before, the ENDO2 nuclease discloses its nucleolytic activity toward dsDNA as a Zn^{2+}/Ca^{2+}-dependent acidic enzyme (**Fig. 4T**). Another enzyme, ENDO1, confirmed the previously identified activity toward dsDNA at neutral pH and in the presence of Mn^{2+} (**Fig. 4H**). However, interestingly, this nuclease in native conditions also revealed its ability to digest dsDNA under the same conditions as ENDO2, i.e. at low pH and in the simultaneous presence of Ca^{2+} and Zn^{2+} (**Fig. 4T**).

All members of the Arabidopsis S1-like family possess RNase activity

The well-characterized plant S1-like nucleases, such as BFN1, are defined as bifunctional enzymes due to their ability to digest both DNA and RNA substrates. To investigate whether this bifunctional activity is characteristic of all Arabidopsis members of the S1-like nuclease family, we conducted a native in-gel assay using ssRNA as a substrate. Since the generally accepted hypothesis states that members of this family are mostly involved in the redistribution of nucleic acid-derived nutrients, we performed this assay with plant total RNA, because its predominant component, rRNA, is the major cellular reservoir of nucleotides. As shown in **Fig. 5**, all Arabidopsis members of S1-like nucleases revealed RNase activity at pH and ion combinations that also activated their DNase activity toward ssDNA. However, it should be noted that the ratios of DNase and RNase activities are somewhat different for individual nucleases. For example, ENDO1 and ENDO4 nucleases, despite their quite strong DNase activity, revealed relatively weak RNase activity (**Fig. 5B**). Moreover, in addition to recombinant nucleases, the native in-gel RNase assay allowed us also to observe some endogenous RNase activities. The gels incubated at low pH

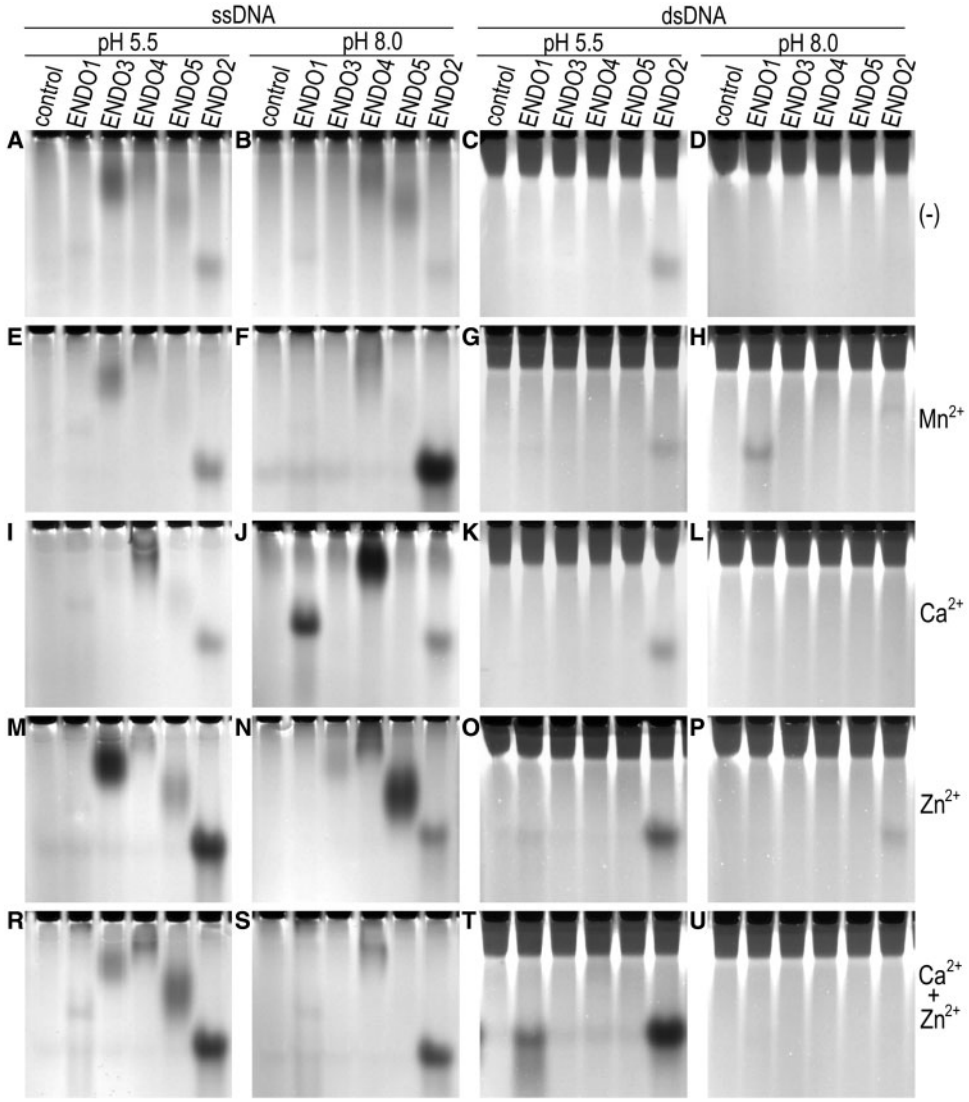

Fig. 4 DNase activities of Arabidopsis members of the S1-like family identified by in-gel nuclease assay conducted under native conditions. Different gels have been arranged as in **Fig. 3**. The protein extract from protoplasts transformed with empty vectors (pSAT6A) was used as a negative control (contr.). The individual gels show the following substrate/ion/pH requirements of Arabidopsis S1-like nucleases: (A) ssDNA/−/pH 5.5, (B) ssDNA/−/pH 8.0, (C) dsDNA/−/pH 5.5, (D) dsDNA/−/pH 8.0, (E) ssDNA/Mn^{2+}/pH 5.5, (F) ssDNA/Mn^{2+}/pH 8.0, (G) dsDNA/Mn^{2+}/pH 5.5, (H) dsDNA/Mn^{2+}/pH 8.0, (I) ssDNA/Ca^{2+}/pH 5.5, (J) ssDNA/Ca^{2+}/pH 8.0, (K) dsDNA/Ca^{2+}/pH 5.5, (L) dsDNA/Ca^{2+}/pH 8.0, (M) ssDNA/Zn^{2+}/pH 5.5, (N) ssDNA/Zn^{2+}/pH 8.0, (O) dsDNA/Zn^{2+}/pH 5.5, (P) dsDNA/Zn^{2+}/pH 8.0, (R) ssDNA/Ca^{2+} + Zn^{2+}/pH 5.5, (S) ssDNA/Ca^{2+} + Zn^{2+}/pH 8.0, (T) dsDNA/Ca^{2+} + Zn^{2+}/pH 5.5, (U) dsDNA/ Ca^{2+} + Zn^{2+}/pH 8.0.

and in the presence of Ca^{2+} or Ca^{2+}/Zn^{2+} ions revealed the activity of a fast-migrating nuclease that does not correspond to any S1-like nucleases (**Fig. 5E, F**). Since we were not able to detect this enzyme in DNase in-gel assays, we conclude that this is a new Ca^{2+}-dependent acidic RNase that, to the best of our knowledge, has not thus far been characterized.

The C-terminal end of Arabidopsis S1-like nucleases is proteolytically cleaved

The presence or absence of a given nuclease activity signal in gels treated under certain conditions may result either from the specific requirements of this enzyme or from the expression level of its encoding transgene. Since the wide range of applied conditions presented above allowed us to detect enzymatic activity in all the examined nucleases, we concluded that all relevant transgenes were efficiently expressed in protoplasts. Nonetheless, because we were also interested in investigating the stability of particular nucleases, the amount of recombinant proteins was estimated by Western blot analysis. For this purpose, we prepared constructs containing Arabidopsis S1 nucleases fused to an enhanced cyan fluorescent protein (ECFP) tag. Since all plant S1 nucleases possess the proteolytically processed N-terminal signal peptide, the ECFP sequence has been fused to their C-termini. As shown in **Fig. 6**, all tagged

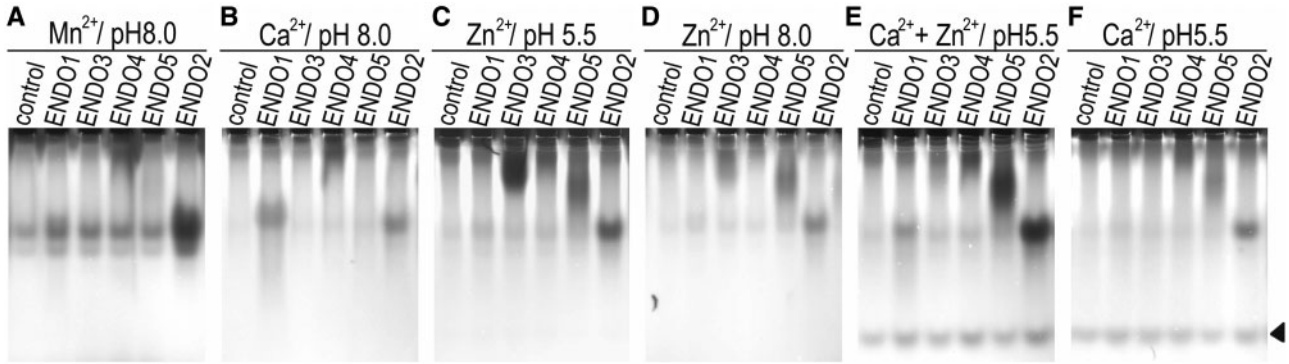

Fig. 5 RNase activities of Arabidopsis members of the S1-like family identified by in-gel nuclease assay conducted under native conditions. (A) ENDO2 activity induced by Mn^{2+} ions at pH 8.0, (B) ENDO1 and ENDO2 activities induced by Ca^{2+} ions at pH 8.0, (C) ENDO3, ENDO5 and ENDO2 activities induced by Zn^{2+} ions at pH 5.5, (D) ENDO5 and ENDO2 activities induced by Zn^{2+} ions at pH 8.0, (E) ENDO5 and ENDO2 activities induced by simultaneously applied Zn^{2+} and Ca^{2+} ions at pH 5.5, (F) The activity of acidic Ca^{2+}-dependent RNase (filled triangle) unrelated to S1-like nucleases.

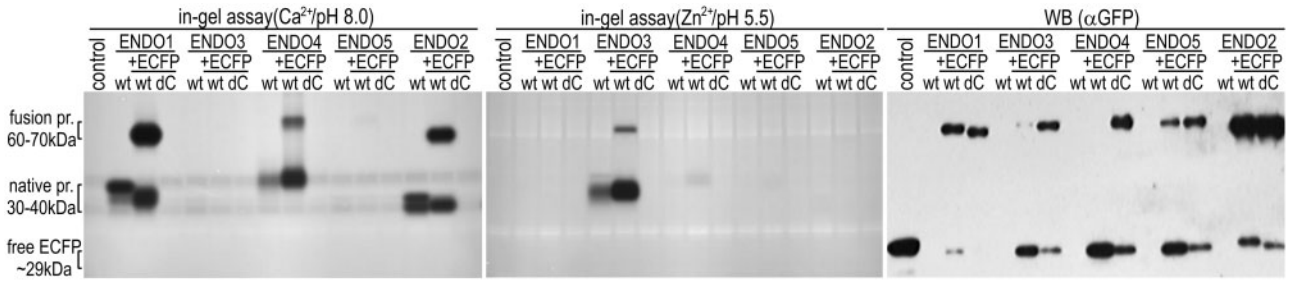

Fig. 6 In-gel nuclease assays and Western blot analysis showing the expression and stability of C-terminal end-tagged Arabidopsis S1-like nucleases. Two in-gel nuclease assays (left and middle panels) and a Western blot assay (right panel) were performed with the same set of overexpressed nucleases and control sample. Each nuclease was analyzed as: wild-type enzyme (wt), wild-type enzyme (wt) fused with ECFP (+ECFP) and C-terminal end deletion mutant (dC) fused with ECFP (+ECFP). As the control (control), the protein extract from protoplasts transformed with the pSAT6A-ECFP vector expressing ECFP alone was used. Molecular mass deduced from gel electrophoresis is indicated on the left. Left: the in-gel nuclease assay performed with ssDNA substrate in the presence of Ca^{2+} at pH 8.0. Middle: the in-gel nuclease assay performed with ssDNA substrate in the presence of Zn^{2+} at pH 5.5. Right: the Western blot analysis performed with anti-GFP antibody.

nucleases overexpressed in protoplasts and analyzed by in-gel nuclease assay maintain enzymatic activities under the same ionic and pH conditions as untagged nucleases, i.e. ENDO1, ENDO2 and ENDO4 are activated by Ca^{2+} at neutral pH while ENDO3 activity is stimulated by Zn^{2+} under acidic conditions. However, it is significant that the electrophoretic separation of samples containing all ECFP-tagged nucleases revealed that, in addition to a band representing the full-length fusion protein (60–70 kDa), a second band appears at the position of the untagged enzyme (30–40 kDa). In our opinion, the presence of these signals could result from proteolytic cleavage occurring at the C-termini of nuclease sequences which leads to the removal of a tagged domain and restoration of an enzyme of the size of a native protein. The comparison of tagged and truncated protein activities shows that this process seems to occur most intensely in ENDO3 and ENDO4 nucleases and less efficiently in ENDO1 and ENDO5.

All the samples whose activities were tested by the in-gel nuclease assays were also analyzed by Western blot using anti-green fluorescent protein (GFP) antibody (**Fig. 6**, right panel). This experiment shows that all tagged S1 nucleases, including the ENDO5 enzyme which was inactive in the denaturing in-gel assay, were effectively expressed in protoplasts. Moreover, the presence of two signals, one migrating with the size of a fused protein and the second at the position of free ECFP (29 kDa), confirms the assumption that the tagged peptide sequence was cleaved out in the portion of the protein pool. It should also be noted that the reciprocal relationship between the intensity of these two bands again shows that ENDO1 and ENDO2 are less efficiently truncated than other nucleases.

To demonstrate the presence of a putative proteolytic cleavage site near the C-terminus of S1 nucleases, all members of this family have been subjected to site-directed mutagenesis. In each case we deleted the terminal 8–19 residues as indicated in **Fig. 1**. On the supposition that the removal of the predicted cleavage site will affect the fusion protein stability, we analyzed this by Western blot together with corresponding, undisturbed

recombinant proteins. The Western blot analysis presented in Fig. 6 confirmed the above assumption, since in all cases the deletion of C-terminal residues significantly decreased the amount of free ECFP in mutant protein samples, simultaneously increasing the intensity of bands representing the full-length fusion protein. In conclusion, all Arabidopsis S1 recombinant nucleases can be efficiently expressed in plant protoplasts, and the observed variability of their activity does not result from the expression level of each cDNA but reflects their different ion/pH/substrate requirements. Moreover, the particular Arabidopsis S1-like nucleases differ in terms of the stability of their C-termini. It should also be noted that this region seems to be crucial for the enzymatic activity of plant S1 nucleases, since the mutation causing changes in this area generates a completely inactive enzyme, as shown by the in-gel assays presented in Fig. 6.

The members of the *Arabidopsis thaliana* S1-like gene family are expressed in different tissues and developmental stages

The observation that individual members of the plant S1-like family display markedly different catalytic properties raises the question of whether or not they have diverse biological functions. To identify the processes which correlate with the expression of plant S1 nucleases, we analyzed the results of microarray experiments collected in the Genevestigator database. Four of five members of the Arabidopsis S1-like family, with the exception of the ENDO4 gene, are present on the Affymetrix ATH1 (22k) genome array.

Generally, S1 nucleases are highly specific for different plant tissues and/or developmental stages. As shown in Supplementary Fig. S3A–C, ENDO5 nuclease gene expression is strictly related to the root. Already in the early stage of seed germination, ENDO5 expression significantly exceeds that of other S1 nucleases in the emerging radicle (Supplementary Fig. S3D) and maintains a relatively high level in the root from the seedling and developed rosette stages until the developed flower stage (Supplementary Fig. S3E, F). All microarray experiments comparing the gene expression in different root regions revealed that ENDO5 is expressed both in the root tip and in elongation end maturation zones (Supplementary Fig. S3G). Similarly, comparison of different root tissues shows that ENDO5 is expressed in root cortex, phloem and xylem cells (Supplementary Fig. S3H). It should be noted that the same experiment revealed that another member of the S1 family, ENDO1, in contrast to ENDO5, is highly specific only for root xylem cells. The involvement of ENDO1 in xylogenesis can also be concluded from the experiment conducted with root cells induced to transdifferentiate into tracheary elements (Supplementary Fig. S3I).

Supplementary Fig. S3B and C shows that expression of ENDO3 nuclease, in contrast to ENDO5, is highly specific to flower development. The ENDO3 expression appears first in the shoot apex of an emerging inflorescence stem (Supplementary Fig. S3A), remains high in young flowers and decreases gradually with the development of siliques (Supplementary Fig. S3J). In addition, as shown in Supplementary Fig. S3K, ENDO3 is preferentially expressed in the pistil and less abundantly in the stamen. The comparison of ENDO3 transcript levels in the whole pistil and separate ovules suggests that ENDO3 activity is mainly related to the ovule (Supplementary Fig. 3L). The most significant decrease in ENDO3 expression occurs between the proglobular and globular stages of seed development (Supplementary Fig. S3M).

Similar to ENDO3, the expression of ENDO1 is also associated with flower development (Fig. 3J, K). However, detailed analysis of different microarray experiments shows that the transcription of these two genes is not correlated with each other. The stamen is the only part of the flower where both of these genes are expressed at a relatively high level, but it should be noted that while the ENDO1 level remains stable, the ENDO3 transcription decreases dramatically with stamen age (Supplementary Fig. S3K). The ENDO1 gene is also transcribed in the sepal and petal, but in contrast to ENDO3 its expression tends to increase, especially in the petal, with flower aging.

Our analysis of a wide range of microarray experiments has shown that ENDO2 nuclease is expressed at a high level mainly in callus and different Arabidopsis cell cultures (data not shown). However, a series of experiments devoted to the study of Arabidopsis seed tissue development (Belmonte et al. 2013) have demonstrated that the expression of this nuclease is also highly specific for chalazal endosperm. As shown in Supplementary Fig. S3N–S, a high level of ENDO2 transcription is maintained in this part of the endosperm from the preglobular to the linear cotyledon stage and rapidly decreases at later stages of seed development. It is interesting that in late endosperm the decrease in ENDO2 is accompanied by a significant increase in ENDO1 nucleases (Supplementary Fig. S3R, S), which may suggest the involvement of ENDO1 in endosperm senescence.

Although transcription of ENDO1 is not associated with a single plant tissue, the microarray data presented above show that expression of this nuclease correlates with processes classified into two categories of PCD, i.e. xylogenesis (Supplementary Fig. S3H, I) and senescence (Supplementary Fig. S3K, R, S). The relationship of this enzyme to senescence also seems to be confirmed by the observation reported in Supplementary Fig. S3A that shows an up-regulation of ENDO1 gene expression in senescent leaves as well as by the study of genes that are specifically expressed in leaves induced to senescence by nitrogen deficiency (Supplementary Fig. S3T).

Evolution of the plant S1-like nuclease gene family

Comparison of different Arabidopsis S1-like activities inspired us to analyze the evolutionary patterns of this enzyme class in land plants. According to the well-established evolutionary scenario, a gene family can be created by duplication events of an ancient gene. Subsequent mutations and selection processes gradually differentiate the family members, which

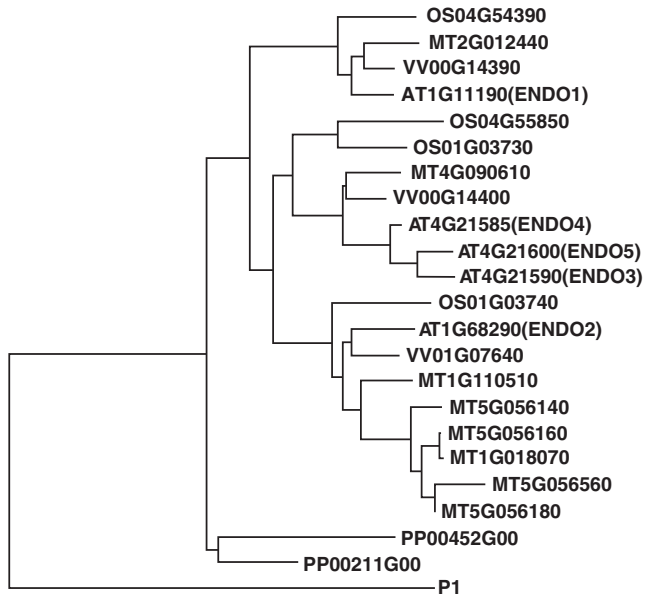

Fig. 7 Reconstruction of the phylogenetic relationship between sequences of the S1-like nuclease family from *Arabidopsis thaliana* (AT), *Medicago truncatula* (MT), *Vitis vinifera* (VV), *Oryza sativa* (OS) and *Physcomitrella patens* (PP).

acquire different molecular properties leading to their distinct functions (Copley et al. 2002). An interesting conclusion can be drawn about the evolutionary trends of the S1-like nuclease family in Arabidopsis based on reconstruction of its phylogenetic relationships in the context of homologous sequences from representative land plant species. As shown in **Fig. 7**, the external placement of *Physcomitrella patens* genes indicates that a common ancestor of land plants contained only one copy of the S1 nuclease. In contrast, the sequences from monocot and dicot plant species form three major clades, each of them represented by at least one gene from selected species. This may suggest early diversification of function and/or expression regulation. An interesting pattern appears when we compare the diversity of catalytic properties of Arabidopsis nucleases and the number of duplicated genes in each of the clades. It appears that sequences encoding enzymes with increased diversity for substrate and reaction conditions (ENDO1 and ENDO2) are represented by single-copy genes, where more basic type of enzymatic activities (ENDO3, ENDO4 and ENDO5; closely resembling the fungal P1 nuclease) need to be present in a higher number of copies, resulting from local, tandem duplications.

Discussion

The numerous nucleolytic activities that have long been observed in plant tissues have usually been classified according to catalytic requirements regarding their ion and pH dependency. This approach allowed a distinction to be made between two main types of nucleases whose representatives are most commonly observed in plants, i.e. Zn^{2+}-dependent acidic and Ca^{2+}-dependent neutral nucleases (Sugiyama et al. 2000). Other less frequently detected activities were classified into additional subgroups. This classification seemed to be consistent with results of later studies that enabled the characterization of individual nucleases based on their known coding sequences. The Zn^{2+}-dependent activity of some plant nucleases showing similarity to S1 and P1 enzymes confirmed the belief that all plant members of this family are characterized basically by the same catalytic requirements as their fungal orthologs. The identification of plant staphylococcal-like nucleases, which exert their activities exclusively in the presence of Ca^{2+} ions at neutral pH, seemed to confirm the assumption that the classification based on catalytic requirements corresponds to the phylogenetic origin of the relevant protein families.

However, some uncertainties regarding the properties of S1-like nucleases encouraged us to perform a more complex study of their activity. In our opinion, adaptation of the general definition describing plant S1-like nucleases requires experiments enabling the simultaneous analysis of different members of this family. General conclusions drawn from the results of different studies may be misleading because activity assays conducted in different laboratories can vary greatly as a result, for example, of different levels of enzyme denaturation or the efficiency of removal of native bound ions.

The results of our study revealed that plant S1-like nucleases are characterized by a surprisingly wide variety of enzyme activity. This diversity sheds new light on the classification of plant nucleases. First of all, it becomes clear that a classification of plant nucleases based on their ion dependency does not coincide with their phylogenetic origin. The term 'nuclease I', considered as a class of plant Zn^{2+}-dependent nucleases, cannot be exclusively identified with S1-like nucleases since, as we prove herein, some members of the plant S1-like nuclease family are induced by ions other than Zn^{2+}, i.e. Ca^{2+} and Mn^{2+}. In addition, the activity of some Arabidopsis S1 nucleases, such as ENDO1 and ENDO2, is in contradiction to the generally accepted classification because in certain circumstances they can be activated by simultaneously used Ca^{2+} and Zn^{2+} ions. The results presented herein are also relevant to an understanding of the substrate specificity of S1-like nucleases. It is often believed that this class of enzymes is generally involved in the hydrolysis of ssDNA and RNA, while their efficiency of dsDNA digestion is much lower. Yet, as we have shown in our study, different members of the S1 nuclease family revealed a radically different affinity toward dsDNA. Three of the five members of this family did not show any activity toward dsDNA. However, it is interesting that two Arabidopsis S1-like nucleases capable of digesting dsDNA in some conditions can catalyze this reaction with an efficiency comparable with or higher than ssDNA.

Research on plant nuclease activity patterns has indicated that different types of PCD often induce the strong activity of numerous Ca^{2+}-dependent nucleases (Mittler and Lam 1995, Lesniewicz et al. 2010). However, until recently, it was uncertain

which genes encode this activity. Now, though, according to results presented herein as well as other studies, it can be concluded that the Ca^{2+}-dependent nucleases in plants belong to at least two unrelated families: one derived from staphylococcal-like and the second related to fungal S1-like enzymes. Despite the different types of their catalytic sites, they exhibit similar properties, since in both cases their calcium-dependent activity is optimal at neutral pH and can be inhibited by Zn^{2+}. It must, however, be noted that, as we have shown herein, plants also possess the Ca^{2+}-dependent acidic RNase that is unrelated to S1- and SNc-like enzymes. Interestingly, this enzyme differs in its properties from the conventional classification that places Ca^{2+}-dependent nucleases with the enzymes acting at neutral pH.

An interesting aspect of our study is the demonstration that Mn^{2+} ions, in addition to Zn^{2+} and Ca^{2+}, can play a role in the activation of some S1-like nucleases. Mn^{2+}-dependent nuclease activity in plant tissues has been reported elsewhere (Lers et al. 2001, He et al. 2003); however, the authors have not determined the genes encoding the respective enzyme. The only known exception is Mn^{2+}-dependent OmBBD nuclease, which is unrelated to S1-type enzymes (You et al. 2010). However, the physiological relevance of the finding that Ca^{2+}-dependent S1-like nucleases can also be activated by Mn^{2+} is difficult to evaluate, because it is known that in some proteins Mn^{2+} could mimic the activity of Ca^{2+} ions (Powis et al. 1996).

The discovery of a wide variety of ions activating or inhibiting plant S1-like nucleases opens up new perspectives for future research into reaction mechanisms catalyzed by these enzymes. The fungal P1 nuclease is considered a model enzyme performing the hydrolysis of phosphate diester bonds via a trinuclear zinc complex. To the best of our knowledge, the mechanism of this reaction has been studied exclusively in relation to zinc ions. All nine amino acids in the P1 active site that coordinate three zinc ions are well conserved in all Arabidopsis S1-like enzymes, and their predicted three-dimensional arrangements are also roughly the same. However, the results of our study clearly show that in plants this kind of active site can also cooperate with Ca^{2+} and Mn^{2+}. Perhaps future crystallography studies in combination with biochemical approaches will be able to explain how the slight differences in their active site structures result in different preferences for metal ion binding.

The analysis of the phylogenetic distribution of Arabidopsis nuclease sequences in the context of homologous molecules from major plant taxa suggests two strategies for S1-like nuclease gene family evolution: one represented by three genes located on chromosome 4, where each of them represents a narrow range of catalytic and expression activities (ENDO3, ENDO4 and ENDO5), and the second that includes solitary genes located on chromosome 1 with much broader catalytic properties (ENDO1 and ENDO2). The analysis shows that a similar scenario was adopted during evolution of *Medicago truncatula* S1 nucleases, where sequences belonging to another clade were highly amplified. It will remain an open question for now whether the barrel medic specific amplification of nuclease genes is also connected with their narrow range of catalytic activity and/or highly specific pattern of expression.

The results presented herein lead to the conclusion that plant members of the S1-like family evolve towards increasing the diversity of their catalytic properties. This hypothesis appears to be justified in respect of the known biological functions of plant S1-like nuclease representatives. In heterotrophic microorganisms, the secretory enzymes, such as fungal S1 and P1 nucleases, play an important role in degradation of extracellular DNA and RNA, facilitating the acquisition of nucleic acid-derived nutrients for biosynthesis (Balabanova et al. 2011, Seper et al. 2011). In contrast, in plants the vast majority of the available data suggest a functional relationship between S1-like nucleases and different types of PCD. Farge-Barhom et al. (2008), who studied in detail the expression of the ENDO1 (BFN1) gene using its promoter fused with a GUS (β-glucuronidase) reporter gene, showed that ENDO1 transcription is highly specific for different processes related to PCD, e.g. leaf and petal senescence, xylem differentiation, and anther and stamen development. The microarray analysis presented here fully confirmed these findings, indicating the same tissues and developmental stages as those characteristic for ENDO1 expression. The function(s) of other Arabidopsis S1-type nucleases were not examined in such detail, but the data presented here suggest that at least one more member of this family, ENDO2, is also involved in PCD-related processes. The microarray experiment revealed that expression of this gene correlates significantly with endosperm development. Published data show that in barley the endosperm nucleic acids are intensively digested by the S1 homolog named BEN1 (Aoyagi et al. 1998). According to the data presented here in Arabidopsis, two members of the S1 family, ENDO2 and ENDO1, are expressed in developing and late endosperm, respectively. The observation that these two S1 nucleases are involved in different stages of endosperm development, the process characterized by significant molecular and structural changes, suggests the diverse functions of these enzymes, related to distinct physiological conditions.

The expression profiles of other Arabidopsis S1 nucleases, e.g. ENDO3 and ENDO5, show that these nucleases are involved in other specific developmental processes different from those of ENDO1 and ENDO2. Similar to ENDO1, the expression profile of ENDO3 established on the basis of microarray experiments is supported by experimental evidence. Gomez-Mana et al. (2004), who studied flower-specific proteins whose expression is regulated by the AGAMOUS homeotic gene, found that ENDO3 is specifically up-regulated in floral meristem and is continued in stamen development. These results, demonstrated by in situ hybridization, correspond exactly to the results of microarray experiments to which we referred herein. It should be noted here that none of the available data concerning ENDO3 expression support the assumption that this gene function is related to any well-defined PCD process. Similarly, the expression of ENDO5 is also highly specific for one organ, i.e. the root, but does not clearly correlate with PCD.

Although the function of particular members of plant S1 nucleases remain to be explained, available data concerning their expression profiles clearly indicate that they are involved in developmental processes occurring in tissues characterized by large morphological and physiological variation. A good example of such diversity can be seen in tissues undergoing different types of PCD. In cells differentiating into tracheary elements, nuclear degradation is triggered by collapse of the vacuole, which results in the release of heavy metal ions and acidification of the cytosol. These conditions, according to Ito and Fukuda (2002), activate Zn^{2+}-dependent ZEN1 nuclease, having an optimum acidic pH. On the other hand, the localization pattern, showing the transfer of BFN1 (ENDO1) nuclease from the endoplasmic reticulum to the nucleus, suggests that this senescence-specific enzyme degrades nucleic acids in cellular environments different from those of acidic nucleases (Farage-Barhom et al. 2011). Moreover, since the DNA degradation seems to be a multistep process, some authors suggest that even one type of PCD may involve several nucleases acting under different conditions characteristic for various stages of this process. These observations show that plant organisms require the presence of a whole set of various nucleases having the ability to hydrolyze the nucleic acids under different conditions. Therefore, it is reasonable to conclude that the evolution of the plant S1-like nuclease family tends to increase the range of the catalytic properties of its members.

Materials and Methods

Bioinformatic analysis

The amino acid sequences encoded by Arabidopsis S1-like nuclease genes (ENDO1, AT1G11190; ENDO2, AT1G68290; ENDO3, AT4G21590; ENDO4, AT4G21585; ENDO5, AT4G21600) and P1 nuclease from *P. citrinum* (P24289) were obtained from NCBI (http://www.ncbi.nlm.nih.gov). For the alignment of the amino acid sequences, the BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2) programs were employed.

In silico analysis of the expression of each S1 nuclease gene was carried out using microarray expression data provided by Genevestigator (Hruz et al. 2008) (https://www.genevestigator.ethz.ch/at). The condition search/samples tool of the Genevestigator software was used to estimate the levels of gene expression in different Arabidopsis tissues. The signal values of individual arrays were transferred to a Microsoft Excel spreadsheet for the calculation of the standard error values and to create charts.

To determine the molecular evolution of the S1-like nuclease gene family, the protein sequences corresponding to the S1-like nuclease family from *A. thaliana*, *M. truncatula*, *Vitis vinifera*, *Oryza sativa* and *P. patens* were downloaded from the Plaza 2.5 database (Plaza 2.5 database gene family HOM001434) (Van Bel et al. 2012). Endonuclease P1 from *P. citrinum* (UniProtKB/Swiss-Prot accession number P24289) was used as the out-group for the analysis. Sequences were aligned using the muscle program (Edgar 2004) using default parameters. PhyML (Guindon et al. 2010) was used for reconstruction and bootstrapping of the phylogenetic tree. All shown branches achieved at least 70% in the bootstrap test. The cladogram was visualized and formatted using Dendroscope viewer (Huson et al. 2012).

Structure modeling of Arabidopsis S1-like nucleases

To investigate the putative protein structures of plant S1-like nucleases, we applied the comparative modeling approach that was based on the known template structure of P1 nuclease (PDB code: 1ak0) from *P. citrinum* (Romier et al. 1998). Consensus structure prediction for all Arabidopsis S1-like nucleases was performed using the MetaServer program at http://bioinfo.pl/meta, which derives and scores consensus predictions from the results of several publicly available online services for fold recognition (Ginalski et al. 2003). The MODELLER software was used to obtain the final models of individual plant nucleases. The figures were generated using Pymol Molecular Graphics Software (http://www.pymol.org).

cDNA cloning and mutagenesis

Total RNA was isolated from 20-day-old *A. thaliana* seedlings using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The mRNA was reverse-transcribed into single-stranded cDNA using an oligo(dT) primer and MMLV reverse transcriptase (Promega) according to the protocol supplied along with the enzyme. PCR was used to amplify cDNAs encoding sequences of all five S1-like nucleases. The amplification was performed with specific pairs of primers designed for all cDNAs using the Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primers used to amplify Arabidopsis S1-like nucleases encoding cDNAs are shown in **Supplementary Table S2.**

The PCR products encoding individual nucleases were cloned into pGEM vector. Encoding sequences were then amplified by PCR performed with primers containing artificial *Sal*I and *Bam*HI restriction sites (**Supplementary Table S2**) and subcloned into pSAT6A-ECFP expression vector (Chung et al. 2005, Tzfira et al. 2005). The constructs designed to overexpress native recombinant nucleases contain a stop codon at their 3′ ends. In turn, the nuclease 3′ end stop codon was removed from the constructs encoding the nuclease–ECFP fusion protein. All the resulting constructs were confirmed by sequencing.

The deletion mutants of S1-like nucleases were generated by PCR. The pSAT6A vectors harboring full-length coding cDNAs for each Arabidopsis S1 nuclease and the primers showed in **Supplementary Table S2** were used to create mutations. Primers flanking the deleted regions were designed to be facing away from each other. The 5′ end sequences of each primer contain restriction sites for *Bam*HI, enabling self-ligation of the resulting PCR product ends and thus the reconstruction

of circular plasmid vectors. The PCRs were performed with Phusion DNA Polymerase (Finnzymes).

Transient gene expression in *Arabidopsis thaliana* protoplasts

Protoplasts from Arabidopsis leaf mesophyll were prepared and transiently transformed by the polyethylene glycol (PEG) method as described by Yoo et al. (2007). Twelve hours after PEG-mediated transformation, the protoplasts were harvested and used to prepare the protein extracts for in vitro assays. Depending on the application of the protein extracts, the protoplasts were lysed in denaturation buffer [30% glycerol (v/v), 160 mM Tris–HCl (pH 6.8); 6% SDS; 5 mM EDTA; 0.01% (w/v) bromophenol blue] or non-denaturating buffer [40% glycerol (v/v); 300 mM Tris (pH 8.8); 0.5% Triton X-100 (v/v)]. An aliquot of each extract was taken to measure the protein concentration using Bradford reagent (Pierce). The remainder of the extracts was then frozen rapidly at −20°C.

Denaturing in-gel nuclease activity assay

The detection of DNase activities was based on the methods of Thelen and Northcote (1989) with the following modifications. The resolving mini gels (10×10 cm) contained sonicated calf thymus DNA at the concentration 0.01 mg ml^{-1}. For single-stranded DNase activity, DNA was boiled for 5 min immediately prior to pouring the resolving gel. Equal amounts of protein (5 μg) were denatured for 5 min at 99°C in sample buffer lacking 2-mercaptoethanol before loading into the gel. Electrophoreses were performed at +4°C, applying 5 V cm^{-1} for about 3 h. After electrophoresis, the resolving gels were soaked twice at room temperature in a buffer containing 20% 2-propanol for 20 min to remove SDS. Subsequently, the gels were washed twice for 15 min and overnight in renaturation/reaction buffer. The following renaturation buffers were used to adjust the various pH values: acid buffer [25 mM sodium acetate (pH 5.5); 1% (v/v) Triton X-100], neutral buffer [20 mM Tris–HCl (pH 8.0); 1% (v/v) Triton X-100]. The above buffers were supplemented with 5 mM $CaCl_2$, 0.1 mM $ZnCl_2$ or 0.5 mM $MnCl_2$, depending on the experiment. After incubations, the gels were washed for 5 min in ice-cold staining buffer (10 mM Tris–HCl pH 8.0; 1 mM EDTA; 0.01 mg ml^{-1} ethidium bromide) to reveal the position of the nucleases. The gels were imaged using the G:Box system (Syngene). Each experiment was repeated at least three times.

Native in-gel nuclease activity assay

To detect the nucleolytic activities of non-denatured proteins, we modified denaturing in-gel nuclease activity assay. Proteins were resolved in 4.5% native polyacrylamide gels containing 0.375 M Tris (pH 8.8), 1% glycerol and 1.8% glycine. Gels were supplemented with ssDNA, dsDNA or *A. thaliana* total RNA (0.01 mg ml^{-1}) depending on the experiment. The electrophoresis was performed using Tris-glycine running buffer (1.8% Tris base and 1.8% glycine). Protein extracts (5 μg) were adjusted to an equal volume of sample containing 0.5 mM EDTA and incubated for 3 min at 45°C to remove residential ions. Subsequently, samples were supplemented with $MnCl_2$ to a final concentration at 1.5 mM and incubated for another 5 min at room temperature. Finally, the samples were mixed with an equal volume of sample buffer [40% glycerol (v/v); 300 mM Tris (pH 8.8); 0.5% Triton X-100; 0.01% bromophenol blue] and loaded without heating onto the gel. Electrophoresis was carried out at 4°C for 3 h at 50 mA constant current. After completion of electrophoresis, gels were incubated twice for 30 min and once for 18 h in 100 ml of renaturation/reaction acid buffer [25 mM sodium acetate (pH 5.5); 1% (v/v) Triton X-100] or neutral buffer [20 mM Tris–HCl (pH 8.0); 1% (v/v) Triton X-100]. The renaturation/reaction buffers were supplemented with various metal ions as described in the Results. After completion of the reaction, gels were stained and imaged as above. Each experiment was repeated at least three times.

SDS–PAGE and Western blot analysis

SDS–PAGE was performed according to standard protocols with the Hoefer Mighty Small II gel system. Proteins were transferred to Amersham Hybond-P PVDV membrane with the Biometra Fastblot B43 semi-dry transfer system (0.8 mA cm^{-2}, 1 h). The membrane was blocked and washed according to the manufacturer's instructions (Amersham). The primary mouse monoclonal anti-GFP antibody (B-2) (Santa Cruz Biotechnology) and secondary anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Sigma) were diluted 1:200 and 1:5,000, respectively. Chemiluminescence detection of proteins was performed with the GE Healthcare ECL Prime Western Blotting Detection Reagents.

Supplementary data

Supplementary data are available at PCP online.

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