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Identification of BFN1, a Bifunctional Nuclease Induced during Leaf and Stem Senescence in Arabidopsis[1]

Miguel A. Pérez-Amador, Michael L. Abler[2], E. Jay De Rocher[3], Debrah M. Thompson[4], Ambro van Hoof[5], Nicole D. LeBrasseur, Amnon Lers[6], and Pamela J. Green*

Department of Energy–Plant Research Laboratory, and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824–1312

Nuclease I enzymes are responsible for the degradation of RNA and single-stranded DNA during several plant growth and developmental processes, including senescence. However, in the case of senescence the corresponding genes have not been reported. We describe the identification and characterization of *BFN1* of Arabidopsis, and demonstrate that it is a senescence-associated nuclease I gene. BFN1 nuclease shows high similarity to the sequence of a barley nuclease induced during germination and a zinnia (*Zinnia elegans*) nuclease induced during xylogenesis. In transgenic plants overexpressing the *BFN1* cDNA, a nuclease activity of about 38 kD was detected on both RNase and DNase activity gels. Levels of *BFN1* mRNA were extremely low or undetectable in roots, leaves, and stems. In contrast, relatively high *BFN1* mRNA levels were detected in flowers and during leaf and stem senescence. BFN1 nuclease activity was also induced during leaf and stem senescence. The strong response of the *BFN1* gene to senescence indicated that it would be an excellent tool with which to study the mechanisms of senescence induction, as well as the role of the BFN1 enzyme in senescence using reverse genetic approaches in Arabidopsis.

Plant senescence is a highly regulated process during which coordinated changes in cell structure, metabolism, and gene expression occur (Gan and Amasino, 1997). An early event during senescence is the breakdown of the chloroplast, with the subsequent degradation of chlorophyll and protein. Upon cell disruption, RNA is degraded and DNA is fragmented (Orzáez and Granell, 1997) and eventually degraded as well.

One of the groups of genes potentially involved in the senescence process are the nuclease I enzymes. Together with other hydrolytic enzymes, nucleases can provide RNA and DNA degradation products to be used in other parts of the plant as part of the mechanism of nutrient salvage that occurs in the plant following cell death (Bleecker, 1998). Nevertheless, and in spite of extensive studies on the gene expression that occurs during plant senescence (Lohman et al., 1994; Oh et al., 1996; Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Weaver et al., 1998), the genes that encode senescence-induced nucleases have not been identified. To better understand plant senescence, it is important to isolate and study the genes responsible for degradation of both bulk RNA and DNA, i.e. genes for nuclease I enzymes.

All living organisms contain enzymes responsible for the degradation of single-stranded nucleic acids (Gite and Shankar, 1995), including nuclease I proteins (EC 3.1.30.1). Nuclease I enzymes are extracellular heat-stable glycoproteins that degrade both RNA and single-stranded DNA endonucleolytically. They have a preference for bonds adjacent to adenine and produce 5′-phosphoryl-oligo and mononucleotides. Several biochemical characteristics define this family of enzymes: they have a molecular mass range between 31 and 42 kD, are highly sensitive to EDTA, have acidic pH optima, and require Zn^{2+} for activation and for stability (Fraser and Low, 1993). Two fungal nuclease I proteins have been extensively characterized, and their sequences have been determined. These are nuclease P1 from *Penicillium citrum* (Maekawa et al., 1991) and nuclease S1 from *Aspergillus oryzae* (Iwamatsu et al., 1991).

Plant nuclease I and other single-strand-specific nucleases are induced during plant growth and developmental processes such as germination, xylem differentiation, the hypersensitive response, stress responses, and senescence (for review, see Bariola and Green, 1997). Many examples of such activities have been reported. They include proteins with nuclease I characteristics from mung bean (Laskowski, 1980), tobacco cell suspension cultures (Oleson et al., 1982), tobacco pollen (Matousek and Tupy, 1984), barley (Brown and Ho, 1986, 1987), zinnia (*Zinnia elegans*)

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[2] Present address: Department of Biology, 3020 Cowley Hall, University of Wisconsin, La Crosse, WI 54601.

[3] Present address: Eden Bioscience Corporation, 11816 North Creek Parkway N., Bothell, WA 98011.

[4] Present address: Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

[5] Present address: Howard Hughes Medical Institute, Department of Molecular and Cellular Biology, University of Arizona, Tucson, AZ 85721.

[6] Present address: Department of Postharvest Science of Fresh Produce, The Volvani Center, P.O. Box 6, Bet Dagan, 50250 Israel.

* Corresponding author; e-mail green@pilot.msu.edu; fax 517–355–9168.

(Thelen and Northcote, 1989), rye (Siwecka et al., 1989; el Adlouni et al., 1993), and *Lentinula edodes* (Kobayashi et al., 1995). In addition, a number of activities with some similarities to the tobacco pollen nuclease I (Matousek and Tupy, 1984) have been identified in pollen from various other plants (Matousek and Tupy, 1985). Finally, single-strand-specific nucleases have also been found in a variety of species, including spinach (Strickland et al., 1991; Yupsanis et al., 1996; Christou et al., 1998), scallion (Uchida et al., 1993), wheat chloroplasts (Kuligowska et al., 1988; Monko et al., 1994), pea seeds (Naseem and Hadi, 1987), and pea chloroplasts (Kumar et al., 1995).

Proteins from Arabidopsis with some of the properties of nuclease I enzymes have been identified using activity gels (Yen and Green, 1991). Specifically, a doublet of about 33 kD appears in both RNase and DNase activity gels. More recently, analysis of altered RNase profile (*arp*) mutants confirmed that nuclease I enzymes exist in Arabidopsis. Several *arp* mutants lack or overproduce one or both of the 33-kD activity bands in RNase and DNase activity gels, with RNase patterns mirroring the DNase patterns in each mutant (M.L. Abler and P.J. Green, unpublished data). These results confirm the presence of bifunctional nuclease activities at 33 kD.

Until recently, sequence information was available only for several fungal nuclease I genes and short N-terminal regions of proteins with properties of nuclease I enzyme from barley and zinnia. In barley, it was found that aleurone layers secrete a nuclease into the endosperm in response to gibberellic acid (Brown and Ho, 1986). The first 17 amino acids corresponding to the NH_2-terminal sequence of the secreted protein were determined (Brown and Ho, 1987). During xylogenesis in cultured cells, zinnia secretes a single-strand-specific nuclease (Thelen and Northcote, 1989). The NH_2-terminal amino acid sequence of this mature protein was also determined and shown to be similar to that of the barley enzyme (Thelen and Northcote, 1989). Only very recently were the cDNA sequences corresponding to the barley and zinnia proteins described above reported (Aoyagi et al., 1998).

Previously, we characterized three Arabidopsis S-like RNase genes, *RNS1*, *RNS2*, and *RNS3*, that are each induced to different extents during leaf senescence. *RNS1* is induced only slightly (Bariola et al., 1994), whereas *RNS2* and *RNS3* are more strongly induced (Taylor et al., 1993; Bariola et al., 1994). Isolation and characterization of Arabidopsis genes encoding nuclease I enzymes, especially those induced during senescence, will enable us to better understand the role of nucleases in senescence.

We report the identification of an Arabidopsis nuclease I cDNA that is induced specifically during leaf and stem senescence. We also identified two zinnia cDNAs that are similar to the Arabidopsis clone. The Arabidopsis gene, designated *BFN1*, encodes a bifunctional nuclease I enzyme, a protein with both RNase and DNase activities. The expression characteristics of *BFN1* suggest a role in nucleic acid degradation to facilitate nucleotide and phosphate recovery during senescence.

MATERIALS AND METHODS

Plant Material

All Arabidopsis tissues described in this report are from the Columbia ecotype. Roots, stems, leaves, flowers, and siliques were harvested from 4- to 8-week-old plants grown in growth chambers in 16 h of light and 50% relative humidity at 20°C. Stems were harvested as bolts (1–3 cm tall), young stems (3–10 cm tall), and mature stems (>10 cm tall). Leaves were harvested as young leaves (1–2 cm in diameter) and mature leaves (fully expanded). Senescent stems were harvested when they exhibited a purple color, while senescent leaves were harvested when at least 50% of the leaf was yellow. For the phosphate starvation experiment, seedlings were grown on P_i-rich and P_i-deficient media and harvested as described previously (Bariola et al., 1994). In the germination experiment, 1.5 g of seeds was surface-sterilized for 7 to 9 min in 50% (v/v) bleach containing 0.02% (v/v) Triton X-100. Seeds were extensively washed with sterile distilled water, and resuspended in 35 mL of sterile liquid Arabidopsis growing medium (4.3 g L^{-1} Murashige-Skoog salts [Life Technologies/Gibco-BRL, Cleveland], 1× B5 vitamins, 1% [w/v] Suc, and 0.5 g L^{-1} 2-(N-morpholino)-ethanesulfonic acid [MES] buffer, pH 5.7 with KOH). Resuspended seeds (5 mL) were plated on a Petri dish in which a sterile filter paper soaked in Arabidopsis growing medium had been placed. At 2, 3, 4, and 5 d after plating, seedlings from a Petri dish were harvested.

Zinnia (*Zinnia elegans* cv Envy) leaves were collected from 2-month-old plants grown in growth chambers in the same conditions as for Arabidopsis plants.

All samples were frozen in liquid N_2 immediately after harvesting and stored at −70°C until analysis.

Arabidopsis *BFN1* cDNA Cloning and Overexpression in Plants

The Arabidopsis expressed sequence tag clone 62B4T7 (accession no. T41625) was identified as a *BFN1* cDNA clone on the basis of homology to other nucleases, as described in "Results." This clone, designated p1504, is a pZL1 plasmid (Life Technologies/Gibco-BRL) generated by in vivo excision from the PRL2 cDNA library (Newman et al., 1994). As described previously, the PRL2 library represents a combination of Arabidopsis tissues, organs, and growth conditions (Newman et al., 1994). The full-length cDNA sequence of clone p1504 was deposited into the EMBL, GenBank, and DDBJ databases with the accession number U90264. To overexpress *BFN1* in plants, a 1.1-kb *Sal*I fragment of *BFN1* cDNA containing the leader, full open reading frame (ORF), and 135 nt of the 3′-untranslated region (UTR) was inserted in the binary vector pBI121 from which the β-glucuronidase (GUS) ORF was removed. Correct orientation was identified by restriction analysis and confirmed by sequencing. In this construct, expression of BFN1 nuclease is under the control of the cauliflower mosaic virus 35S promoter, and terminated by the 3′ sequence of the nopaline synthase gene. The new plasmid was designated p1626.

Plasmid p1626 and unmodified pBI121 as a control were introduced into *Agrobacterium tumefaciens* GV3101 C58C1 Rif^r (pMP90) (Koncz and Schell, 1986) by electroporation using a Gene-Pulse apparatus (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. Arabidopsis plants were transformed with T-DNA by the vacuum infiltration method of Bechtold et al. (1993) with the modifications described in van Hoof and Green (1996), and at http://www.bch.msu.edu/pamgreen/vac.htm. T_1 seeds from these plants were plated on solid Arabidopsis growing medium (containing 0.8% [w/v] phytagar), which contained 50 μg mL^{-1} kanamycin for selection of transformants and 500 μg mL^{-1} vancomycin to limit the growth of *A. tumefaciens*. One kanamycin-resistant plant from seeds derived from each originally infiltrated plant was transferred to soil. T_1 plants were grown to maturity and T_2 seeds were collected. T_2 seeds were sterilized and plated on Arabidopsis growing medium with 50 μg mL^{-1} kanamycin. After 2 weeks, seedlings were harvested and analyzed for expression of the *BFN1* transgene by RNA blot analysis. Selected lines were also analyzed by RNase and DNase activity gels (Yen and Green, 1991).

Sequence Analysis

Database searches were performed with the BLAST program (Altschul et al., 1997) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequence alignment was created with the PileUp program of the Genetics Computer Group (Madison, WI). The phylogenetic tree was generated with PROTDIST and NEIGHBOR of the Phylogeny Inference Package, version 3.5c (J. Felsenstein, 1993, Department of Genetics, University of Washington, Seattle) from 2,000 bootstrapped data sets.

Isolation of Zinnia cDNAs Homologous to *BFN1*

All DNA probes used to screen phage plaques, or DNA and RNA gel blots, were labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983). Labeled probes were separated from unincorporated nucleotides using probe purification columns (NucTrap, Stratagene, La Jolla, CA).

A zinnia cDNA library from tracheary elements differentiated in vitro (Ye and Varner, 1993) was screened by plaque hybridization (Sambrook et al., 1989) using a ^{32}P-labeled *BFN1* probe. Positive plaques were purified, and the corresponding cDNAs were sequenced. *ZEN2* and *ZEN3* cDNA sequences were deposited into the EMBL, GenBank, and DDBJ databases as NucZe1 accession number U90265 and NucZe2 accession number U90266.

RNA Extraction and Northern Hybridization

Total RNA from Arabidopsis samples was extracted as previously described (Newman et al., 1993). Total RNA from zinnia leaves was extracted according to the method of Bugos et al. (1995). RNA (10 μg per lane) was separated by electrophoresis in 3% (w/v) formaldehyde/1.2% (w/v) agarose gels and blotted to nylon membrane (Nytran Plus, Schleicher & Schuell, Keene, NH). The RNA blots were hybridized as described in Taylor and Green (1991) using a ^{32}P-labeled *BFN1* probe. As a loading control, the same RNA blots were also hybridized with a ^{32}P-labeled cDNA probe for the Arabidopsis translation initiation factor *eIF4A* (Taylor et al., 1993). For this, blots were first stripped in 0.1% (w/v) SDS at 90°C to 95°C with two changes for 1 h at room temperature. Quantification of *BFN1* and *eIF4A* hybridization was achieved using a phosphor imager (Molecular Dynamics, Sunnyvale, CA) analysis.

DNA Extraction and Genomic DNA Gel-Blot Analysis

Genomic DNA was extracted from total aboveground tissue of mature Arabidopsis plants using the method of Dellaporta et al. (1983). After digestion with restriction endonucleases, 20 μg of DNA was separated by electrophoresis in 1.0% (w/v) agarose gels and blotted to nylon membrane (Nytran Plus, Schleicher & Schuell). Prehybridization and hybridization were as described previously (Taylor and Green, 1991).

The Arabidopsis recombinant inbred lines between ecotypes Columbia and Landsberg *erecta* were used to map the *BFN1* gene (Lister and Dean, 1993). DNA samples from 30 recombinant inbred lines were digested with *Hin*cII and analyzed by DNA gel blot as described above, using *BFN1* cDNA from p1504 as a probe. Results were scored with RFLP markers using MAPMAKER (Lander et al., 1987) at the Nottingham Arabidopsis Stock Center (http://nasc.nott.ac.uk/new_ri_map.html).

Protein Extraction and Detection of RNase and DNase Activities

Total protein was extracted from tissues basically as described previously (Yen and Green, 1991), except that the extraction buffer consisted of 250 mM $NaPO_4$, pH 7.4, 5 mM EDTA, 4 mM phenylmethylsulfonyl fluoride, 25 μg mL^{-1} leupeptin, and 25 μg mL^{-1} antipain. Approximately 200 mg of tissue was homogenized with 200 μL of extraction buffer at room temperature. Homogenates were clarified by centrifugation, and soluble protein was quantified by the Bradford method (Bradford, 1977).

RNase and DNase activities were assayed using activity gels basically as described previously (Yen and Green, 1991). After electrophoresis and before incubation, gels were washed in 100 mM Tris-HCl, pH 7.0, containing 2 μM $ZnCl_2$ for 50 min to restore the Zn^{2+} needed for nuclease activity. With this method, nuclease activity appears as a clear band on a dark background.

RESULTS

BFN1, an Arabidopsis Nuclease I cDNA Clone

Figure 1A shows the N-terminal sequences that were determined previously by peptide microsequencing of nuclease I enzymes from barley (Brown and Ho, 1986, 1987) and zinnia (Thelen and Northcote, 1989). Both proteins

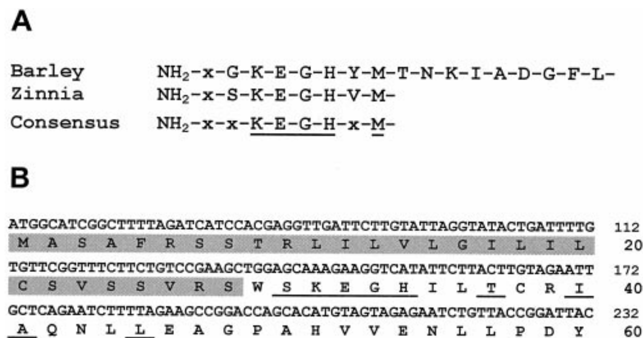

Figure 1. A, Known peptide sequences used to clone *BFN1*. N-terminal sequences determined via peptide microsequencing of barley and zinnia nuclease I proteins are shown (Brown and Ho, 1986, 1987; Thelen and Northcote, 1989). Sequences were aligned and a consensus sequence was determined. B, Nucleotide and peptide sequence corresponding to the first 60 amino acid residues of Arabidopsis *BFN1*. The putative signal peptide (28 amino acids) is shaded. Amino acid residues identical to barley and/or zinnia nucleases are underlined.

show high similarity at their N terminus, with a consensus sequence NH_2-Xaa-Xaa-Lys-Glu-Gly-His-Xaa-Met. However, the sequence determined from barley is longer and includes the residues Thr-Asn-Lys-Ile-Ala-Asp-Gly-Phe-Leu (Brown and Ho, 1987). To identify cDNAs from Arabidopsis that encoded nucleases, we searched the Arabidopsis database using this sequence information. We identified an Arabidopsis cDNA clone (62B4T7, accession no. T41625) from the expressed sequence tag project at Michigan State University (Newman et al., 1994) that contained the sequence Lys-Glu-Gly-His in the 5′ region of the ORF. The gene corresponding to this clone was designated *BFN1*. The cDNA was completely sequenced on both strands, and nucleotide and deduced peptide sequences were deposited in the database with accession number U90264.

The *BFN1* cDNA is 1,161 nucleotides long, with the longest ORF from position 53 to 967, encoding a protein of 34.9 kD. Figure 1B shows the first 60 amino acid residues and the corresponding nucleotide sequence of this cDNA. The hydrophobicity profile of the deduced protein identifies a highly hydrophobic potential signal sequence at the N terminus, with a predicted cleavage site (Nielsen et al., 1997) between residues 28 and 29 (highlighted in Fig. 1B). The predicted molecular mass for the mature protein after the cleavage of the signal is 31.9 kD. In addition, three putative *N*-glycosylation sites are present, at positions 122, 140, and 214 (94, 112, and 186 of the mature protein), based on the presence of the consensus sequence Asn-Xaa-Ser/Thr (Marshall, 1972). These results suggest that BFN1 is a glycosylated protein that is targeted to the secretory pathway.

ZEN2 and *ZEN3*, Two New Zinnia Nuclease I Clones

When we isolated the cDNA for the Arabidopsis *BFN1* gene, no other cDNAs corresponding to a nuclease I protein were available from plants. To help determine which structural features of fungal nuclease I were conserved in plants, we sought to identify a nuclease I cDNA from zinnia. Zinnia was chosen because biochemical studies had indicated that at least one such gene must be present (Thelen and Northcote, 1989). For this purpose, we screened a zinnia cDNA library (Ye and Varner, 1993) using *BFN1* cDNA as a probe. Two different cDNA clones, *ZEN2* and *ZEN3* (accession nos. U90265 and U90266, respectively), were isolated. Although these cDNAs do not correspond to the previously described zinnia nuclease I, they are clearly in the nuclease I family (see Fig. 2A). Recently, a cDNA corresponding to the previously described zinnia nuclease I has been reported and named *ZEN1* (Aoyagi et al., 1998). The three proteins are highly similar in the N-terminal half and in a region corresponding to the last 30 amino acid residues of ZEN2. The percentage of similarity is lower between residues 162 and 238 of the ZEN2 protein. Overall, ZEN2 and ZEN3 are 49% and 44.1% similar to ZEN1, respectively. In addition, the two new zinnia cDNA clones isolated have a signal peptide at the N terminus, as predicted by sequence analysis (data not shown).

Comparison of BFN1 with Related Nucleases

The BFN1 deduced amino acid sequence without the putative signal peptide was used to search for similar sequences in the database. Several proteins with significant similarity to BFN1 were identified. Figure 2A shows a pileup alignment of the deduced amino acid sequences of nucleases BFN1 from Arabidopsis, SA6 from the daylily *Hemerocallis* sp., ZEN1, ZEN2, and ZEN3 from zinnia, BEN1 from barley, nuclease S1 from *Aspergillus oryzae*, nuclease P1 from *Penicillium citrinum*, 3′nucleotidase/nuclease (3′NTNU) from *Leishmania donovani* (Debrabant et al., 1995), and N-terminal amino acid sequences of a nuclease from *Lentinula edodes* (le3) (Kobayashi et al., 1995). The putative signal peptides of BFN1, SA6, ZEN1, ZEN2, ZEN3, and BEN1, and the first 125 amino acid residues of the *L. donovani* protein are not shown.

Similarity among these proteins is dispersed throughout the entire sequence. BFN1 and SA6 are the most similar, sharing 74% of identical amino acids. Compared with the zinnia nucleases, BFN1 is more similar to ZEN1 than to ZEN2 or ZEN3, even though the latter two nucleases were identified using *BFN1* cDNA as a probe (70%, 52%, and 48% identity, respectively). Among plant nucleases, BFN1 is most distantly related to BEN1, with 46% identity. The PROTDIST/NEIGHBOR programs of the Phylogeny Inference Package were used to generate a gene genealogy with these nuclease I genes (Fig. 2B). According to the consensus tree generated from 2,000 bootstrapped data sets, plant nucleases can be divided in two groups. BFN1 and SA6, along with ZEN1, form one of them. The other group is formed by ZEN2 and ZEN3, together with the monocot BEN1. As expected, fungal nucleases fall in a separate cluster.

BFN1, ZEN2, and ZEN3 contain the major sequence features characteristic of the fungal nuclease I proteins, as

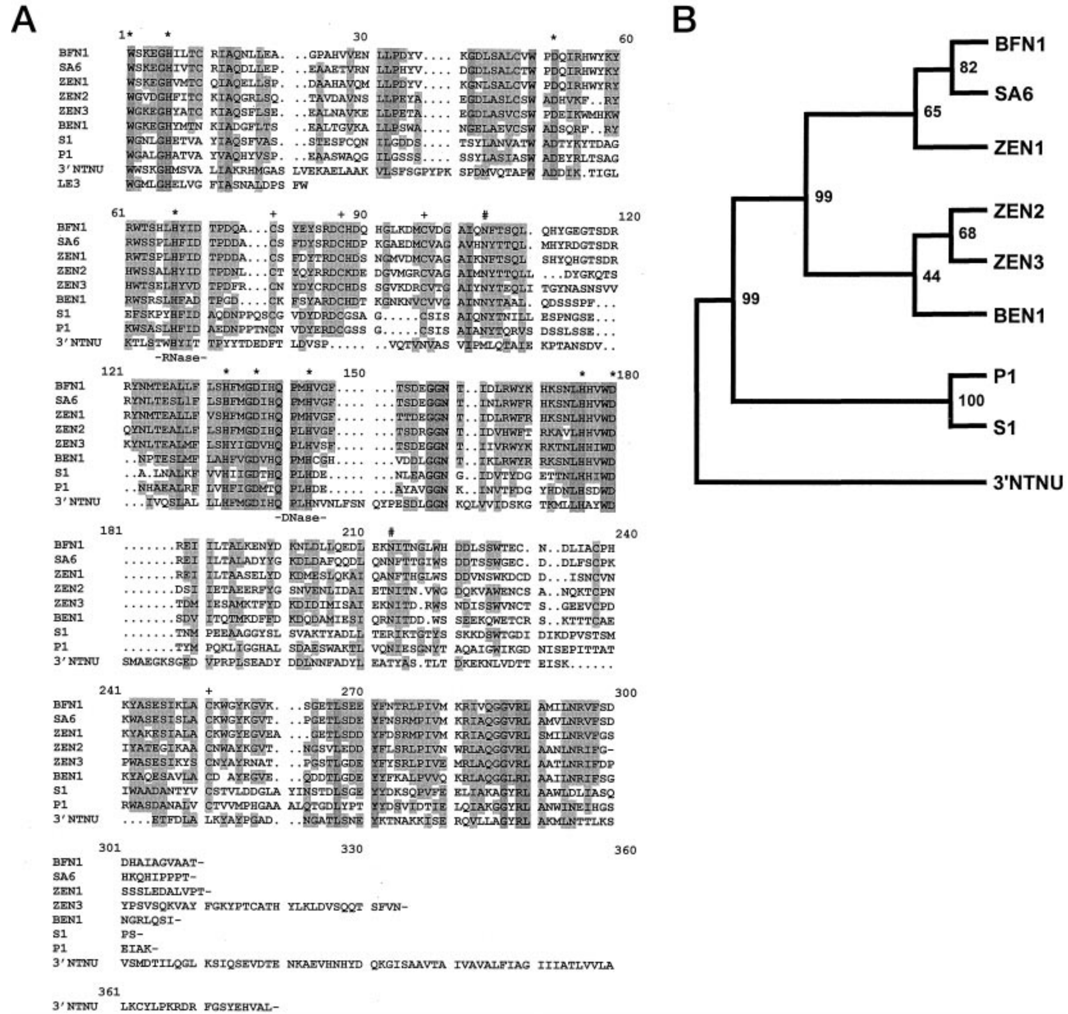

Figure 2. Alignment of the deduced amino acid sequences of nuclease I enzymes. A, Nuclease *BFN1* from Arabidopsis (accession no. U90264) and *ZEN2* and *ZEN3* (accession nos. U90265, and U90266, respectively) from zinnia, are compared with the deduced amino acid sequences from nuclease *SA6* from daylily (accession no. AF082031), nuclease *ZEN1* from zinnia (accession no. AB003131) (Aoyagi et al., 1998), nuclease *BEN1* from barley (accession no. D83178) (Aoyagi et al., 1998), nuclease *S1* from *A. oryzae* (accession no. P24021) (Iwamatsu et al., 1991), nuclease *P1* from *P. citrinum* (accession no. P24289) (Maekawa et al., 1991), 3′nucleotidase/nuclease (3′NTNU) from *L. donovani* (accession no. L35078) (Debrabant et al., 1995), and the N-terminal amino acid sequence of nuclease *LE3* from *L. edodes* (accession no. PC4030) (Kobayashi et al., 1995). Sequences were aligned using the PileUp program (Genetics Computer Group) with default settings. Dark gray shading, Residues that are identical in all sequences; light gray shading, residues that are functionally identical (A,S,T; N,Q; D,E; I,L,M,V; H,K,R; and F,W,Y) in at least five of the nine full-length sequences; periods, gaps introduced to produce the alignment; asterisks (*), residues involved in the binding of zinc atoms in nuclease P1; plus signs (+), residues involved in forming disulfide bonds in nuclease P1; number symbols (#), structurally important glycosylation sites in nuclease P1. Active sites for RNase and DNase activities in nucleases P1 and S1 are also indicated under the alignment. Not shown are putative signal peptides of BFN1, SA6, ZEN1, ZEN2, ZEN3, and BEN1, and the first 125 amino acid residues of the 3′NTNU. B, Gene genealogy of the nuclease I family. The consensus tree was generated with the PROTDIST and NIGHBOR programs of the Phylogeny Inference Package using 2,000 bootstrapped data sets. Numbers in branches are bootstrap values and indicate the percentage of trees in which the proteins in the branch cluster together. Designations for each nuclease are as in Figure 2A.

highlighted in Figure 2A. Two regions of high similarity include residues surrounding the active sites for both RNase and DNase activities in nucleases P1 and S1 (His residues at positions 60 and 132, respectively, in the *BFN1* mature protein) (Maekawa et al., 1991). Nucleases P1 and S1 contain four Cys residues that form two disulfide bonds that are responsible for the tertiary structure of the protein (Iwamatsu et al., 1991; Maekawa et al., 1991). These residues are conserved in the Arabidopsis and zinnia nucleases (+ symbol in Fig. 2A). Similarly, several Trp, His, and Asp residues, which have been implicated in the binding of zinc atoms in nucleases P1 and S1, are also conserved (asterisks in Fig. 2A) (Maekawa et al., 1991; Volbeda et al., 1991; Gite and Shankar, 1992; Gite et al., 1992). Finally, two of the Asn

residues glycosylated in the P1 nuclease (Maekawa et al., 1991) are conserved in BFN1 and zinnia nucleases (# symbol in Fig. 2A).

Genomic Organization of *BFN1*

As mentioned above, Arabidopsis contains several proteins with RNase and DNase activities characteristic of nuclease I enzymes. To help assess whether *BFN1* is a member of a gene family, we performed genomic DNA-blot analysis using the *BFN1* cDNA as a probe (Fig. 3). DNA digestion with *Bam*HI, *Eco*RI, and *Xba*I resulted in the identification of unique DNA fragments between 3 and 4.5 kb in size that hybridized with *BFN1* cDNA. Digestion with *Hin*cII revealed three DNA fragments. Analysis at low stringency did not reveal additional bands hybridizing with *BFN1* (data not shown). The *BFN1* cDNA sequence does not contain *Bam*HI, *Eco*RI, *Hin*cII, or *Xba*I sites. This indicates that *BFN1* is present in the genome of Arabidopsis as a small gene family or, more probably, as a single gene. The identification of three bands after digestion with *Hin*cII indicates that the *BFN1* gene contains at least one intron with two *Hin*cII sites in it. Nevertheless, there is a sequence in chromosome 4 of the Arabidopsis genome with the potential to encode one or two proteins with limited similarity to BFN1 (AL0022603, genes F18E5210 and F18E5220). Hybridization of *BFN1* cDNA to this gene is not apparent in Figure 3, based on fragment prediction from the available DNA sequence.

The *BFN1* gene was mapped using 30 Columbia/Landsberg recombinant inbred lines digested with *Hin*cII, which generates a RFLP. *BFN1* was found to be located close to the top of chromosome 1 (−9.86 cM), between markers mi443 (−9.31 cM) and ATTS0477 (−10.44 cM). While this manuscript was under review, a genomic sequence corresponding to *BFN1* was released (BAC T28P6 from position 65,333–67,938). Restriction analysis of the genomic sequence fully confirmed data in Figure 3.

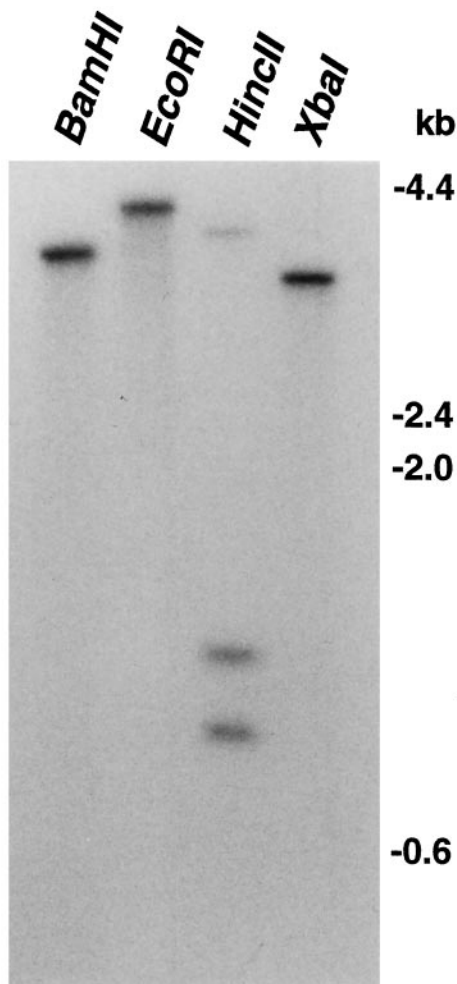

Figure 3. Genomic DNA gel-blot analysis of the *BFN1* gene. Genomic DNA (20 μg per lane) from Arabidopsis was digested with *Bam*HI, *Eco*RI, *Hin*cII, or *Xba*I, electrophoresed in agarose gels, blotted, and probe with ^{32}P-labeled *BFN1* cDNA. DNA marker sizes are indicated on the right (in kb).

BFN1 Has RNase and DNase Activity

To determine if the *BFN1* gene encodes a bifunctional nuclease, we overexpressed this cDNA in transgenic Arabidopsis plants and assayed nuclease activity by RNase and DNase activity gels (Yen and Green, 1991). The construct used, p1626 (Fig. 4A), was derived from the binary plasmid pBI121, with the GUS coding region replaced with a fragment of the *BFN1* cDNA, including the leader, ORF, and 135 nt of the 3′-UTR. Figure 4B shows mRNA levels for *BFN1* in transgenic Arabidopsis lines pBI121 and p1626. As expected, the *BFN1* mRNA transcript of 1.2 kb was present at high levels in line p1626. A weak hybridization signal of the same size, likely corresponding to the endogenous *BFN1* transcript, was also detected in line pBI121.

Protein extracts from transgenic pBI121 and p1626 plants were assayed for RNase and DNase activity in activity gels as described in "Materials and Methods" (Fig. 4C) (Yen and Green, 1991). A highly intense band of RNase activity of approximately 38 kD was detected in transgenic p1626 plants (Fig. 4C, top). At the same position, no activity was detected in transgenic pBI121 plants. Similarly, a unique band of DNase activity of approximately 38 kD present in transgenic p1626 plants did not appear in extracts from transgenic pBI121 plants (Fig. 4C, bottom). This result demonstrates that *BFN1* encodes a bifunctional nuclease from Arabidopsis capable of degrading RNA and DNA.

Overexpression of *BFN1* cDNA does not result in any obvious visible phenotype. When plants from transgenic Arabidopsis lines p1626 and pBI121 were grown to maturity in parallel under the same growth conditions, no evident differences in morphological characteristics were detected. In addition, neither timing nor the onset of senescence was altered (data not shown), indicating that overexpression of *BFN1* in Arabidopsis does not affect normal plant growth and development.

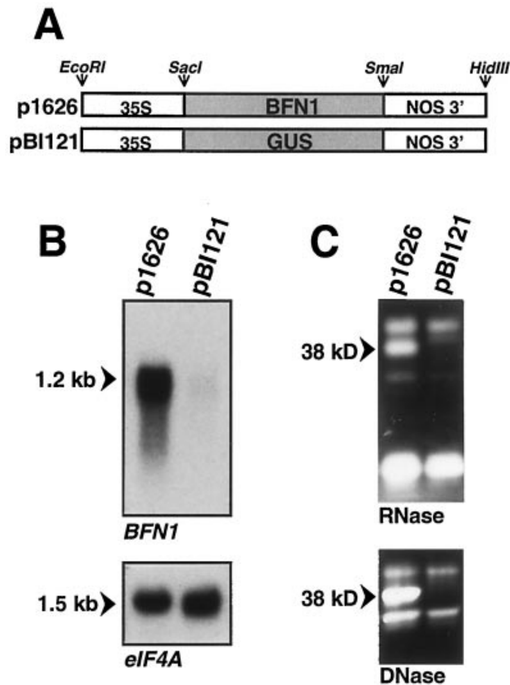

Figure 4. Overexpression of the *BFN1* cDNA in Arabidopsis. A, *BFN1* expression construct and GUS control construct in plant transformation vectors p1626 and pBI121, respectively. 35S, 35S promoter from cauliflower mosaic virus; NOS 3′, 3′-UTR from the nopaline synthase gene. B, RNA gel-blot analysis. Total RNA (10 μg) from T2 plants transformed with p1626 (*BFN1*) or pBI121 (control) was hybridized sequentially with ^{32}P-labeled *BFN1* (top) and Arabidopsis translation initiation factor *eIF4A* (bottom). Transcript size is indicated. C, RNase (top) and DNase (bottom) activity gels. Lanes contain protein extracts (100 μg) from T_2 plants transformed with p1626 or pBI121. Enzyme activities that degrade the RNA or single-stranded DNA substrate in the gel appear as clear bands on a dark background.

BFN1 Is Induced during Senescence But Not by Phosphate Starvation or Germination

Because nucleases have been implicated in several plant growth and developmental processes, including senescence, phosphate starvation, and germination (for review, see Bariola and Green, 1997), determining whether *BFN1* is regulated by any of these processes is of significant interest.

Figure 5 shows *BFN1* mRNA levels in leaves and stems at different developmental stages. A unique mRNA of about 1.2 kb was detected using *BFN1* cDNA as a probe. The abundance of the *BFN1* mRNA was extremely low in young or mature leaves (lanes YL and ML) but was induced to a high level during senescence (lane SL). Induction was also observed in senescing stems, albeit to a lower extent than in leaves (compare lanes YB, YS, MS, and SS). Relative to *eIF4A* mRNA, which was used as an internal standard (Taylor et al., 1993; Bariola et al., 1994), *BFN1* mRNA levels increased 10- and 2-fold during leaf and stem senescence, respectively.

Figure 6 shows RNase and DNase activity gels of Arabidopsis tissues during leaf and stem development and senescence. No RNase activity of the size of BFN1 was detected in young or mature leaves (Fig. 6A, lanes YL and ML) or during stem growth and development (Fig. 6A, lanes YB, YS, and MS). However, such an RNase activity did appear during leaf and stem senescence (Fig. 6A, lanes SL and SS). To correlate this activity with that of BFN1, protein extracts from transgenic p1626 plants overexpressing *BFN1* cDNA were electrophoresed in the same gel. BFN1 RNase activity co-migrated with the RNase activity induced during leaf and stem senescence. During stem and especially leaf senescence, additional RNases of approximately 40, 33, and 26 to 23 kD were strongly induced. This made it difficult to observe BFN1 RNase activity in senescent leaves (Fig. 6A). To circumvent this problem, less protein (40 μg compared with 100 μg per lane) from senescent leaves (lane SL) or from plants transgenic for p1626 or pBI121 was applied to the RNase activity gel (Fig. 6B). In this gel, BFN1 RNase activity could be resolved from the major 40-kD activity.

Results from DNase activity gels were very similar, as shown in Figure 6C. DNase activity of approximately 38 kD was not detected in young or mature leaves, but was induced during senescence. A less-intense DNase activity of the same size detected during bolting and stem growth and development was strongly induced during stem senescence. This DNase activity co-migrated with BFN1 DNase activity overexpressed in plants transgenic for p1626. It is not clear why levels of BFN1 mRNA and nuclease activities were not parallel in senescent leaves and stems. Senescing stems have more BFN1 activity but less mRNA than senescing leaves. Perhaps there is some impact of translational or post-translational control on BFN1 in one or both organs. Nevertheless, these data indicate that BFN1 RNase and DNase activities are induced during leaf and stem senescence in both the mRNA and nuclease activity levels in Arabidopsis.

In contrast to senescence, phosphate starvation did not lead to induction of *BFN1*. When seeds were germinated on complete medium with phosphate for 2 d and then trans-

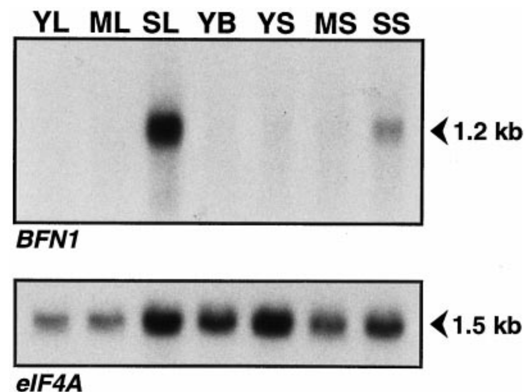

Figure 5. RNA gel-blot analysis of *BFN1* expression during leaf and stem growth and senescence. Lanes contain 10 μg of total RNA extracted from young leaves (YL), mature green leaves (ML), senescent leaves (SL), young bolts (stems 1–3 cm long) (YB), young stems (4–6 cm long) (YS), mature stems (MS), and senescent stems (SS) of Arabidopsis. The blot was hybridized sequentially with ^{32}P-labeled *BFN1* (top) and *eIF4A* (bottom).

ferred to fresh plates with or without phosphate for 14 d, *BFN1* mRNA was not detected in either sample (data not shown). Also, *BFN1* mRNA was not detected during germination or during early seedling growth (data not shown). The earliest time point analyzed was 2 d after plating. Under our growth conditions, the seed coat was broken and the radicle had already emerged at this stage. These results provide strong evidence that *BFN1* expression is highly specific for leaf and stem senescence and is not detected during phosphate starvation or germination.

We also determined the expression of *ZEN2* and *ZEN3* expression during zinnia leaf senescence. Interestingly, *ZEN3* and, to a lesser extent, *ZEN2* mRNA levels were also elevated during leaf senescence (data not shown). This indicates that induction during senescence is a common feature of a number of plant nuclease I enzymes.

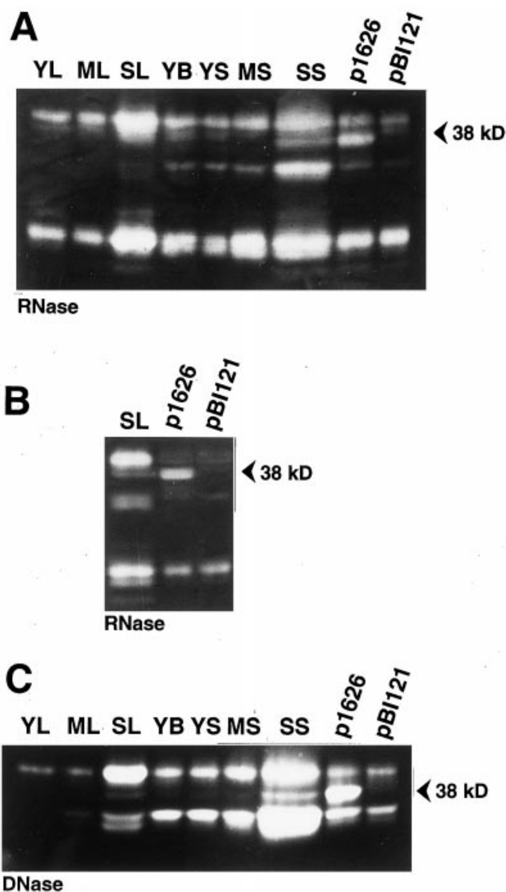

Figure 6. Examination of RNase and DNase activities during leaf and stem growth and senescence. Protein extracts from leaves and stems of wild-type plants at the same stages as in Figure 5 are compared. Protein extracts from transgenic T_2 plants transformed with the control plasmid pBI121 or the *BFN1* overexpression construct p1626 are also shown. A, RNase activity gel with 100 μg of total protein per lane. B, RNase activity gel with 40 μg of total protein per lane. C, DNase activity gel with 100 μg of total protein per lane. Abbreviations are as in Figure 5.

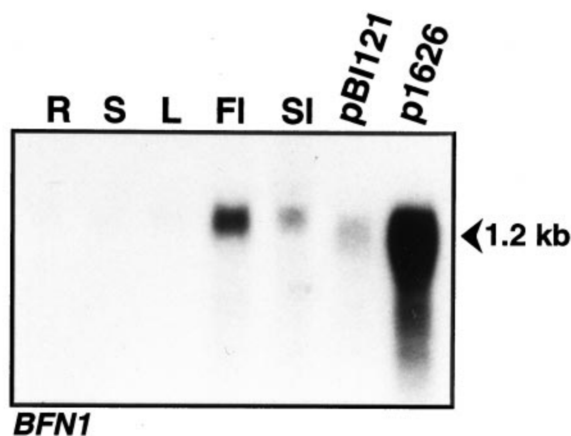

Figure 7. BFN1 expression in organs of Arabidopsis. Total RNA (10 μg) from roots (R), stems (S), leaves (L), flowers (Fl), and green siliques (Sl) of wild-type plants and from seedlings transgenic for pBI121 or p1626 were subjected to RNA gel-blot analysis. The blot was hybridized with ^{32}P-labeled *BFN1*.

Tissue Specificity of *BFN1* Expression

To detect the expression of *BFN1* in different organs and tissues of the adult Arabidopsis plant, RNA levels in mature roots, leaves, stems, flowers, and green developing siliques, as well as from p1626 and pBI121 transgenic Arabidopsis plants were compared. RNA blots were generated and hybridized to the *BFN1* probe. Extremely low levels of mRNA for *BFN1* were detected in roots, leaves, stems, and siliques (Fig. 7). In contrast, relatively high levels could be detected in flowers. This indicates that *BFN1* is not expressed or is expressed at very low levels during normal growth of vegetative tissues. The high expression in flowers is unlikely to be due exclusively to the presence of senescent tissues such as sepals and petals in the preparations, because *BFN1* was also highly expressed in young flowers (data not shown).

DISCUSSION

Bifunctional nucleases in the nuclease I class that degrade both RNA and DNA, have been known to exist in plants for many years, but their molecular analysis began only recently. *BFN1*, described here, is the first example to our knowledge of a senescence-associated gene encoding a nuclease I enzyme, and is also the first nuclease I cloned and characterized from Arabidopsis. The properties of this gene indicate that *BFN1* will be a useful tool in the study of senescence and the degradation of nucleic acid that occurs during this process.

Regulation and Implications of *BFN1* Expression

Senescence is an important and complex phase in the plant life cycle that is thought to contribute to fitness through the recycling of nutrients to actively growing regions (Buchanan-Wollaston, 1997). Senescence is also a highly regulated process during which many hydrolytic enzymes are activated in order to remobilize cell compo-

nents. Among these hydrolytic enzymes, those with DNase and/or RNase activity are important for the degradation of nucleic acid.

Our observations indicate that BFN1 helps fulfill this role in Arabidopsis. We observed a 10-fold increase in *BFN1* mRNA levels during leaf senescence and a 2-fold induction in senescing stems. Concomitant with mRNA accumulation, an induction of BFN1 activity was observed in the corresponding senescing tissue. In Arabidopsis, senescence of leaves and stems occurs when the plant is flowering and producing fruits, a time at which the released nutrients likely contribute to completion of fruit development and seed maturation (Nooden, 1988). Thus, it seems likely that BFN1 participates in this process.

A number of other plant nucleases characterized at the protein level have been implicated in senescence. Wheat produces several single-strand-specific nucleases during leaf senescence (Blank and McKeon, 1989). Induction of these nuclease activities can be detected by RNase activity gels at the onset of senescence, just when chlorophyll loss is initiated (Blank and McKeon, 1989). In addition, at least three RNase activities of 20 to 27 kD are induced during wheat senescence (Blank and McKeon, 1991). In Arabidopsis, *RNS2* and *RNS3*, which encode S-like RNases (Taylor and Green, 1991), increase in abundance during senescence (Taylor et al., 1993; Bariola et al., 1994). Another S-like RNase induced during senescence is RNase LX of tomato (Lers et al., 1998). All of these S-like RNases are expressed in non-senescing tissues and therefore their induction does not appear to be senescence specific (Taylor et al., 1993; Bariola et al., 1994; Lers et al., 1998).

Similar to *BFN1*, the two zinnia nuclease I genes described in this report, *ZEN2* and *ZEN3*, are also induced during senescence at the RNA level. It is not known whether *ZEN1*, the xylogenesis-associated (Thelen and Northcote, 1989) nuclease I gene isolated previously (Aoyagi et al., 1998), is induced during senescence. According to the notation listed with its GenBank entry, a daylily nuclease I gene, *SA6* (accession no. AF082031), may exhibit induction during petal senescence, but no characterization of this gene or its expression has been published. Nevertheless, at least a subset of nuclease I enzymes are senescence associated if not senescence specific.

In addition to senescence, there are several other processes or conditions, including germination, xylogenesis, and phosphate starvation, during which it would be advantageous for the plant to induce nucleic-acid-degrading activities. The bifunctional nucleases, RNases, and DNases, presumably work together with phosphatases and phosphodiesterases to release phosphate from DNA and RNA for remobilization (Glund and Goldstein, 1993). Some enzymes or their genes are known to be induced by more than one of these conditions, such as *RNS2* of Arabidopsis, which responds to both senescence and phosphate starvation.

In contrast, our data are consistent with a senescence-specific role for *BFN1* in vegetative tissues. *BFN1* mRNA was not detected in seedlings grown in phosphate-depleted medium. This is consistent with a previous study that did not detect a RNases of 38 kD in activity gels following phosphate starvation (Bariola et al., 1994). Our data further indicate that, unlike a barley nuclease I (Brown and Ho, 1986, 1987), *BFN1* expression is not induced during germination. We did observe *BFN1* expression in flowers that cannot solely be explained by the presence of senescing tissues in those preparations. Still other explanations are possible, especially in light of RNS1, which is barely induced by senescence in leaves but markedly expressed in flowers. Another role suggested for nucleic-acid-degrading activities in flowers is to protect that organ, more specifically the style, from invasion by pathogens (Bariola et al., 1994).

Insight from *BFN1* Structure

Genomic DNA gel-blot and mapping analysis indicated that *BFN1* is represented in the genome of Arabidopsis as a single gene on chromosome I. Nevertheless, there are at least two other putative proteins in the database with some similarity to BFN1. They correspond to two contiguous predicted genes on chromosome 4 (accession no. AL0022603, genes F18E5210 and F18E5220). Gene F18E5210 is 29.4% identical to BFN1, and has two repeats of the consensus sequence around the His-134 residue implicated in DNase activity in the porcine pancreatic DNase I (Paudel and Liao, 1986). Gene F18E5220 is 24.2% identical to BFN1 and, in addition to the DNase consensus sequence repeat, includes a region with similarity to His-119 and surrounding sequences in the active site of pancreatic RNase A (Blackburn and Moore, 1982; Cuchillo et al., 1997). This information indicates that other nuclease-I-type enzymes may be present in Arabidopsis.

Sequences encoding nuclease I enzymes have been conserved throughout evolution. The sequence of Arabidopsis nuclease BFN1, as well as nucleases ZEN2 and ZEN3 from zinnia, are highly similar to the two other plant nucleases that have been cloned (Aoyagi et al., 1998) and to other nucleases from fungi described previously (Iwamatsu et al., 1991; Maekawa et al., 1991). Even though the plant and fungal nucleases differ in length, they contain several conserved regions, including the catalytic sites for the porcine pancreatic DNase I (Paudel and Liao, 1986) and pancreatic RNase A (Blackburn and Moore, 1982; Cuchillo et al., 1997) mentioned above. In addition, all nuclease I enzymes reported to date enter the secretory pathway and are known to be extracellular. The deduced amino acid sequence of the BFN1, ZEN2, and ZEN3 proteins start with a typical signal peptide, indicating that these proteins also enter the secretory pathway. They also lack a KDEL-like sequence for retention in the endoplasmic reticulum or an obvious C- or N-terminal vacuolar targeting signal (Bar-Peled et al., 1996), so they too may be extracellular. It is not yet clear whether the amino acid sequences of senescence-associated nuclease I enzymes have any distinct features, but this issue should be resolved once more genes are characterized.

The deduced BFN1 protein sequence predicts a mature protein of 32 kD after cleavage of the signal peptide. However, expression of the *BFN1* cDNA in Arabidopsis results in the production of a unique 38-kD protein with both

RNase and DNase activity. It is likely that the size discrepancy occurs because BFN1 is glycosylated at Asn residues during its transit through the secretory pathway. Predicted glycosylated residues based on the consensus sequence Asn-Xaa-Ser/Thr, where Xaa indicates any amino acid residue (Marshall, 1972), are located at positions 94, 112, and 186 of the mature BFN1 protein. At least two of these align with glycosylated Asn residues in nuclease P1 (Maekawa et al., 1991). Another Asn residue in the *BFN1* sequence that is not predicted to be an *N*-glycosylation site (position 142) is conserved in nuclease P1 protein as an Asn residue that is *N*-glycosylated. Thus, it is highly likely that BFN1 is modified post-translationally by the addition of two or three carbohydrate moieties. A number of the other nucleases, including nuclease S1 (Iwamatsu et al., 1991) and nucleases from mung bean (Laskowski, 1980), pea seed (Naseem et al., 1987), barley seed (Brown and Ho, 1986, 1987), rye germ ribosome (Siwecka et al., 1989), and spinach (Strickland et al., 1991), are glycoproteins, with carbohydrate contents that account for 17% to 29% of the final relative M_r (for review, see Gite and Shankar, 1995).

Now that the *BFN1* gene has been isolated and its activity identified, the role of its potential glycosylation sites and its location within the secretory system can be investigated. Further, the strong and specific response of *BFN1* to senescence indicates that it should be an excellent tool with which to study the mechanisms of senescence induction, as well as the role of the enzyme in senescence using reverse genetic approaches and other methodologies in Arabidopsis.

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