FEBS Letters 429 (1998) 134–138 FEBS 20328

BEN1 and *ZEN1* cDNAs encoding S1-type DNases that are associated with programmed cell death in plants

Shigemi Aoyagi[a,b], Munetaka Sugiyama[a,*], Hiroo Fukuda[c]

[a] *Botanical Gardens, Graduate School of Science, The University of Tokyo, Hakusan 3-7-1, Bunkyo-ku, Tokyo 112-0001, Japan*
[b] *Biological Institute, Graduate School of Science, Tohoku University, Aramaki Aza Aoba, Aoba-ku, Sendai 980-8578, Japan*
[c] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan*

Received 27 March 1998; revised version received 5 May 1998

Abstract We describe the isolation and characterization of cDNAs encoding two DNases that may be involved in the programmed death of plant cells: a 35-kDa nuclease of barley, which had been reported to be secreted from the aleurone layer into the endosperm during germination and may be responsible for the digestion of nuclear DNA in the course of degradation (cell death) of endosperm, and a 43-kDa nuclease of zinnia, which appears transiently in association with differentiation to tracheary elements and is likely to participate in the autolysis at the final step of the differentiation. Genes for these nucleases of barley and zinnia were designated *BEN1* and *ZEN1*, respectively. The amino acid sequence of BEN1 protein deduced from the nucleotide sequence of *BEN1* cDNA consisted of 288 residues with a putative signal sequence of 23 residues. RNA gel blot analysis revealed that *BEN1* mRNA increased in the embryo-less half seeds of barley in response to the application of gibberellic acid. The deduced amino acid sequence of ZEN1 protein consisted of 303 residues with a putative signal sequence of 25 residues. Temporal accumulation of *ZEN1* mRNA was detected during transdifferentiation of zinnia mesophyll cells into tracheary elements. Significant similarities were found among the amino acid sequences of BEN1, ZEN1, nuclease S1 from *Aspergillus oryzae*, and two other S1-type nucleases.

Key words: DNase; Programmed cell death; Aleurone layer; Tracheary element; *Hordeum vulgare*; *Zinnia elegans*

1. Introduction

Programmed cell death is an indispensable process in the normal development and growth of plants as well as animals. In various aspects of the life cycle of plants, cells die under strict control. As examples, cell death is observed in association with endosperm degradation during germination, tracheary element differentiation, aerenchyma formation, leaf senescence, tapetum break-down during pollen development, and hypersensitive reaction against microbial attacks.

Programmed cell death includes active degradation of nucleus as a very important step; this is believed to require a specific kind of DNase. In plants, two enzymes have been purified and characterized as promising candidates for such a DNase. One is a 35-kDa nuclease of barley (*Hordeum vulgare*) which is secreted from the aleurone layer into the endosperm in response to gibberellic acid during germination [1], and the other is a 43-kDa nuclease of zinnia (*Zinnia elegans*), which appears transiently at the time of autolysis during transdifferentiation of mesophyll cells into tracheary elements [2]. Both enzymes have endonucleolytic activity on dsDNA, ssDNA, and RNA in the presence of Zn^{2+} ion. The partial amino acid sequences at their N-terminal regions were found to be rather similar. These findings suggest that nucleases of the same kind may participate in programmed cell death associated with different aspects of plant development. Thus, analysis of these two nucleases at the molecular level could clarify common and variable features among nucleases involved in programmed cell death in plants.

In the present article, we report the first isolation of cDNAs for these two plant DNases, the barley 35-kDa nuclease and the zinnia 43-kDa nuclease, and indicate significant similarities in amino acid sequences among these two plant nucleases, nuclease S1 from *Aspergillus oryzae* and two other S1-type nucleases. The possible involvement of S1-type DNases in developmentally controlled death of plant cells is discussed.

2. Materials and methods

2.1. Plant materials

Seeds of barley (*H. vulgare* L.) cultivars Himalaya and Hoshimasari were kindly donated by Prof. Kazuyoshi Takeda, Okayama University, and by Miss Tomoko Sakura, Tokyo University of Agriculture, respectively. Seeds of zinnia (*Z. elegans* L.) cultivar Canary Bird were purchased from Takii Shubyo, Kyoto, Japan.

2.2. Molecular cloning of cDNA fragments for the 35-kDa nuclease of barley

Embryo-less half seeds of barley cultivar Himalaya were surface-sterilized and pre-incubated on the moistened sea sand for 3 days at approximately 25°C in the dark. After pre-incubation, aleurone layers were isolated and cultured in 10 mM sodium acetate buffer (pH 5.3) that contained 2 mM $CaCl_2$ and 1 μM GA_3 on a rotary shaker at 27°C in the dark [3]. Aleurone layers cultured for 48, 60, and 72 h were frozen in liquid nitrogen and combined. Poly(A)$^+$ RNA was prepared from this sample using FastTrack mRNA Isolation Kit Ver. 3.5 (Invitrogen).

3′-RACE (rapid amplification of cDNA end) and 5′-RACE were carried out according to the method of Frohman et al. [4] with modifications. For 3′-RACE, first strand cDNA was reverse-transcribed from poly(A)$^+$ RNA with dT_{17}-LL-BamA as a primer (see Table 1). A degenerate primer specific for the 35-kDa nuclease of barley, HV.N-2 was designed for the partial amino acid sequence at the N-terminal region of this nuclease [1]. Putative cDNA fragments for the 35-kDa nuclease were amplified from first strand cDNA by PCR with primers, HV.N-2 and LL-BamA. After size-fractionation by agarose gel electrophoresis, DNA of 1.0 kbp in length was cloned into pBluescript KS$^+$ (Stratagene).

*Corresponding author. Fax: +81 (3) 3814 0139.
E-mail: sugim@hongo.ecc.u-tokyo.ac.jp

Abbreviations: PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; RT-PCR, reverse transcription-polymerase chain reaction

Data depositions: The nucleotide sequences reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers of D83178 (*BEN1*) and AB003131 (*ZEN1*).

PII S0014-5793(98)00563-8

Table 1
Primers used in this study

Name	Sequence
LL-BamA	5′-GATTAGGATCCACTAATATC-3′
dT_{17}-LL-BamA	5′-GATTAGGATCCACTAATATCTTTTTTTTTTTTTTTT-3′
HV.N-2	5′-CAYTAYATGACNAAYAARAT-3′
BN-R-223	5′-ACCACGCACACGTCCTTGTT-3′
BN-R-311	5′-AAGTGCGCCAGGAACATCA-3′
NucR	5′-TCCCAIACRTGRTGIARRTT-3′
ZE.N	5′-AARGARGGNCAYGTNATG-3′
ZN-F-339	5′-GCTTTGCTATTTGTATCACA-3′
ZN-R-199	5′-CTTGTATAATCAAAGGAGCA-3′

N = A, G, C, T; R = A, G; Y = C, T.

On the basis of the nucleotide sequences of putative cDNA fragments for 35-kDa nuclease, which were obtained through 3′-RACE, two backward primers BN-R-311 and BN-R-223 were designed for subsequent 5′-RACE. First strand cDNA was synthesized from poly-$(A)^+$ RNA with BN-R-311, and subjected to dA-tailing reaction. PCR amplification from dA-tailed cDNA was performed with three primers, dT_{17}-LL-BamA, LL-BamA, and BN-R-223, to yield DNA of 0.41 kbp. This PCR product was cloned into pBluescript KS^+.

2.3. Molecular cloning of cDNA fragments for the 43-kDa nuclease of zinnia

Mesophyll cells of zinnia were isolated from surface-sterilized, first true leaves of 14-day-old seedlings and cultured in a liquid medium that contained 0.2 mg/l N^6-benzyladenine and 0.1 mg/l 1-naphthaleneacetic acid (D medium) for the induction of transdifferentiation into tracheary elements, according to Fukuda and Komamine [5] with a slight modification. After 42, 48, and 54 h in culture, cells that underwent transdifferentiation were harvested. From a mixed sample of these cells, poly$(A)^+$ RNA was prepared by use of FastTrack mRNA Isolation Kit Ver. 3.5.

Putative cDNA fragments for the 43-kDa nuclease of zinnia were amplified from poly$(A)^+$ RNA by RT-PCR with ZE.N and NucR primers, which were designed for the partial amino acid sequence of the 43-kDa nuclease [2] and for the conserved sequence common to the amino acid sequences of 35-kDa nuclease of barley (deduced from *BEN1*) and nuclease S1 of *A. oryzae* [6], respectively. PCR products were electrophoresed in an agarose gel. DNA of 0.46 kbp in length was withdrawn from the gel and cloned into a BSII TSK vector [7]. On the basis of the nucleotide sequence of this DNA, two more primers, ZN-F-339 and ZN-R-199, were designed. For 3′-RACE, first strand cDNA was reverse-transcribed from poly$(A)^+$ RNA with dT_{17}-LL-BamA as a primer and subjected to PCR amplification with LL-BamA and ZN-F-339 primers. For 5′-RACE, first strand cDNA was synthesized with NucR as a primer and tailed by dA. PCR was performed with this cDNA as a template and with dT_{17}-LL-BamA, LL-BamA, and ZN-R-199 as primers. Putative cDNA end fragments for zinnia nuclease obtained through 3′-RACE and 5′-RACE were cloned into pBluescript KS^+.

2.4. Sequence analysis of cDNA fragments

Nucleotide sequences of cloned cDNA fragments were determined using an ABI 373A DNA Sequencer (Applied Biosystems) or A.L.F. DNA Sequencer (Pharmacia Biotech). For the sequence reaction, Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems), Dye Terminator Cycle Sequencing Kit (Applied Biosystems), and AutoRead Sequencing Kit (Pharmacia Biotech) were employed. Computer analysis of nucleotide and deduced amino acid sequences was performed with the GENETYX-MAC Ver. 7.0 computer software (Genetyx). DDBJ (DNA Data Bank of Japan) was utilized for homology search.

2.5. RNA gel blot analysis

Total RNA samples of barley were prepared from embryo-less half seeds of cultivar Hoshimasari, which were cultured for various times in 10 mM sodium acetate buffer (pH 5.3) that contained 2 mM $CaCl_2$ with or without 1 μM GA_3 after pre-incubation for 3 days on the moistened sea sand, according to the procedure described by Palmiter [8]. Total RNA samples from mesophyll cells of zinnia, which were cultured for various times in D medium for the induction of transdifferentiation into tracheary elements or in Cp medium containing 0.001 mg/l benzyladenine and 0.1 mg/l naphthaleneacetic acid as a control that did not induce transdifferentiation, were kindly contributed by Dr. Taku Demura, the University of Tokyo. After separation by agarose gel electrophoresis, total RNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) and subjected to hybridization with digoxigenin-labelled RNA probes that were prepared from cDNA clones using DIG RNA Labeling Kit (Boehringer Mannheim). Hybridization signals were visualized by the immunological method with anti-digoxigenin-AP Fab fragments (Boehringer Mannheim) according to the manufacturer's protocol.

3. Results and discussion

3.1. Isolation and characterization of cDNA for the 35-kDa nuclease of barley

Brown and Ho purified and characterized a 35-kDa nuclease (DNase/RNase) of barley that was secreted from the aleurone layer in response to gibberellic acid [1]. Exploiting the procedures of 3′-RACE followed by 5′-RACE, putative cDNA fragments for this nuclease were obtained from aleurone layers of barley incubated in the presence of GA_3. Analyses of these cDNA fragments led to the determination of a contiguous nucleotide sequence that covers an open reading frame for 288 amino acid residues (Fig. 1). In the deduced amino acid sequence, a region nearly identical to the partial sequence reported for the barley 35-kDa nuclease [1] was found. Accordingly, this cDNA was concluded to encode

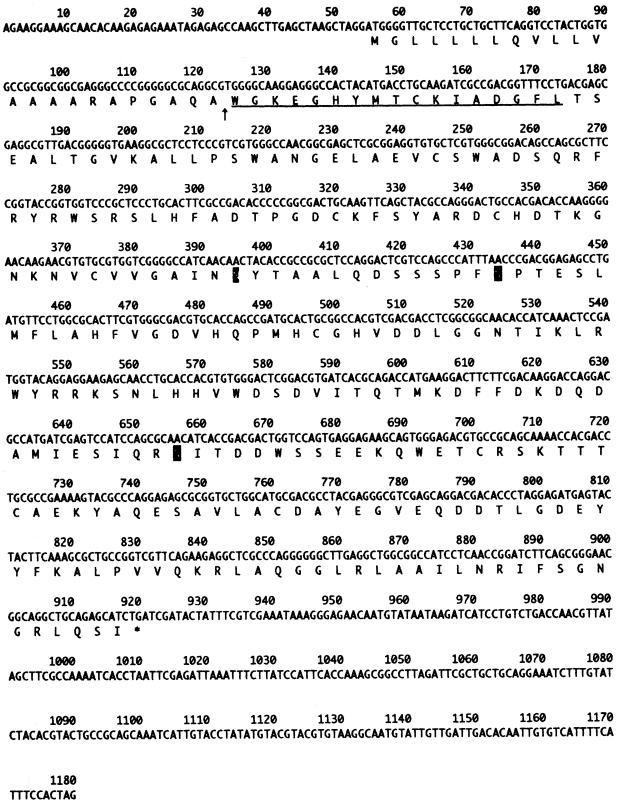

Fig. 1. Nucleotide and deduced amino acid sequences of *BEN1*. The amino acid sequence corresponding to the partial sequence reported for the 35-kDa nuclease of barley [1] is underlined. The putative cleavage site is indicated by an arrow. The boxed Asn residues (white letters on black background) are potential sites of N-glycosylation.

the 35-kDa nuclease of barley that had been characterized by Brown and Ho. The gene represented by this cDNA is designated *BEN1* (barley endonuclease 1). This is the first plant DNase whose cDNA has been cloned and sequenced.

Comparison between the amino acid sequence of the translation product of *BEN1* (BEN1 protein) and the partial sequence of the purified 35-kDa nuclease revealed that the N terminus of the mature protein is located at amino acid 24 in the sequence of BEN1 protein. This indicates that BEN1 is cleaved between amino acids 23 and 24 prior to maturation, which is consistent with (−3, −1)-rule [9], because amino acids 21 and 23 are small, nonpolar amino acids, Ala. The N-terminal region of BEN1 upstream of the putative cleavage site is highly hydrophobic. This region is likely to act as a signal sequence for secretion, in the light of our knowledge that the 35-kDa nuclease is secreted from aleurone cells. BEN1 protein contains three Asn residues that are potentially N-glycosylated. This is in agreement with the 35-kDa nuclease being a glycoprotein [1].

Accumulation of *BEN1* mRNA during incubation of embryo-less half seeds of barley was examined by RNA gel blot analysis and shown to be induced by treatment with GA_3 (Fig. 2). Taking these results together with the studies by Brown and Ho on the 35-kDa nuclease [1,10], we can reasonably postulate the following hypothesis with respect to changes in *BEN1* and the 35-kDa nuclease during germination of barley seeds: de novo expression of *BEN1* induced by gibberellic acid leads to an increase in its translation product BEN1 in the aleurone layer. BEN1 is transported to the endoplasmic reticulum with the aid of its N-terminal signal sequence, cleaved to lose this sequence, glycosylated in the Golgi apparatus to become the mature form of the 35-kDa nuclease, and finally secreted into the endosperm. Since the 35-kDa nuclease was the only DNase detected among the nucleases secreted from the aleurone layer of barley [10], this nuclease is presumed to play a central role in the digestion of nuclear DNA during endosperm degradation.

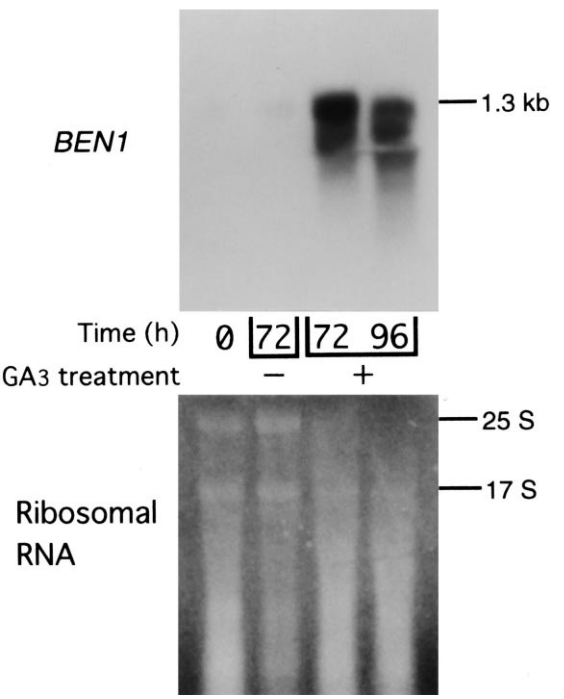

Fig. 2. RNA gel blot analysis on *BEN1* expression during culture of embryo-less half seeds of barley. Total RNA was prepared from embryo-less half seeds of barley that were cultured for 0, 72, or 96 h in the presence (+) or absence (−) of GA_3. Twenty micrograms of RNA was electrophoresed in a 1.2% agarose gel and blotted onto a nylon membrane. Hybridization was performed with a fragment (nucleotide 505–1180 in Fig. 1) of *BEN1* cDNA as a probe. The ethidium bromide-stained gel is shown below.

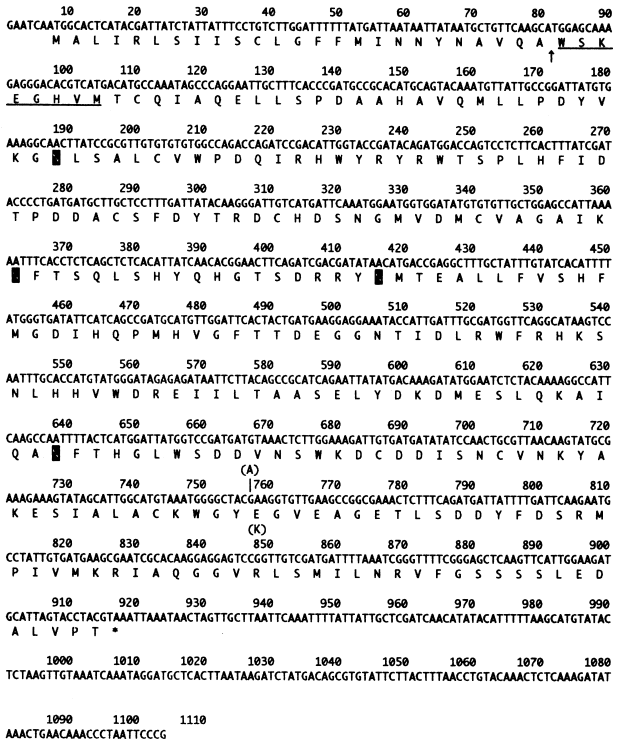

Fig. 3. Nucleotide and deduced amino acid sequences of *ZEN1*. The amino acid sequence corresponding to the partial sequence reported for the 43-kDa nuclease of zinnia [2] is underlined. The putative cleavage site is indicated by an arrow. The boxed Asn residues (white letters on black background) are potential sites of N-glycosylation. Within the first 80 nucleotides, some differences probably due to polymorphism were found among 5′-RACE clones. The sequence of one of such clones is presented here as a representative.

3.2. Isolation and characterization of cDNA for the 43-kDa nuclease of zinnia

Thelen and Northcote purified and characterized a 43-kDa nuclease (DNase/RNase) of zinnia whose activity increased in cultured mesophyll cells in close parallel with autolysis during their transdifferentiation into tracheary elements [2]. Putative cDNA fragments for this nuclease were isolated from transdifferentiating cells of zinnia by RT-PCR, 3′-RACE, and 5′-RACE. The nucleotide sequence of a cDNA contigue of 1104 bp was determined, in which an open reading frame for 303 amino acid residues was found (Fig. 3). The deduced amino acid sequence contained a region identical to the partial sequence reported for the zinnia 43-kDa nuclease [2]. This strongly suggests that the present cDNA encodes the 43-kDa nuclease of zinnia described by Thelen and Northcote. The gene represented by this cDNA is designated *ZEN1* (zinnia endonuclease 1).

ZEN1 protein possesses a putative signal sequence of 25 amino acid residues at the N terminus as does BEN1. Since the 43-kDa nuclease was shown to be present not in the cul-

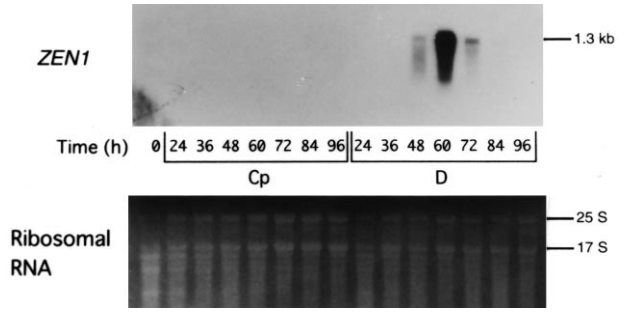

Fig. 4. RNA gel blot analysis on *ZEN1* expression during transdifferentiation of mesophyll cells of zinnia into tracheary elements. Total RNA samples from mesophyll cells of zinnia cultured for indicated times in Cp or D medium were electrophoresed in a 1.2% agarose gel (10 μg/lane) and blotted onto a nylon membrane. Hybridization was performed with a fragment (nucleotide 88–560 in Fig. 3) of *ZEN1* cDNA as a probe. The ethidium bromide-stained gel is shown below.

ture medium but in the cells [2], the putative signal sequence of ZEN1 does not seem to guide this protein to secretion. It is more likely that ZEN1 is transported to the vacuole. Assuming that ZEN1 protein accumulates in the vacuole in cells differentiating to tracheary elements as the mature and active form, this can reasonably explain the observation by electron microscopy that break-down of the tonoplast precedes degradation of the nucleus during autolysis in association with the differentiation [11]. The ZEN1 protein contains four Asn residues that can be N-glycosylation sites, which is consistent with the fact that the 43-kDa nuclease is a glycoprotein [2].

RNA gel blot analysis revealed a close correlation between the level of *ZEN1* mRNA and transdifferentiation of mesophyll cells into tracheary elements (Fig. 4). In the differentiation-inducing culture, transient accumulation of *ZEN1* mRNA was observed at the 48–60th hour, when morphological changes linked to the differentiation (secondary cell wall thickening) became obvious; no such accumulation of *ZEN1* mRNA was found in the control culture, in which few cells differentiated into tracheary elements. This pattern of changes in the level of *ZEN1* mRNA was very similar to that reported for the activity of the 43-kDa nuclease [2]. Thus, it can be inferred from these results that the regulation of gene expression of *ZEN1* is responsible for the increase of the 43-kDa nuclease specific for the differentiation to tracheary elements.

During transdifferentiation of zinnia cells into tracheary elements, nuclei disappear approximately 6 h after visible thickening of secondary cell walls (data not shown). Since the marked increase in the level of *ZEN1* mRNA was almost simultaneous with the onset of secondary wall thickening in the cell population, the accumulation of *ZEN1* mRNA is considered to precede nuclear break-down by at least several hours in individual differentiating cells.

3.3. Homology analysis on the amino acid sequences of BEN1 and ZEN1 proteins

As a result of a homology search, significant similarity was detected among the amino acid sequences of BEN1, ZEN1, nuclease S1 from *A. oryzae*, nuclease P1 from *Penicillium citrinum*, and the 3′-nucleotidase/nuclease of *Leishmania donovani* (Fig. 5). The latter three enzymes have been well characterized. All of these enzymes are glycoproteins with molecular masses of approximately 40 kDa that catalyze hydrolysis of RNA, DNA, and the 3′-phosphoester linkage of ribonucleoside monophosphates [12–14]. These enzymatic properties are in principle shared by the 35-kDa nuclease of barley [1] and the 43-kDa nuclease of zinnia [2] which are predicted to be produced from *BEN1* and *ZEN1* genes, respectively. Accordingly, these five nucleases that are similar in their primary structures can be classified enzymologically into the same group, S1-type nuclease.

By searching plant EST and BAC end sequence databases, four clones were found to show high sequence similarity with *BEN1* or *ZEN1* cDNA: A rice cDNA clone 97GS1100 (accession number AA754180), a watermelon cDNA clone EST00149 (AA660099), an arabidopsis cDNA clone 62B4T7 (T41625), and an arabidopsis genomic clone T18D7 (B26739). This suggests that genes encoding S1-type nucleases may be present generally in diverse plant species. Since the partial sequences deposited for the arabidopsis clones 62B4T7 and T18D7 do not overlap one another, it is unclear whether they correspond to the same gene or different genes.

An important feature common to S1-type nucleases is the requirement for divalent cation Zn^{2+} in the reaction. Analysis of the crystal structure of P1 nuclease revealed that its binding

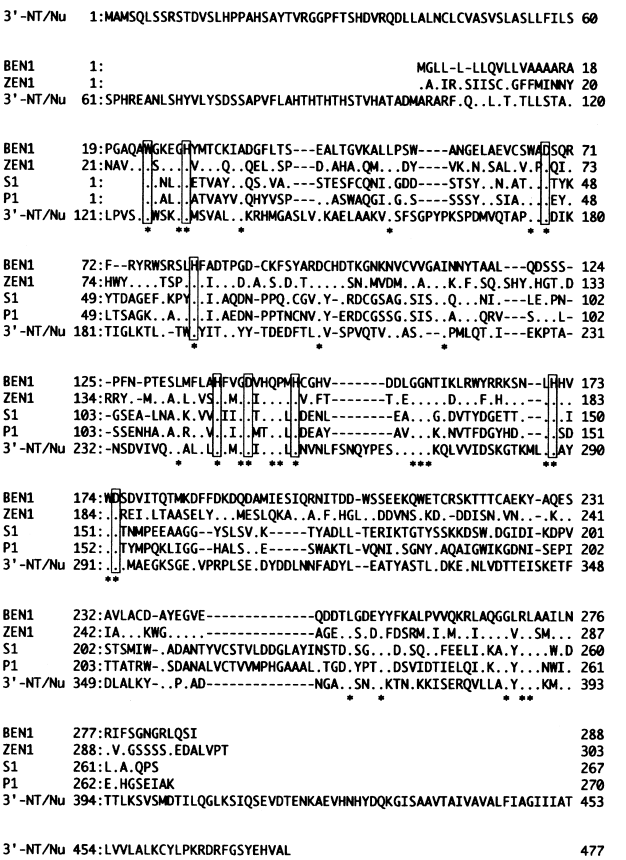

Fig. 5. Alignment of amino acid sequences of S1-type nucleases, nuclease S1 from *Aspergillus oryzae* [6], nuclease P1 from *Penicillium citrinum* [21], and 3′-nucleotidase/nuclease of *Leishmania donovani* [22], with deduced sequences of BEN1 and ZEN1 proteins. Hyphens represent gaps introduced to optimize sequence alignment. Amino acid residues identical to the corresponding ones in BEN1 are indicated by dots. Conserved residues among all five sequences are marked with asterisks. Boxes show amino acid residues that have been reported to be involved in binding of nuclease P1 to Zn^{2+} [15].

to Zn^{2+} involves Trp-1, His-6, Asp-45, His-60, His-116, Asp-120, His-126, His-149, and Asp-153 residues [15]. All of these residues are conserved among the presented five nucleases (Fig. 5), in good agreement with their requirement for Zn^{2+}.

The activity of S1-like nucleases has been reported in several plant species in association with various physiological aspects. In leaves of wheat, a single strand-preferring nuclease of S1-type increased during senescence [16]. As senescence is a kind of programmed death of leaf cells, it is possible that this nuclease degrades nucleic acids under the control of the program toward cell death as do the 35-kDa nuclease of barley and the 43-kDa nuclease of zinnia. There have also been some reports of S1-type nucleases of plants that are unlikely to have any relationship to cell death. For example, the activity of the single strand-specific endonuclease of mung bean, a famous plant nuclease of S1-type, was found to be highest in the root tip of developing seedling, and assumed to be linked with cell division [17]. Thus, instead of the unique S1-type nuclease being specific to cell death, a few or many S1-type nucleases with different biological roles may exist in a single plant species.

Recently, the endonucleolytic activity of tobacco induced upon cell death in the course of a hypersensitive reaction was characterized and shown to be stimulated by Ca^{2+} and inhibited by Zn^{2+} [18]. Such effects of divalent cations indicate a striking distinction between this nuclease and S1-type nucleases. It is possible that programmed death of plant cells may be classified into at least two categories: one involves an S1-type, Zn^{2+}-requiring nuclease and another involves a Ca^{2+}-requiring nuclease.

To our knowledge, the activity of S1-type nucleases has not been reported from mammalian cells. DNase γ, which was found in nuclei of rat thymocytes that were induced to undergo apoptosis, requires Ca^{2+} and Mg^{2+} for reaction and is inhibited by Zn^{2+} [19]. This property of DNase γ represents that it is clearly different from S1-type nucleases and similar to the tobacco nuclease associated with hypersensitive cell death. A caspase-activated DNase (CAD) was recently identified in the cytoplasmic fraction of mouse lymphoma cells as an endonuclease responsible for DNA fragmentation during apoptosis [20]. CAD was shown to exist as an inactive complex with its specific inhibitor (ICAD) in living cells, which makes CAD conspicuous among various DNases. Sequence analysis detected no significant similarities between the CAD gene and any known genes including S1-type nuclease genes. Thus, in the light of possible involvement of S1-type nucleases in endosperm degradation and tracheary element differentiation, such developmentally controlled cell death in plants can be distinguished from apoptosis of mammalian cells. Further investigation on the roles of S1-type nucleases in programmed cell death would reveal unique aspects of plant cell death distinct from apoptosis.

Acknowledgements: We would like to thank Prof. K. Takeda, Okayama University, and Miss T. Sakura, Tokyo University of Agriculture, for their kind gifts of barley seeds, Dr. T. Demura, the University of Tokyo, for providing RNA samples of zinnia, and Dr. Junji Yamaguchi, Nagoya University, for his useful suggestion about RNA isolation from barley. This study was supported by Grants-in-Aid (06278103, 07454215) to H.F. from the Ministry of Education, Science and Culture, Japan.

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