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Internucleosomal DNA fragmentation in wild emmer wheat is catalyzed by S1-type endonucleases translocated to the nucleus upon induction of cell death

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ABSTRACT

Leaves of cereal plants display nucleosomal fragmentation of DNA attributed to the action of nucleases induced during program cell death (PCD). Yet, the specific nuclease activity responsible for generating double strand DNA breaks (DSBs) that lead to DNA fragmentation has not been fully described. Here, we characterized a Ca^{2+}/Mg^{2+}-dependent S1-type endonuclease activity in leaves of wild emmer wheat (*Triticum dicoccoides* Köern.) capable of introducing DSBs as demonstrated by the conversion of supercoiled plasmid DNA into a linear duplex DNA. In-gel nuclease assay revealed a nuclease of about 35 kDa capable of degrading both single stranded DNA and RNA. We further showed that the endonuclease activity can be purified on Concanavalin A and treatment with peptide-N-glycosidase F (PNGase F) did not abolish its activity. Furthermore, ConA-associated endonuclease was capable of generating nucleosomal DNA fragmentation in tobacco nuclei. Since S1-type endonucleases lack canonical nuclear localization signal it was necessary to determine their subcellular localization. To this end, a cDNA encoding for a putative 34 kDa S1-type nuclease, designated TaS1-like (TaS1L) was synthesized based on available sequence data of *Triticum aestivum* and fused with RFP. Introduction into protoplasts showed that TaS1L-RFP is cytoplasmic 24 h post transformation but gradually turn nuclear at 48 h concomitantly with induction of cell death. Our results suggest that DNA fragmentation occurring in leaves of wild emmer wheat may be attributed to S1-type endonuclease(s) that reside in the cytoplasm but translocate to the nucleus upon induction of cell death.

1. Introduction

DNA fragmentation is commonly studied with respect to programmed cell death (PCD) both in plants and animals [1,2]. This process is often characterized by the activation of endogenous endonucleases that cleave chromatin DNA at sites between nucleosomes (linker DNA) resulting in a typical nucleosomal DNA ladder [reviewed in 3]. Internucleosomal DNA fragmentation was reported in plants following exposure to biotic and abiotic stresses including pathogen-induced hypersensitive response as well as following treatment with potassium cyanide [4], during death of toxin-treated tomato protoplasts [5], or exposure to D-mannose [6] or ozone [7]. Also, TUNEL assay showed that PCD-induced nucleosomal DNA fragmentation occurs during tracheary element differentiation in pea [8] and in aleurone cells of germinating barley [9]. Some of the nucleases associated with PCD in plants have been characterized. Accordingly, a nuclear-localized nuclease of about 30 kDa was induced by gibberellin in wheat aleurone cells undergoing PCD [10]. The in-gel activity assay showed that the nucleolytic activity of this nuclease requires both Ca^{2+} and Mg^{2+}; nuclease activity was inhibited in the presence of EDTA. Later, this group reported on nuclease activity in both the cytoplasm and the nucleus of maternal cells of developing wheat grain [11], which are corresponding to 35 and 50 kDa proteins, respectively. Interestingly, both nuclease activities require Ca^{2+} and Mg^{2+}, while in the presence of Zn^{2+} nucleolytic activities were completely inhibited [11]. The inhibitory effect of Zn^{2+} on nuclease activities was also reported for the hypersensitive response (HR)-associated nuclease in tobacco [12]. Furthermore, nuclear extracts from nucellar cells undergoing PCD were capable of inducing DNA fragmentation and dismantling of nuclei derived from endosperm cells or from human cells [11]. Also the scutellum of wheat grains undergoes PCD following germination, which was associated with DNA fragmentation and the presence of a nuclear-localized acid- and Zn^{2+}-dependent nuclease of about 70 kDa [13].

Most peculiar is the occurrence of DNA fragmentation in DNA preparation from healthy leaves of cereal plants, which was attributed to PCD [14,15]. The timing of appearance of DNA fragmentation during

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growth and development differs among cereals. Clear fragmentation was observed in DNA extracted from 10-day-old leaves of wheat and triticale [a hybrid between wheat (*Triticum*) and rye (*Secale cereale*)]; in barley and oat, DNA fragmentation was observed in DNA extracts derived from 25-day-old leaves [15]. The observed nucleosomal DNA fragmentation was suggested to be a consequence of PCD induced during the development of the leaf such as during differentiation of tracheary elements and formation of intercellular spaces [15] or could well be the result of cell death [16] that commonly occurs in old tissue of the leaf (the leaf tip in grasses) [17]. General activity of nucleases was characterized in the coleoptile and the first leaf of developing etiolated wheat seedlings [18]. Furthermore, S1-type endonucleases were isolated from *Zinnia* and barley designated ZEN1 and BEN1, respectively, whose expression was found to be associated with PCD [19]. Later, ZEN1 was found to be a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements [20].

A bifunctional nuclease (BFN1) of about 38 kDa in *Arabidopsis* is the best-studied PCD-associated nuclease in plants [21]. BFN1 expression was almost undetectable in roots, leaves, and stems but significantly induced in flowers and during leaf and stem senescence that showed increased nuclease activity. Analysis of BFN1 promoter showed its ability to direct the expression of a GUS reporter gene in tissues engaged in PCD including senescing leaves, abscission zone of flowers and xylem as well as in developing anthers and seeds, and in floral organs after fertilization [22]. Interestingly, subcellular localization of BFN1 transiently expressed in tobacco protoplasts revealed initial localization in filamentous structures spread throughout the cytoplasm, which appear to be of ER origin, but became clustered around nuclei as protoplasts senesced [23]. Yet, it is not clear whether BFN1 is a general nucleic acid degrading enzyme or an enzyme responsible for DNA fragmentation occurring during leaf senescence [16].

We characterized an S1-type endonuclease from leaves of wild emmer wheat (*Triticum dicoccoides* Köern.) capable of introducing double strand DNA breaks (DSBs) and may be responsible for the well-described phenomenon of fragmentation of genomic DNA extracted from leaves. This endonuclease activity can be recovered on concanavalin A and removal of glycans by peptide-N-glycosidase F (PNGase F) did not abolish its activity. A cDNA encoding for an S1-like nuclease designated TaS1-like (TaS1L) was synthesized based on DNA sequence of *Triticum aestivum* and its dynamic subcellular localization was demonstrated.

2. Materials and methods

2.1. Seed collection and plant growth conditions

Spikes of wild emmer wheat (*T. dicoccoides* Köern.) were collected from four sites in Israel, namely, the "Evolution Canyon" north facing slope (32°42′55″N 34°58′33″E) and south facing slope (32°42′46″N 34°58′32″E), Sea of Galilee on terra rosa soil (32°53′37″N 35°33′02″E) and Sea of Galilee on basalt soil (32°53′34″N 35°33′04″E) [24]. Spikes were dried for 6 weeks at 37°C, and stored at room temperature for several weeks until used. Seeds of various plant species were sown on solid media and grown in a growth room at 22 °C ± 2°C under long day photoperiod. In some experiments, 2-week-old seedlings were subjected to heat stress (37 °C) for 2 h, or exposed to dark for 5 days.

2.2. DNA and protein extraction, immunoblotting, in vitro endonuclease assay and in vitro nucleosomal fragmentation assay

DNA was extracted from leaves of wheat species, *Arabidopsis* and tobacco as described [25]. DNAs were separated on 1% agarose gel stained with ethidium bromide. Total proteins were extracted from leaves of wild emmer wheat or from leaves and protoplasts of *Arabidopsis thaliana* with NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8, and 0.5% NP-40) buffer supplemented with protease inhibitor cocktail (Sigma). Immunoblotting was performed using antibodies to HSPs, namely, anti-HSP70 (Agrisera, AS08 371) and anti-HSP17.6 (Agrisera, AS07 254). In vitro nuclease assay was performed essentially as described [26].

Nucleosomal DNA fragmentation assay was performed on nuclei isolated from tobacco (*Nicotiana tabacum*) leaves in the presence of nuclei digestion buffer [27] supplemented with 10 mM $CaCl_2$ and 100 μg of total proteins extracted from wild emmer wheat leaves. Reactions were performed at 37°C for 0, 30, 60 and 120 min. DNA was extracted from nuclei and separated on 1.2% agarose gel as described previously [27].

2.3. In-gel nuclease assay, concanavalin a-agarose chromatography and peptide-N-glycosidase F (PNGase F) reaction

Nuclease assay was performed essentially as described [28] in polyacrylamide gel containing 300 μg/ml denatured salmon sperm DNA or ribonucleic acid from torula yeast (Sigma) for RNases activity. Briefy, 10 μg proteins extracted from various tissues (indicated in the text) were incubated with sample buffer containing 2% SDS, 62.5 mM Tris pH 6.8 and 10% glycerol and bromophenol blue for 1 h at 37 °C followed by separation on SDS/PAGE (samples were not boiled). The gel was washed twice, each time for 30 min, at room temperature in buffer containing 10 mM Tris–HCl pH 7.5 and 25% isopropanol, followed by washing twice 15 min each with 10 mM Tris–HCl pH 7.5. Nuclease activity was performed by incubating the gel with 10 mM Tris–HCl pH 7.5 containing divalent cations (10 mM $MgSO_4$, 10 mM $CaCl_2$) for 75 min at 37 °C. The gel was stained for 60–80 min with 10 mM Tris–HCl pH 7.5 containing 2 μg/ml ethidium bromide and inspected under UV light.

Purification of glycoproteins was performed on ConA-agarose (Sigma). Total proteins extracted from wild emmer wheat leaves were loaded on ConA-agarose column in PBS buffer containing 500 mM NaCl and eluted by 10 mM Tris pH 7.5 and 500 mM mannose. Peptide -*N*-glycosidase F (PNGase, purchased from New England BioLabs) reactions were performed at 37 °C for 2 h in G7 reaction buffer according to the manufacturers' protocol except that treatment with glycoprotein denaturing buffer was omitted.

2.4. Cloning of S1-type endonuclease from T. aestivum and transformation into Arabidopsis protoplasts

The cDNA of *T. aestivum* (accession no. AK334922) encoding for an S1-type endonuclease designated TaS1L was synthesized and cloned into pUC57 (GeneScript). The TaS1L sequence was cleaved with *Bam*HI and *Sma*I and subcloned into *Bgl*II-*Sma*I sites of pUC19 downstream from the 35S promoter and in frame with RFP to generate pUC19-35S-TaS1-RFP. This construct was transformed into *Arabidopsis* protoplasts by the PEG method essentially as described [29]. Commonly, transformation success was at the range of 1–10%. The endoplasmic reticulum marker calnexin fused to GFP (pBI221-CNX-GFP) was kindly provided by Dr. Liwen Jiang [30].

3. Results

3.1. Internucleosomal DNA fragmentation in leaves of wild emmer wheat is correlated with high activity of S1-type endonucleases

Genomic DNA was prepared from green leaves of wild emmer wheat ("Evolution Canyon", north facing slope ecotype), domesticated wheat as well as from *N. tabacum* and *A. thaliana* and analyzed on agarose gel (Fig. 1). Results showed an apparent fragmentation of genomic DNA in extracts from green leaves of both domesticated bread wheat (*T. aestivum* L.) and wild emmer wheat but not in DNA extracted from tobacco and *Arabidopsis* leaves. Although wild emmer wheat leaves looked green and viable, we speculated that DNA fragmentation might have resulted from cell death-associated endonuclease activity capable