Expression analysis of the BFN1 nuclease gene promoter during senescence, abscission, and programmed cell death-related processes

Sarit Farage-Barhom1,2, Shaul Burd1, Lilian Sonego1, Rafael Perl-Treves2 and Amnon Lers1,*

1 Department of Postharvest Science of Fresh Produce, Volcani Center, Agricultural Research Organization, Bet Dagan 50250, Israel
2 Faculty of Life Science, Bar Ilan University, Ramat Gan, Israel

Received 8 April 2008; Revised 19 May 2008; Accepted 3 June 2008

Abstract

Little is known about the biological role of nucleases induced during plant senescence and programmed cell death (PCD). Arabidopsis BFN1 has been identified as a senescence-associated type I nuclease, whose protein sequence shares high homology with some other senescence- or PCD-associated plant nucleases. To learn about BFN1 regulation, its expression pattern was analysed. A 2.3 kb portion of the 5′ promoter sequence of BFN1 was cloned and its ability to activate the GUS reporter gene was examined. Transgenic Arabidopsis and tomato plants harbouring this chimeric construct were analysed for GUS expression. In both, the BFN1 promoter was able specifically to direct GUS expression in senescent leaves, differentiating xylem and the abscission zone of flowers. Thus, at least part of the regulation of BFN1 is mediated at the transcriptional level, and the regulatory elements are recognized in the two different plants. In tomato, specific expression was observed in the leaf and the fruit abscission zones. The BFN1 promoter was also active in other tissues, including developing anthers and seeds, and in floral organs after fertilization. PCD has been implicated in all of these processes, suggesting that in addition to senescence, BFN1 is involved in PCD associated with different development processes in Arabidopsis.

Key words: Abscission, BFN1, nuclease, programmed cell death, promoter, senescence.

Introduction

Leaf senescence is an endogenously controlled degenerative process leading to cell death. It is an active, energy-requiring, genetically controlled process (Nooden et al., 1997; Guo and Gan, 2005; Lim et al., 2007) which, in plants, is believed to be a form of programmed cell death (PCD) (van Doorn and Woltering, 2004). However, it is viewed as a special type of PCD which does not share all of PCD’s typical characteristics (Thomas et al., 2003). Nevertheless, senescence is likely to be distantly related to other plant PCD processes (Thomas et al., 2003). During senescence, the leaf’s cellular structure, metabolic activities, and physiological role are greatly altered. Chloroplasts degenerate and the photosynthetic apparatus is disassembled (Hortensteiner, 2006).

Senescence is characterized by a wide and significant change in the pattern of gene expression (Buchanan-Wollaston et al., 2005; Van der Graaff et al., 2006): the expression of many genes, such as those associated with photosynthesis, is repressed, while that of many other genes, termed senescence-associated genes (SAGs), is induced. Microarray analyses have demonstrated that >800 genes are distinctively up-regulated during senescence, illustrating the dramatic alteration in cellular physiology that underlies leaf senescence (Guo et al., 2004; Buchanan-Wollaston et al., 2005).

The molecular mechanisms governing senescence regulation are poorly understood. They have been suggested to form a complex network responsible for activation of the different SAGs (Guo and Gan, 2005). Various SAGs exhibit differential expression in different tissues and in
response to different senescence-promoting factors, including hormones, salicylic acid, ozone, UV radiation, hydration, and dark incubation (Park et al., 1998; Weaver et al., 1998; Morris et al., 2000). At least part of SAG regulation occurs at the transcriptional level (Hanfrey et al., 1996; Oh et al., 1996; Noh and Amasino, 1999). Genes with a possible regulatory role in senescence have been reported, including members of the WRKY transcription factor family (Robatzek and Somssich, 2002; Miao and Zentgraf, 2007; Ulker et al., 2007), a NAC family transcription factor (Guo and Gan, 2006), and a zinc-finger protein (Kong et al., 2006).

In senescence, intensive catalytic processes leading to macromolecule degradation occur while the leaf becomes a source of mobilized carbon, nitrogen, phosphate, and other minerals (Fischer, 2007). Some of the SAGs encode hydrolytic enzymes, such as proteases and nucleases (Buchanan-Wollaston et al., 2005), which are likely to be involved in macromolecule degradation during senescence. Specific nuclease activities that can degrade both RNA and DNA have been reported to be induced in parallel to the advancement of senescence in leaves (Blank and McKeon, 1989; Wood et al., 1998; Lers et al., 2001; Canetti et al., 2002) and flower petals (Panavas et al., 1999; Xu and Hanson, 2000; Langston et al., 2005). The general aim of this study was to understand both the function and regulation of the senescence-associated BFN1 nuclease in Arabidopsis. The BFN1 gene has been cloned, and levels of its transcript have been found to be induced during leaf and stem senescence (Perez-Amador et al., 2000). The BFN1 protein sequence is highly similar to the petal senescence DSA6 nuclease (Panavas et al., 1999) and the PCD-associated ZEN1 nuclease (Ito and Fukuda, 2002), and can be classified as a type I nuclease.

The type I nucleases, also termed S1-like nucleases, are single-strand-specific endonucleases that degrade both RNA and single-stranded DNA. They have been described in many different organisms, from microorganisms to mammals (Desai and Shankar, 2003); however, knowledge of their biological functions is limited. In plants, two major classes of these endonucleases have been proposed, Zn$^{2+}$-dependent and Ca$^{2+}$-dependent (Sugiyama et al., 2000). Endonucleases have been isolated from various plant cell compartments such as the nucleus, vacuole, chloroplast, endoplasmic reticulum, and the Golgi apparatus (Bariola and Green, 1997; Desai and Shankar, 2003). Induction of plant endonucleases has been observed during growth and developmental processes such as cell division (Graf and Larkins, 1995), as well as in response to environmental stress (Muramoto et al., 1999; Yupsanis et al., 2001). In addition to senescence, nuclease induction is strongly associated with a variety of different plant PCD processes (Sugiyama et al., 2000), including the hypersensitive response (HR) (Mittler and Lam, 1997), aleurone cell death (Fath et al., 1999; Dominguez et al., 2004), endosperm development (Young and Gallie, 1999), and tracheary element (TE) differentiation (Thelen and Northcote, 1989; Aoyagi et al., 1998). However, the only direct evidence of nuclease function in PCD was reported for the zinnia nuclease ZEN1, which was demonstrated to be responsible for nuclear DNA degradation during PCD associated with xylem development (Ito and Fukuda, 2002).

The Arabidopsis endonuclease BFN1 may be involved in the nucleic acid degradation that takes place during senescence, as inferred by its senescence-associated expression (Perez-Amador et al., 2000). To learn more about BFN1 gene regulation and the function of its encoded BFN1 endonuclease, detailed analysis of the BFN1 promoter’s pattern of induction was performed in both Arabidopsis and tomato. The results indicate that the BFN1 endonuclease is involved in developmental PCD as well as senescence.

**Materials and methods**

**Construction of the BFN1 promoter::GUS gene fusion and generation of transgenic plants**

To construct the BFN1 promoter::GUS gene fusion, a 2.3 kb DNA fragment containing the BFN1 promoter (AT1G11190: 3752746–3755053) was PCR-amplified from genomic DNA using gene-specific primers: forward 5'-TCTCAAGGTCGACATATGCAC-3' and reverse 5'-GTCTTCTCTCTCTTCTCTATACACCTCATCG-3'. The amplified DNA fragment was cloned into the Smal site located in front of the GUS (β-glucuronidase) gene-coding region in the binary vector pCAMBIA1381Z (CAMBIA, Black Mountain, Australia), in which the plant selection gene hptII had previously been replaced with nptII to enable selection with kanamycin instead of hygromycin (S Burd, unpublished). This was achieved by excising the XhoI fragment containing the nptII-coding sequence from pCAMBIA2301 and inserting into pCAMBIA1381Z following release with XhoI of the hptII-coding sequence. The resulting vector, which included the chimeric BFN1 promoter::GUS fusion and the nptII selectable marker gene, was named pFPB.

Transformation of pFPB into Arabidopsis thaliana (ecotype Col-0) plants was performed by the Agrobacterium-mediated vacuum infiltration method (Bechtold et al., 1993). Tomato transformation with pFPB was performed via Agrobacterium tumefaciens strain EHA105 using cotyledons of Solanum lycopersicum variety VF36 according to McCormick (1991). Transformants were selected on kanamycin, and antibiotic-resistant T₀ plants were analysed by PCR with specific BFN1 promoter and GUS primers to verify the presence of the chimeric gene. Homozygous lines were established, and T₂ or T₃ lines were used for the experiments.

**Plant growth conditions**

Arabidopsis seeds were sown, after a 2 d vernalization treatment at 4 °C, on half-strength Murashige and Skoog (MS) medium and grown at 22 °C under a 16 h/8 h light/dark cycle. After 10 d, the seedlings were transferred to soil. Tomato seeds were germinated on perlite support at 26 °C in the dark, and after 3 d, were transferred to the light. About a week later, when cotyledons were fully developed, the seedlings were transferred to 12 cm containers filled with HR1 potting mixture (Hagarin Ltd, Yavne, Israel). The plants
were grown in the greenhouse under a controlled temperature of 25 °C and natural daylight. Two independent transgenic lines of \textit{Arabidopsis} and tomato were selected and used for detailed analysis.

**GUS activity assays**

Localization of reporter gene expression was visualized by \textit{in situ} histochemical staining. Transgenic plants harbouring the \textit{BFN1} promoter::\textit{GUS} fusion were grown on agar medium or in soil as described above. Plant tissues, at different developmental stages, were submerged in a staining solution [50 mM sodium phosphate pH 7.0, 0.1% (v/v) Triton X-100, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide, 1 mM Na\textsubscript{2}EDTA pH 8.0, 20% (v/v) methanol, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-\textit{\beta}-D-glucuronid acid (X-gluc; Duchefa, Haarlem)] and subjected to a vacuum for 3 min. Samples were incubated at 37 °C for several hours to overnight (depending on the tissue type and colour development rate) followed by chlorophyll removal, by submerging the samples in ethanol [70% (v/v)] (Jefferson et al., 1987). GUS staining was visualized using an MZFIIII stereoscope (Leica, Heerbrugg, Switzerland) or a BMLB light microscope (Leica). Control non-transformed plants were analysed for GUS activity in order to exclude non-specific staining resulting from endogenous activity (Sudan et al., 2006).

Quantitative measurements of GUS activity were made by fluorometric GUS assay, using 4-methylumbelliferyl glucuronide (MUG) as the substrate, which is converted by GUS enzyme into the fluorescent product 4-methyl umbelliferone (4-MU) (Jefferson et al., 1987). Leaf samples were ground in GUS extraction buffer [50 mM NaH\textsubscript{2}PO\textsubscript{4}, 1 mM EDTA, 0.1% Triton X-100, 0.1% (w/v) sarcosine and 10 mM dithiothreitol (DTT)] and, following removal of tissue debris by centrifugation at 10 000 g for 10 min at 4 °C, the crude total protein extract was used to measure GUS activity using an FL600 fluorometer (BIOTEX). Standard curves were prepared with 4-MU, and GUS activity was expressed as pmol 4-MU mg\textsuperscript{-1} FW\textsuperscript{-1} min\textsuperscript{-1} (Jefferson et al., 1987). Chlorophyll content was determined in the same sample used for the GUS assay. Chlorophyll was extracted from a sample of the solution with 80% (v/v) acetone and its content was measured spectrophotometrically (Porra et al., 1989).

**Results**

**Production of BFN1–GUS transgenic plants**

To study the regulation and function of \textit{BFN1}, its spatial and temporal expression pattern was analysed. A 2.3 kb \textit{Arabidopsis} genomic DNA fragment, including the 5’ sequences upstream of the \textit{BFN1} gene transcription initiation site, was cloned and fused upstream of the coding sequence of the \textit{GUS} reporter gene. It was assumed that this 2.3 kb fragment includes promoter elements and sequences required for \textit{BFN1} regulation. The \textit{BFN1} promoter::\textit{GUS} chimeric construct (pBFN1-GUS) was transformed into \textit{Arabidopsis} plants and the resultant transgenic plants were analysed for \textit{BFN1} promoter activity during the development of different tissues, either by histochemical staining to follow specific tissue localization, or by GUS activity fluorometric assay for quantitative analyses. To examine the functionality of the \textit{BFN1} promoter in a heterologous plant system, the pBFN1-GUS construct was also transformed into tomato plants. Initially, three independent transgenic lines each of \textit{Arabidopsis} and tomato plants were analysed; for some of the more detailed histochemical staining, two independent representative lines were used from each species. In all parallel analyses performed with control plants, no non-specific endogenous activity was visualized.

**Pattern of BFN1 expression during natural leaf senescence**

The pattern of \textit{BFN1} promoter expression was examined in \textit{Arabidopsis} leaves at different developmental stages, from fully green young leaves to leaves at a late senescent stage in which >75% of the leaf area was yellow. Using histochemical staining, specific GUS activity resulting from \textit{BFN1} promoter activation was observed in the senescing tissue, but not in the green sections of leaves which had begun senescing (Fig. 1A). In young, green leaves, no GUS activity was observed, while in leaves at an advanced stage of senescence, high level activity was detected throughout the leaf tissue (data not shown). To follow the kinetics of activation of the \textit{BFN1} promoter, quantitative analysis was performed by measuring GUS activity in extracts of leaves at different stages of senescence, as indicated by the level of chlorophyll. Following homogenization, the leaf tissue extracts were used for both quantifying the chlorophyll level and measuring GUS activity by quantitative fluorometric assay. This analysis revealed clear induction of the \textit{BFN1} promoter as leaf senescence progressed, as reflected by the decrease in leaf chlorophyll content (Fig. 1B). GUS activity was also measured in extracts of \textit{Arabidopsis} flowers and roots. This activity was higher than that measured in young leaves, indicating that the \textit{BFN1} promoter is developmentally induced in these organs as well. Similar quantitative analysis in transgenic tomato plants harbouring the pBFN1-GUS construct revealed the same type of inverse correlation between \textit{BFN1} promoter induction, as indicated by GUS activity, and the advancement of leaf senescence, as reflected by the decrease in chlorophyll content (Fig. 1C). The results indicated that in tomato, as in \textit{Arabidopsis}, the \textit{BFN1} promoter is activated as natural leaf senescence progresses. To examine whether \textit{BFN1} promoter activation occurs continuously during the advancement of leaf senescence or is activated at a specific and distinct stage of the process, the measured level of GUS activity was plotted versus the chlorophyll level determined from measurements performed on leaves at different stages of senescence from a few different plants. The results, shown in Fig. 2, suggest that the induction of the \textit{BFN1} promoter, resulting in elevated GUS activity, does not increase linearly with the decrease in chlorophyll content, but occurs during a more restricted late stage of senescence.
the senescence process in both Arabidopsis (Fig. 2A) and tomato (Fig. 2B) leaves.

**Pattern of BFN1 expression in vascular tissues**

Expression of the BFN1 promoter was examined in the developing vascular tissues of 4- to 14-d-old Arabidopsis seedlings. At all ages, histochemical GUS staining revealed that the BFN1 promoter directs GUS expression in the vascular tissues of the developing seedling (Fig. 3A–E). In the stem and cotyledons, GUS activity, representing BFN1 promoter activation, was observed discontinuously in parts of the vascular tissue (Fig. 3A, B). In order to localize GUS activity more specifically in the different stem tissues, cross-sections were taken of stems of BFN1 promoter::GUS-transformed Arabidopsis plants at different developmental stages. This histochemical analysis revealed GUS-stained cells in the primary xylem bundles in stems of young plants (Fig. 3C), while in stem sections of more mature plants, GUS activity was localized to the vascular cambium cells, which produce secondary xylem (Fig. 3D). Thus, specific BFN1 promoter activation occurs in cells that are probably undergoing differentiation into xylem. Histochemical GUS staining of developing roots of young seedlings also revealed activation of GUS expression in some sections of the root’s central zone, which are likely to be differentiating into xylem (Fig. 3E). Expression of the BFN1 promoter in the stems of transgenic tomato plants was also examined. Following histochemical GUS staining of the pBFN1-GUS tomato stems, they were embedded in paraffin, sectioned, and counterstained with safranin O to visualize cell structures. Similar to the findings in Arabidopsis, GUS expression was detected specifically in the differentiating xylem cells (Fig. 3F).

**Pattern of BFN1 expression in flowers and fruits**

Expression of the BFN1 promoter was examined in Arabidopsis flowers at different developmental stages, as classified by Ferrandiz et al. (1999). In flowers at stage 13 or 14, GUS activity was expressed in the anthers (Fig. 4A), stigma, and transmitting tract cells (Fig. 4B). At this developmental stage, anthers dehisce, and pollination and fertilization take place. GUS activity was also followed after fertilization and during fruit and seed
development; when the siliques expand, flower organs wither (stages 15 and 16). Clear and specific GUS staining was visualized during these stages in the developing seeds (Fig. 4C). GUS activity was also observed in senescent petals, sepals, and stamens (data not shown). In mature siliques, GUS activity was observed in the pod dehiscence zone (DZ) (Fig. 5, stages 18 and 19) and in the valves that separate from the dry siliques (not shown).

The expression of GUS activated by the BFN1 promoter was also examined in the reproductive organs of transgenic tomato plants. At anthesis day, GUS activity was detected in the petal margins of flowers, as well as in the anthers (Fig. 6). In senescent flowers, GUS activity was visualized all over the petals (data not shown). In the green fruit, GUS activity was detected specifically in the developing seed (Fig. 4D, E).

**Pattern of BFN1 expression in pith autolysis**

Pith autolysis is a widespread phenomenon in some plants, resulting in the elimination of parenchyma cells in the pith and formation of hollow stems (Carr *et al.*, 1995). In some species, it is positively correlated with rapid stem elongation and increasing sink strength of the reproductive organs or with abiotic stress conditions such as high temperature (Lu *et al.*, 1991). In transgenic pBFN1-GUS tomatoes, GUS activity was observed in the pith of mature stems. The staining intensity was correlated with the advancement of the pith autolysis process: initially, before...
any autolysis was visible, only a few cells in the central part of the pith exhibited GUS activity (Fig. 7A). Later, when cavities had formed as a result of autolysis, more intense GUS activity was visualized in the cells surrounding the cavities (Fig 7B, C). These cells eventually underwent autolysis as the process progressed.

**BFN1 expression in abscission zones**

During analysis of **BFN1** promoter expression in flowers, GUS activity was observed in the abscission zone (AZ) of the flower organs. The abscission process is responsible for controlled separation of the plant organs from the main plant body, including leaves, flower or flower organs, and fruits. In *Arabidopsis* pBFN1-GUS plants, reporter gene expression was examined during the natural shedding of flower organs after fertilization. GUS activity was detected at the AZ of stamens, petals, and sepals (Fig. 8A, B). **BFN1** promoter expression was also examined in the AZs of leaves and fruits in the pBFN1-GUS transgenic tomato plants. GUS activity was observed specifically around the AZ tissue of the senescing leaves (Fig. 9A–C). In ripe fruits, GUS activity was observed in the mid-pedicel AZ of the fruit which initially appeared to be localized in a ring of cells around the pith and vascular tissue (Fig. 9H). A similar expression pattern was described for the promoter of the polygalacturonase (PG) gene in tomato flower AZs (Hong *et al.*, 2000). GUS staining was also observed in the mid-pedicel AZ of non-fertilized flowers in which the abscission process had begun (Fig. 9D, E, F, G). GUS staining was detected in cells on both the proximal and distal sides of the AZ located in an external ring section of the pedicel (Fig. 9F, G).

**Discussion**

**BFN1** expression was previously found to be associated with senescence in *Arabidopsis* (Perez-Amador *et al.*, 2000). However, the specific function of this nuclease in senescence or other processes can only be hypothesized at this stage. As a first step to gaining better insight into **BFN1** function, a temporal and spatial characterization of **BFN1** promoter activity was performed by using the **GUS**
The specificity of BFN1 gene expression to senescing tissue was demonstrated by histochemical analysis. BFN1 promoter-activated GUS expression was visualized only in leaf sectors that were at an advanced stage of senescence, as reflected by their yellowing (Fig. 1). In general, a very good association was observed between BFN1 promoter activation and tissue senescence, since expression was not detected in nearby green tissue in the same leaf, which was mature and of the same chronological age. This indicated that the dominant factor governing BFN1 promoter activity is senescence stage and not chronological age. The relationship between the increase in BFN1 promoter activity and the decrease in chlorophyll level, used as a marker for senescence progression, identifies BFN1 as a late senescence-associated gene, whose function is probably required in the latest stages of the senescence process. When the increase in BFN1 promoter activity, represented by the level of GUS activity, was plotted against the decline in chlorophyll level, rather than a continuous linear relationship, a sharp increase in GUS activity was observed at a particular stage of the decrease in chlorophyll level. This phenomenon held true for both Arabidopsis and tomato (Fig. 2), and supports the existence of a regulatory mechanism that activates the BFN1 promoter at a specific late stage of the senescence process. The existence of a common mechanism controlling SAG expression among different plants is supported by previous observations in which promoters of SAGs retained their senescence-specific expression in other plants. For example, the Arabidopsis SAG12 promoter was efficiently used in different plant systems to activate senescence-specific expression of different target genes (Guo and Gan, 2007).

The induction of nucleases is tightly associated with plant senescence, as was demonstrated for BFN1, but it is also associated with different PCD processes (Sugiyama et al., 2000), including the HR (Mittler and Lam, 1997), aleurone cell death (Fath et al., 1999), endosperm development (Young and Gallie, 1999), and TE differentiation (Thelen and Northcote, 1989; Aoyagi et al., 1998). However, the only direct evidence of nuclease function in PCD was reported for the zinnia nuclease ZEN1, responsible for nuclear DNA degradation during PCD associated with TE differentiation during xylem development (Ito and Fukuda, 2002). Note that it is still not clear whether the above-mentioned nucleases are involved in PCD processes other than the specific ones they were associated with originally. PCD is a genetically encoded, active process, whereby cells organize their own destruction, crucial to the development and survival of plants. There are two broad categories of PCD in plants, namely developmentally regulated PCD and environmentally induced PCD (Rogers, 2005; van Doorn and Woltering, 2005; Gunawardena, 2007; Hofius et al., 2007). Developmental PCD has been found to occur during various plant developmental processes, such as xylem differentiation, anther dehiscence, organ senescence, seed and embryo development, root cap shedding, and leaf morphogenesis. Developmentally regulated PCD occurs at a predictable time and location, and is induced by internal factors (Rogers, 2005). In contrast, environmentally induced PCD, such as the HR triggered by pathogen invasion (Greenberg and Yao, 2004), is initiated in response to external abiotic or biotic signals. As already mentioned, increased activities of nucleases have been
associated with different plant PCD processes (Sugiyama et al., 2000).

The activation pattern of the BFN1 promoter observed in this study suggests that in addition to its involvement in senescence, BFN1 is involved in PCD processes. Interestingly, in the present study, BFN1 promoter activation was visualized only during developmental PCD processes; so far, it has not been possible to demonstrate its activation during PCD processes associated with biotic stress. For example, activation of the BFN1 promoter was not detected when the plant was challenged with agents known to induce HR-associated PCD, such as fumonisin B1 (Asai et al., 2000) (data not shown). Although senescence can be induced prematurely by environmental stress, it is considered to be a developmental type of PCD (Thomas et al., 2003; van Doorn and Woltering, 2004). Thus, activation of BFN1 in senescence fits with the hypothesis that this nuclease is associated with developmental PCD processes in Arabidopsis.

Vascular cell differentiation is one of the best characterized developmental PCD processes in plants, required for TE differentiation (Fukuda, 2004). During the final stage of PCD associated with TE differentiation, the enzymatic machinery which is responsible for the autolytic digestion of the cells is activated, resulting in the development of water-conducting vascular tissue. Specific autolysis-related hydrolases (cysteine and serine proteases, nucleases, and RNase) are recruited to carry out this cell-autonomous, active, and regulated cell death (Roberts and McCann, 2000).

Activation of the BFN1 promoter was visualized during this process in both Arabidopsis and tomato. BFN1 promoter expression was pronounced in the vascular tissues of roots and leaves of young Arabidopsis seedlings (Fig. 3). Furthermore, strong and highly specific BFN1 promoter activity was observed in primary xylem bundles and in vascular cambium cells, which produce secondary xylem, in both Arabidopsis and tomato (Fig. 3). This observation is supported by a microarray analysis in which BFN1 was found to be up-regulated in xylem relative to non-vascular tissue (Mitsuda et al., 2005).

In zinnia, a particular nuclease, ZEN1, has been shown to be responsible for nuclear DNA degradation during TE differentiation-associated PCD (Ito and Fukuda, 2002). ZEN1 belongs to the S1-type nuclease family (Aoyagi et al., 1998) and shares high similarity with BFN1, exhibiting ~70% identity at the amino acid sequence level (Perez-Amador et al., 2000). BFN1 may also be
involved in nuclear DNA degradation during PCD associated with TE differentiation in Arabidopsis, as well as in other developmental processes associated with PCD. Whereas according to the present analysis, BFN1 is involved in both senescence and TE differentiation, ZEN1 was specifically expressed in PCD associated with TE differentiation: it was not expressed in leaf senescence or in stress-induced cell death. Two other zinnia S1-type nuclease genes, ZEN2 and ZEN3, have been reported to be expressed during senescence (Perez-Amador et al., 2000). In Arabidopsis, BFN1 seems to be the only S1-type nuclease gene induced during leaf senescence based on the database search using the Genevestigator software (Zimmermann et al., 2005).

PCD is known to be associated with seed development, whereby several seed tissues undergo PCD as part of their normal development, including development of the endosperm layer (Young and Gallie, 2000; Rogers, 2005) and cell death of the inner integument during the development of the seed coat (Wan et al., 2002; Nakaune et al., 2005). In cereals, the progression of endosperm PCD is accompanied by an increase in nuclease activity and the internucleosomal degradation of nuclear DNA (Young et al., 1997). Here, the BFN1 promoter was activated during seed development (Fig 4) and the pattern of GUS staining in the seed suggested that the observed expression of the BFN1 gene is related to the PCD that occurs during endosperm development.

The BFN1 promoter was also activated in the transmitting tract of the carpel in Arabidopsis (Fig. 4). Normal transmitting tract development has also been shown to involve PCD (Wang et al., 1996; Crawford et al., 2007). The involvement of BFN1 in this developmental process is supported by transcript profiling showing that its expression is lower in an Arabidopsis mutant whose transmitting tract cells were genetically ablated (Tung et al., 2005). A high level of GUS activity was observed in the stamens, indicating high BFN1 promoter activation in this tissue (Fig. 4). PCD is known to occur progressively during the development of the different anther tissues (Wu and Cheung, 2000; Rogers, 2006). The involvement of BFN1 in the process underlying dehiscence. The pattern of BFN1 promoter activation appears to occur well before the initiation of the abscission process (T Bar, L Sonego, and A Lers, unpublished results). Although GUS staining does not identify the precise cells in which BFN1 is activated, GUS expression coincides with cells in the AZ layer. Interestingly, activation of the BFN1 promoter was also detected during the dehiscence process in mature Arabidopsis siliques (Fig. 5), where expression localized around the pod DZ and in the valves that separate from the dry silique. This activation was observed only in the mature siliques, suggesting an association with the cell separation process underlying dehiscence. The pattern of BFN1 promoter-activated GUS expression is very similar to that observed for the promoters of different origin endopolygalacturonase (PG) genes examined in Arabidopsis, which were activated in the floral organ AZ and in the mature silique (Christiansen et al., 2002; Gonzalez-Carranza...
et al., 2002, 2007). The apparent co-localization of BFN1 and PG expression further supports the involvement of BFN1 in abscission and dehiscence. Similar to abscission, dehiscence is a cell separation process. Both processes, at least in Arabidopsis, seem to be controlled by a common regulatory mechanism, as demonstrated by the involvement of the positive activator INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) in both (Stenvik et al., 2006). Cell separation in dehiscence occurs via breakdown of the middle lamella between the cells of the separation layer of the DZ, resulting in loss of cellular cohesion, which, together with subsequent cell death, creates a detachment line between the valves and replum (Spence et al., 1996). Based on electron microscopy analysis of the soybean pod DZ, it has been suggested that cells on both sides of the opened pod’s DZ may have undergone PCD (Christiansen et al., 2002).

Overall, high correlation had been observed between BFN1 promoter-regulated GUS expression and all examined developmental processes associated with PCD, and no expression was detected elsewhere. Thus, the present results suggest that BFN1 is involved in developmental PCD-related processes in Arabidopsis, as well as senescence. The specific function of BFN1 in these developmental processes has not yet been demonstrated. It is likely that BFN1 is involved in nuclear DNA degradation, as was demonstrated for the related ZEN1 nuclease in zinnia TE differentiation (Ito and Fukuda, 2002). To gain further insight into the function of BFN1 in PCD, Arabidopsis mutants that are deficient in BFN1 gene expression are currently being examined for the consequences of such mutation to the different PCD-related developmental processes in the plant.

Acknowledgements

This research was supported by the Israel Science Foundation of the Israel Academy of Sciences and by Research Grant No. IS-3645-04 from BARD, the United States–Israel Binational Agricultural Research and Development Fund.

References

Aoyagi S, Sugiyama M, Fukuda H. 1998. BEN1 and ZEN1 cDNAs encoding S1-type DNases that are associated with programmed cell death in plants. FEBS Letters 429, 134–138.

Asai T, Stone JM, Heard JE, Kottun Y, Yorget P, Sheen J, Ausubel FM. 2000. Fumonisin B1-induced cell death in Arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. The Plant Cell 12, 1823–1835.

Bariola PA, Green PJ. 1997. Plant ribonucleases. In: Riordan JF, Ferrandiz C, Pelaz S, Yanofsky MF, eds. Ribonucleases: structure and function. Orlando, FL: Academic Press, 163–190.

Bechtold N, Ellis J, Pelletier G. 1993. In-planta Agrobacterium-mediated gene-transfer by infiltration of adult Arabidopsis thaliana plants. Comptes Rendus de l’Academie des Sciences 316, 1194–1199.

Beers EP. 1997. Programmed cell death during plant growth and development. Cell Death and Differentiation 4, 649–661.

Blank A, McKeon TA. 1989. Single-strand-prefering nuclease activity in wheat leaves is increased in senescence and is negatively photoregulated. Proceedings of the National Academy of Sciences, USA 86, 3169–3173.

Buchanan-Wollaston V, Page T, Harrison E, et al. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. The Plant Journal 42, 567–585.

Canetti L, Lomaniec E, Elkind Y, Lers A. 2002. Nuclease activities associated with dark-induced and natural leaf senescence in parsley. Plant Science 163, 873–880.

Carr SM, Jaffe MJ. 1995. Autolysis in herbaceous, dicotyledonous plants—experimental manipulation of pith autolysis in several cultivated species. Annals of Botany 75, 587–592.

Carr SM, Seifert M, Delabaere B, Jaffe MJ. 1995. Pith autolysis in herbaceous dicotyledonous plants—a physiological ecological study of pith autolysis under native conditions with special attention to the wild plant Impatiens capensis meeb. Annals of Botany 76, 177–189.

Christiansen LC, Dal Degan F, Ulsvik P, Borkhardt B. 2002. Examination of the dehiscence zone in soybean pods and isolation of a dehiscence-related endopolygalacturonase gene. Plant, Cell and Environment 25, 479–490.

Crawford B, Ditta G, Yanofsky M. 2007. The NTT gene is required for transmitting-tract development in carpsels of Arabidopsis thaliana. Current Biology 17, 1101–1108.

Desai NA, Shankar V. 2003. Single-strand-specific nucleases. FEMS Microbiology Reviews 26, 457–491.

Domínguez F, Moreno J, Cejudo FJ. 2004. A gibberellin-induced nuclease is localized in the nucleus of wheat aleurone cells undergoing programmed cell death. Journal of Biological Chemistry 279, 11530–11536.

Evans DE. 2004. Aerenchyma formation. New Phytologist 161, 35–49.

Fath A, Bethke PC, Jones RL. 1999. Barley aleurone cell death is not apoptotic: characterization of nuclease activities and DNA degradation. The Plant Journal 20, 305–315.

Ferrandiz C, Pelaz S, Yanofsky MF. 1999. Control of carpel and fruit development in Arabidopsis. Annual Review of Biochemistry 68, 321–354.

Fischer AM. 2007. Nutrient remobilization during leaf senescence. In: Gan S, ed. Senescence processes in plants, Vol. 26. Oxford: Blackwell Publishing, 87–107.

Fukuda H. 2004. Signals that control plant vascular cell differentiation. Nature Reviews Molecular Cell Biology 5, 379–391.

Ge X, Dietrich C, Matsuno M, Li G, Berg H, Xia Y. 2005. An Arabidopsis aspartic protease functions as an anti-cell-death component in reproduction and embryogenesis. EMBO Reports 6, 282–288.

Gonzalez-Carranza ZH, Elliott KA, Roberts JA. 2007. Expression of polygalacturonases and evidence to support their role during cell separation processes in Arabidopsis thaliana. Journal of Experimental Botany 58, 3719–3730.

Gonzalez-Carranza ZH, Whitelaw CA, Swarup R, Roberts JA. 2002. Temporal and spatial expression of a polygalacturonase during leaf and flower abscission in oilseed rape and Arabidopsis. Plant Physiology 128, 534–543.

Grafi G, Larkins BA. 1995. Activity of single-stranded DNA endonucleases in mung bean is associated with cell division. Plant Molecular Biology 29, 703–710.

Greenberg JT, Yao N. 2004. The role and regulation of programmed cell death in plant–pathogen interactions. Cellular Microbiology 6, 201–211.
Gunawardena AH. 2008. Programmed cell death and tissue remodeling in plants. Journal of Experimental Botany 59, 445–451.

Guo Y, Cai Z, Gan S. 2004. Transcriptome of Arabidopsis leaf senescence. Plant Cell and Environment 27, 521–549.

Guo Y, Gan S. 2005. Leaf senescence: signals, execution, and regulation. Current Topics in Developmental Biology 71, 83–112.

Guo YF, Gan S. 2006. ANAP, a NAC family transcription factor, has an important role in leaf senescence. The Plant Journal 46, 601–612.

Hanfrey C, Fife M, Buchanan-Wollaston V. 1996. Leaf senescence in Brassica napus: expression of genes encoding pathogenesis-related proteins. Plant Molecular Biology 30, 597–609.

Hoebenrichts FA, de Jong AJ, Woltering EJ. 2007. Chlorophyll degradation during senescence-related processes in plants. Oxford: Blackwell, 304–322.

Hanfrey C, Fife M, Buchanan-Wollaston V. 1996. Apoptotic-like cell death marks the early stages of gypsophila (Gypsophila paniculata) petal senescence. Postharvest Biology and Technology 35, 229–236.

Hofius D, Tsitsigiannis DI, Jones JDG, Mundy J. 2007. Inducible cell death in plant immunity. Seminars in Cancer Biology 17, 166–187.

Hong SB, Sexton R, Tucker ML. 2000. Analysis of gene promoters for two tomato polygalacturonases expressed in abscission zones and the stigma. Plant Physiology 123, 869–881.

Hortonsteiner S. 2006. Chlorophyll degradation during senescence. Annual Review of Plant Biology 57, 55–77.

Ito J, Fukuda H. 2002. ZEN1 is a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements. The Plant Cell 14, 3201–3211.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. Gus fusions—beta-glucuronidase as a sensitive and versatile gene fusion marker in higher-plants. EMBO Journal 6, 3901–3907.

Kong Z, Li M, Yang W, Xu W, Xue Y. 2001. Temperature-induced cavities and DNA fragmentation induced upon hypersensitive response dehiscence. Current Topics in Developmental Biology 71, 309–315.

Lers A, Lomaniec E, Burd S, Khalchitski A. 2001. The role of nuclear DNA during programmed cell death of tracheary elements. Physiologia Plantarum 115–136.

Langston BJ, Bai S, Jones ML. 2005. Increases in DNA fragmentation and induction of a senescence-specific nuclease are delayed during corolla senescence in ethylene-insensitive (etr-1) transgenic petunias. Journal of Experimental Botany 56, 15–23.

Lers A, Lomaniec E, Burd S, Khalchitski A. 2001. The characterization of LeNUC1, a nuclease associated with leaf senescence of tomato. Physiologia Plantarum 112, 176–182.

Lers A, Sonego S, Green PJ, Burd S. 2006. Suppression of LK ribonuclease in tomato results in a delay of leaf senescence and abscission. Plant Physiology 142, 710–721.

Liu PO, Hye JK, Nam HG. 2007. Leaf senescence. Annual Review of Plant Biology 58, 115–136.

Lu PZ, Gladish D, Rost TL. 1991. Temperature-induced cavities and specialized parenchyma cells in the vascular cylinder of pea roots. American Journal of Botany 78, 729–739.

McCormick S. 1991. Transformation of tomato with Agrobacterium tumefaciens. In: Linscley H, ed. Plant tissue culture manual. Dordrecht: Kluwer Academic Publishers, 1–9.

Miao Y, Zentgraf U. 2007. The antagonist function of Arabidopsis WRKY53 and ESR/ESP in leaf senescence is modulated by the jasmonic and salicylic acid equilibrium. The Plant Cell 19, 819–830.

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M. 2005. The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. The Plant Cell 17, 2993–3006.

Mittler R, Lam E. 1997. Characterization of nuclease activities and DNA fragmentation induced upon hypersensitive response cell death and mechanical stress. Plant Molecular Biology 34, 209–221.

Morris K, Mackerness SAH, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V. 2000. Salicylic acid has a role in regulating gene expression during leaf senescence. The Plant Journal 23, 677–685.

Muramoto Y, Watanabe A, Nakamura T, Takabe T. 1999. Enhanced expression of a nuclease gene in leaves of barley plants under salt stress. Gene 234, 315–321.

Nakane S, Yamada K, Kondo M, Kato T, Tabata S, Nishimura M, Hara-Nishimura I. 2005. A vacuolar processing enzyme, delta VPE, is involved in seed coat formation at the early stage of seed development. The Plant Cell 17, 876–887.

Noh YS, Amasino RM. 1999. Identification of a promoter region responsible for the senescence-specific expression of SAG12. Plant Molecular Biology 41, 181–194.

Nooden LD, Guiamet JJ, John I. 1997. Senescence mechanisms. Physiologia Plantarum 101, 746–753.

Oh SA, Lee SY, Chung IK, Lee CH, Nam HG. 1996. A senescence-associated gene of Arabidopsis thaliana is distincively regulated during natural and artificially induced leaf senescence. Plant Molecular Biology 30, 739–754.

Panavas T, Pikula A, Reid PD, Rubinstein B, Walker EL. 1999. Identification of senescence-associated genes from daylily petals. Plant Molecular Biology 40, 237–248.

Park JH, Oh SA, Kim YH, Woo HR, Nam HG. 1998. Differential expression of senescence-associated mRNAs during leaf senescence induced by different senescence-inducing factors in Arabidopsis. Plant Molecular Biology 37, 445–454.

Perez-Amador MA, Abler ML, De Rocher EJ, Thompson DM, van Hoof A, LeBrasser ND, Lers A, Green PJ. 2000. Identification of BFN1, a bifunctional nuclease induced during leaf and stem senescence in Arabidopsis. Plant Physiology 122, 169–179.

Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-A and chlorophyll-B extracted with 4 different solvents—verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy. Biochimica et Biophysica Acta 975, 384–394.

Robatsek S, Somssich IE. 2002. Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes and Development 16, 1139–1149.

Roberts K, McCann MC. 2000. Xylogenesis: the birth of a corpus. Current Opinion in Plant Biology 3, 517–522.

Rogers HJ. 2005. Cell death and organ development in plants. Current Topics in Developmental Biology 71, 225–261.

Rogers HJ. 2006. Programmed cell death in floral organs: how and why do flowers die? Annals of Botany 97, 309–315.

Sanders PM, Bui AQ, Le BH, Goldberg RB. 2005. Differentiation and degeneration of cells that play a major role in tobacco anther dehiscence. Sexual Plant Reproduction 17, 219–241.

Spence J, Vercher Y, Gates P, Harris N. 1996. ‘Pod shatter’ in Arabidopsis thaliana, Brassica napus and B. juncea. Journal of Microscopy 181, 195–203.

Stenvik GE, Butenko MA, Urbanowicz BR, Rose JKC, Aulen RB. 2006. Overexpression of INFLORESCENCE DEFICIENT IN ABSCISION activates cell separation in vestigial abscission zones in Arabidopsis. The Plant Cell 18, 1467–1476.

Sudan C, Prakash S, Bhomkar P, Jain S, Bhalla-Sarin N. 2006. Ubiquitous expression of β-glucuronidase (GUS) in plants and its regulation in some model plants. Planta 224, 853–864.

Sugiyama M, Ito J, Aoyagi S, Fukuda H. 2000. Endonucleases. Plant Molecular Biology 44, 387–397.
Thelen MP, Northcote DH. 1989. Identification and purification of a nuclease from *Zinnia elegans* L—a potential molecular marker for xylogenesis. *Planta* 179, 181–195.

Thomas H, Ougham HJ, Wagstaff C, Stead AD. 2003. Defining senescence and death. *Journal of Experimental Botany* 54, 1127–1132.

Tung C, Dwyer K, Nasrallah M, Nasrallah J. 2005. Genome-wide identification of genes expressed in Arabidopsis pistils specifically along the path of pollen tube growth. *Plant Physiology* 138, 977–989.

Ulker B, Mukhtar MS, Somssich IE. 2007. The WRKY70 transcription factor of Arabidopsis influences both the plant senescence and defense signaling pathways. *Planta* 226, 125–137.

Van der Graaff E, Schwacke R, Schneider A, Desimone M, Flugge UI, Kunze R. 2006. Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. *Plant Physiology* 141, 776–792.

Wang M, Hoekstra S, van Bergen S, Lamers GEM, Oppedijk BJ, van der Heijden MW, de Priester W, Schilperoort RA. 1999. Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. *Plant Molecular Biology* 39, 489–501.

Weaver LM, Gan SS, Quirino B, Amasino RM. 1998. A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Molecular Biology* 37, 455–469.

Wellner F, Riechmann JL, Alves-Ferreira M, Meyerowitz EM. 2004. Genome-wide analysis of spatial gene expression in Arabidopsis flowers. *The Plant Cell* 16, 1314–1326.

Wood M, Power JB, Davey MR, Lowe KC, Mulligan BJ. 1998. Factors affecting single strand-prefering nuclease activity during leaf aging and dark-induced senescence in barley (*Hordeum vulgare* L.). *Plant Science* 131, 149–159.

Wu HM, Cheung AY. 2000. Programmed cell death in plant reproduction. *Plant Molecular Biology* 44, 267–281.

Xu Y, Hanson MR. 2000. Programmed cell death during pollination-induced petal senescence in petunia. *Plant Physiology* 122, 1323–1333.

Young TE, Gallie DR. 1999. Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. *Plant Molecular Biology* 39, 915–926.

Young TE, Gallie DR. 2000. Programmed cell death during endosperm development. *Plant Molecular Biology* 44, 283–301.

Young TE, Gallie DR, DeMason DA. 1997. Ethylene-mediated programmed cell death during maize endosperm development of wild-type and shrunken2 genotypes. *Plant Physiology* 115, 737–751.

Yupsanis T, Kefalas PS, Eleftheriou P, Kotinis K. 2001. RNase and DNase activities in the alfalfa and lentil grown in iso-osmotic solutions of NaCl and mannitol. *Journal of Plant Physiology* 158, 921–927.

Zimmermann P, Hennig L, Gruissem W. 2005. Gene-expression analysis and network discovery using Genevestigator. *Trends in Plant Science* 10, 407–409.