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RESEARCH PAPER

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

Sarit Farage-Barhom[1,2], Shaul Burd[1], Lilian Sonego[1], Rafael Perl-Treves[2] and Amnon Lers[1,*]

[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*

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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

* To whom correspondence should be addressed. E-mail: alers@volcani.agri.gov.il
Abbreviations: AZ, abscission zone; DZ, dehiscence zone; GUS, β-glucuronidase; HR, hypersensitive response; PCD, programmed cell death; PG, polygalacturonase; SAG, senescence-associated gene; TE, tracheary element.

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 catabolic 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 (Grafi 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′-TCTCAACGCTCAGACATATGCAC-3′ and reverse 5′-GTCTTCTCTTCTCTTGTCTATAACAACCT-CATCG-3′. The amplified DNA fragment was cloned into the *Sma*I 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 *hpt*II had previously been replaced with *npt*II to enable selection with kanamycin instead of hygromycin (S Burd, unpublished). This was achieved by excising the *Xho*I fragment containing the *npt*II-coding sequence from pCAMBIA2301 and inserted into pCAMBIA1381Z following release with *Xho*I of the *hpt*II-coding sequence. The resulting vector, which included the chimeric *BFN1* promoter*::GUS* fusion and the *npt*II 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 EH105 using cotyledons of *Solanum lycopersicum* variety VF36 according to McCormick (1991). Transformants were selected on kanamycin, and antibiotic-resistant T_0 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 T_3 or T_4 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 *Arabidopsis* and tomato were selected and used for detailed analysis.

GUS activity assays

Localization of reporter gene expression was visualized by *in situ* histochemical staining. Transgenic plants harbouring the *BFN1* promoter*::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_2EDTA pH 8.0, 20% (v/v) methanol, and 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β-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 MZFIII 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_2PO_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 FW^{-1} min^{-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 spectroscopically (Porra *et al*., 1989).

Results

Production of BFN1–GUS transgenic plants

To study the regulation and function of *BFN1*, its spatial and temporal expression pattern was analysed. A 2.3 kb *Arabidopsis* genomic DNA fragment, including the 5′ sequences upstream of the *BFN1* gene transcription initiation site, was cloned and fused upstream of the coding sequence of the *GUS* reporter gene. It was assumed that this 2.3 kb fragment includes promoter elements and sequences required for *BFN1* regulation. The *BFN1* promoter::*GUS* chimeric construct (pBFN1-GUS) was transformed into *Arabidopsis* plants and the resultant transgenic plants were analysed for *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 *BFN1* promoter in a heterologous plant system, the pBFN1-GUS construct was also transformed into tomato plants. Initially, three independent transgenic lines each of *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 *BFN1* promoter expression was examined in *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 *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 *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 *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 *Arabidopsis* flowers and roots. This activity was higher than that measured in young leaves, indicating that the *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 *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 *Arabidopsis*, the *BFN1* promoter is activated as natural leaf senescence progresses. To examine whether *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 *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

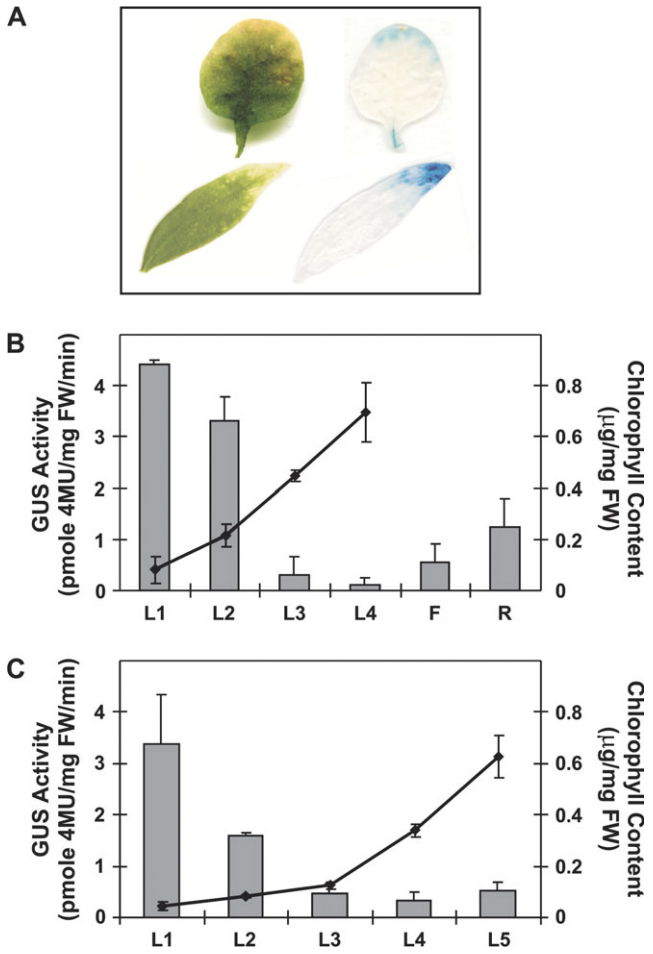

Fig. 1. GUS activity in transgenic *Arabidopsis* and tomato plants during leaf senescence. (A) Senescing *Arabidopsis* leaves before (left) and after (right) histochemical staining of GUS activity and extraction of pigments. (B) Columns: quantitative fluorometric assays of GUS activity in extracts of *Arabidopsis* leaves at different stages of natural senescence, from fully green young leaves (L4) to leaves in late senescence (L1) and in extracts of flowers (F) and roots (R). Line plots: chlorophyll content of leaf samples. (C) Gus activity and chlorophyll content in tomato leaf extracts; legend as in (B). L5, youngest leaf stage.

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

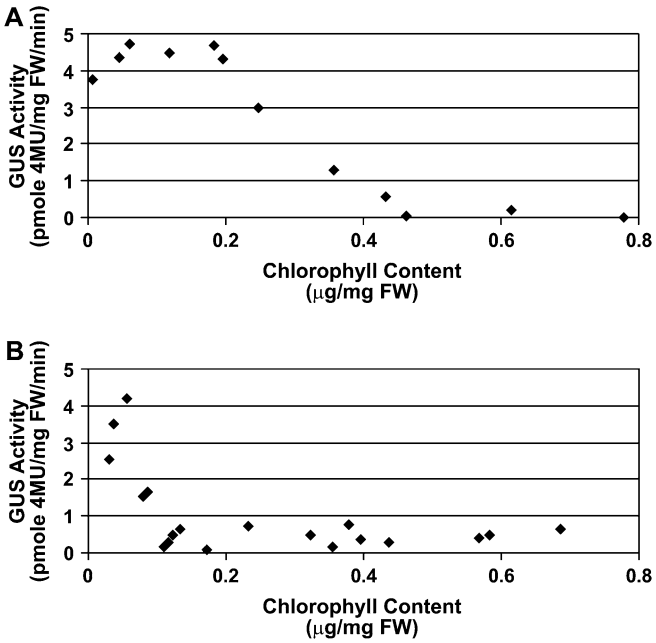

Fig. 2. The relationship between GUS activity and chlorophyll content. GUS activity and chlorophyll content were measured in the same tissue extracts from leaves at different stages of senescence obtained from several *Arabidopsis* (A) and tomato (B) plants.

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

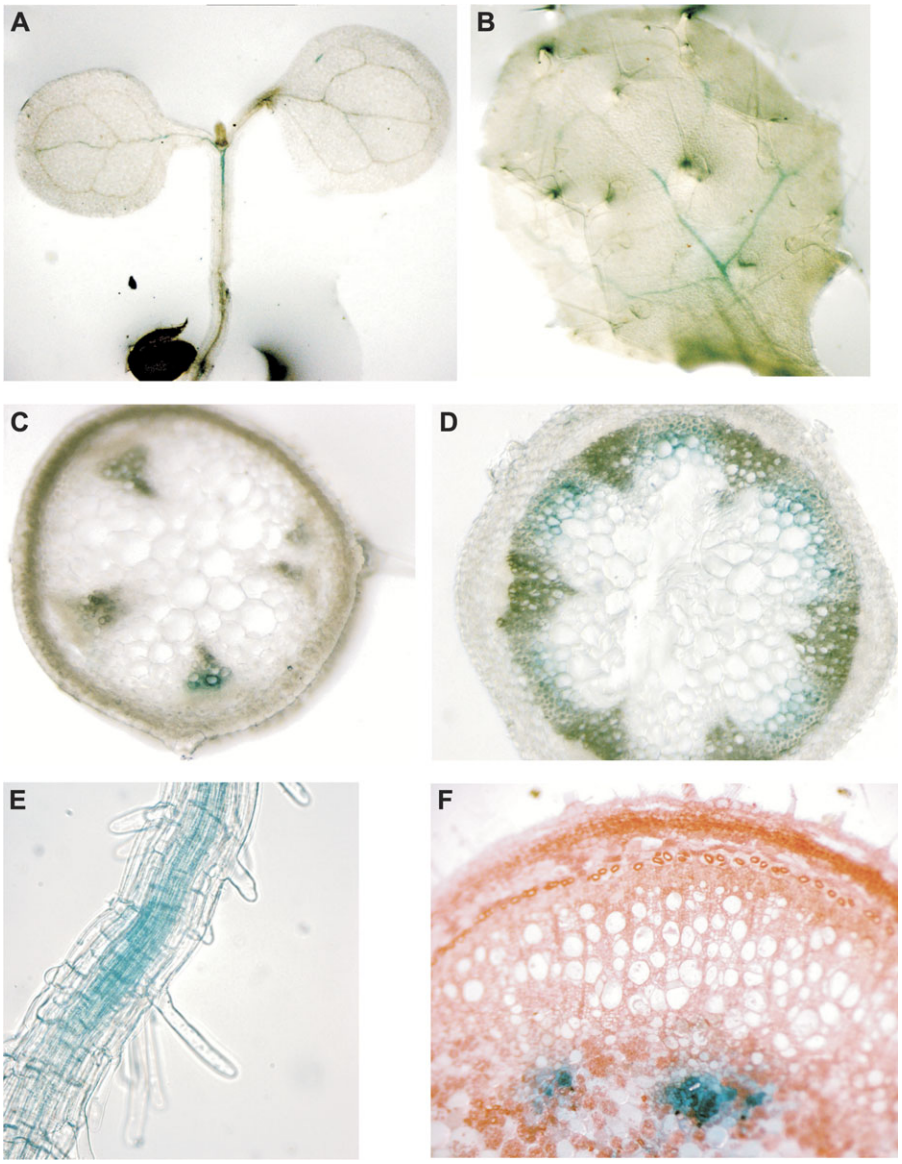

Fig. 3. Histochemical staining of GUS activity in vascular tissues of *Arabidopsis* and tomato. (A) *Arabidopsis* seedling, 4 d after germination. (B) Young rosette leaves of *Arabidopsis*, 13 d after germination. (C, D). Cross-sections of young (C) and mature (D) *Arabidopsis* inflorescence stems. (E) *Arabidopsis* root vascular tissues. (F) Cross-section of tomato stem, counter-stained with safranin.

development; when the silique expands, 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 silique (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

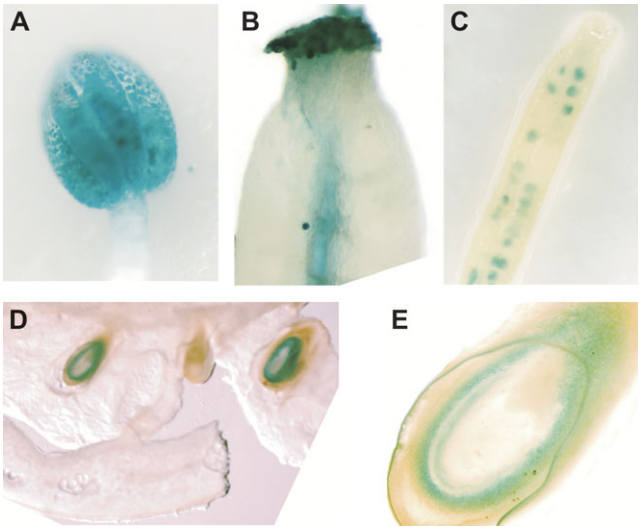

Fig. 4. Histochemical staining of GUS activity in *Arabidopsis* floral organs and tomato fruit. (A) *Arabidopsis* anther. (B) Silique after pollination. (C) Young silique showing the developing seeds. (D) Cross-section of green tomato. (E) Magnification of one of the seeds.

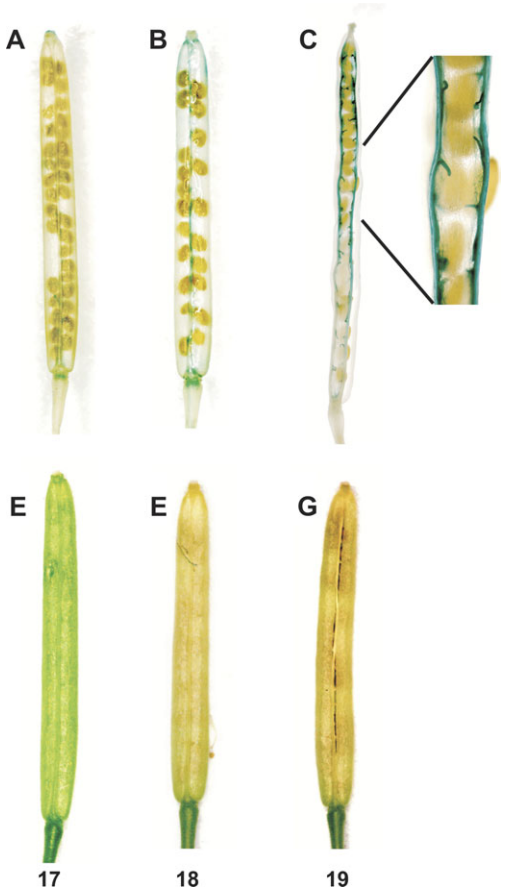

Fig. 5. Histochemical staining of GUS activity in *Arabidopsis* siliques. *Arabidopsis* siliques at different developmental stages [indicated as stages 17, 18, and 19 of development according to Ferrandiz *et al.* (1999)]. Bottom: unstained siliques. Top: the same siliques stained for GUS. Magnification of a stage 19 silique is presented for better visualization of the dehiscence zone.

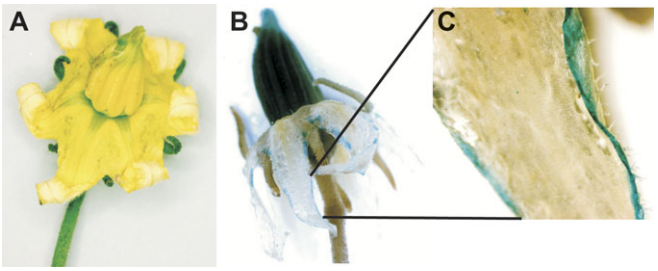

Fig. 6. Histochemical staining of GUS activity in tomato flowers. (A) Tomato flowers at anthesis. (B) The same flower stained for GUS activity. (C) Magnification of petal margins.

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*

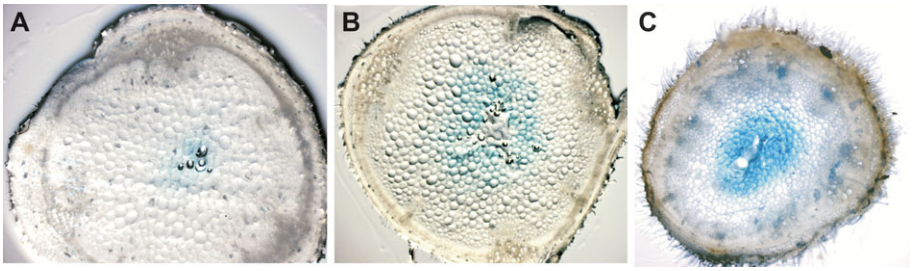

Fig. 7. Histochemical staining of GUS activity in tomato stem. (A–C) Cross-sections of stems at progressive stages of pith development.

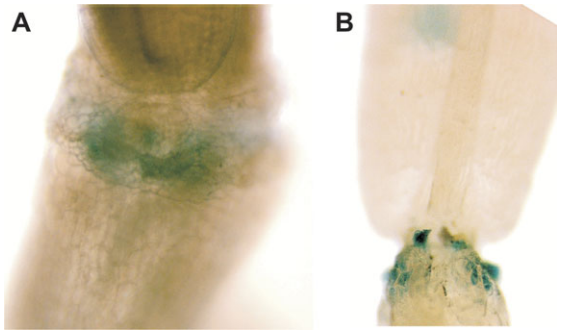

Fig. 8. Histochemical staining of GUS activity in the abscission zones (AZs) of *Arabidopsis* floral organs. (A) *Arabidopsis* petal AZ. (B) Stamen AZ.

reporter gene. The *BFN1–GUS* transgene maintained a similar type of senescence-specific expression in both *Arabidopsis* and tomato transgenic plants, demonstrating that *BFN1* regulation is mediated, at least in part, at the transcriptional level by a mechanism which is conserved between the two different species.

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

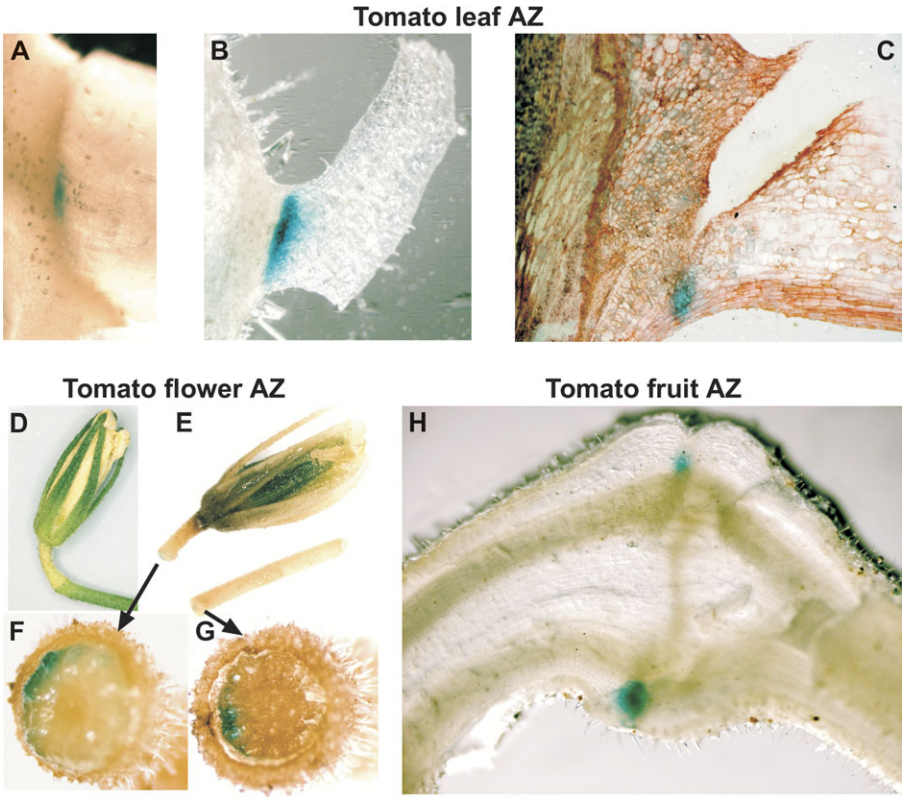

Fig. 9. Histochemical staining of GUS activity in tomato abscission zone (AZ). (A–C) Longitudinally halved section of a tomato leaf AZ at progressive stages of abscission, from early (A), to intermediate (B), to advanced (C). (D, E) Tomato flower (D, unstained; E, GUS stained) undergoing senescence and abscission due to non-fertilization. (F, G) Magnification of the two AZ surfaces of the abscised flower in E, stained for GUS. (H) Fruit pedicel AZ following longitudinal sectioning.

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). It is first triggered in the tapetum, sustaining microspore to pollen development (Wang *et al*., 1999; Varnier *et al*., 2005), while at a later stage, PCD of specified cells of the anther wall is required for anther dehiscence and the release of mature pollen (Ge *et al*., 2005; Sanders *et al*., 2005). Microarray analysis of spatial gene expression in *Arabidopsis* flowers identified specific expression of *BFN1* in stamens (Wellmer *et al*., 2004). Further analysis is required to identify specific stamen-related PCD processes in which BFN1 is involved.

The clear and significant association that was observed between developmental PCD processes and activation of the *BFN1* promoter suggests that this promoter can be used as a marker for PCD. In transgenic tomato plants, intense activation of the *BFN1* promoter was observed in the pith of mature stems in cells targeted for autolysis, which resulted in longitudinal stem cavities (Fig. 7). While it involves cell death (Beers, 1997), hallmarks of PCD have not yet been described for this process. Pith autolysis is a widespread phenomenon which in some species is positively correlated with rapid stem elongation or with abiotic stress conditions (Carr and Jaffe, 1995; Carr *et al*., 1995), and may occur in roots as well (Lu *et al*., 1991). Pith autolysis is very similar to lysigenous aerenchyma, known to form as a consequence of PCD in the roots and shoots of wetland species and in some dryland species under adverse flooding conditions (Evans, 2004).

Activation of *BFN1* in the tomato petal margins may indicate the occurrence of PCD in this tissue (Fig. 6). This activation appears to occur well before the initiation of petal senescence. The early occurrence of nuclear DNA degradation in flower petals, before visible signs of senescence are exhibited, was demonstrated in gypsophila (Hoeberichts *et al*., 2005). It has also been suggested that in *Alstroemeria* petals, PCD processes are initiated extremely early at a similar location on the petals to that observed for expression of the *BFN1* promoter in tomato (Wagstaff *et al*., 2003). By the time *Alstroemeria* flowers have opened, mesophyll cells at the petal and sepal margins have completely degenerated, indicating that some cell death is occurring from the earliest stages of flower development (Wagstaff *et al*., 2003).

Specific activation of the *BFN1* promoter in several types of examined AZs in both *Arabidopsis* and tomato supports the hypothesis of a PCD process involved in plant abscission (Lers *et al*., 2006). It has previously shown that inhibition of the RNase gene *LX*, associated with senescence and PCD in tomato, results in a marked delay of leaf abscission. At that time, it was hypothesized that PCD is involved in abscission and that *LX* probably plays a role in the process. In addition to the demonstrated activation of the *BFN1* promoter in AZs, an induction of nuclease activity has been detected in AZ following 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.

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