Expression of *AtCAN1* and *AtCAN2* genes of the plant SNc nuclease family correlates with programmed cell death and endoreduplication, indicating their role in the recycling of nucleic acid components.

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Abstract

Background

Controlled degradation of genomic DNA is a common phenomenon observed in all known cases of programmed cell death (PCD) in both animals and plants. In plants, nucleic acid degradation during PCD facilitates the redistribution of their constituent building blocks. Previous studies have shown that nucleases from the S1/P1 family are involved in this process; however, due to its complexity, it has been hypothesized that nucleases from other families, including staphylococcal-like nucleases (SNc), may also participate. In *Arabidopsis*, this family comprises two nucleases with an atypical plasma membrane localization for this type of enzymes: AtCAN1 and AtCAN2.

Results

Using the promoter-driven GUS reporter assay, we showed that the genes encoding SNc nucleases are expressed in tissues that can be grouped into three main categories. The first category includes plant structures that are clear examples of organs undergoing PCD, such as the root cap, vascular bundle elements, the tapetum, maturing seed pods, and senescent leaves. The second category comprises cells whose function involves interaction with the external environment and which are susceptible to pathogen attack. This group includes root hairs, stomatal guard cells, and hydathodes. The third group of plant structures showing SNc nuclease activity consists of elements characterized by endoreduplication, i.e., stipules, trichomes, and the basal parts of the hypocotyl.

Conclusions

Our studies show that SNc nucleases are as broadly involved in the DNA degradation during plant PCD as the previously reported S1/P1 proteins. The frequent overlap in their expression profiles suggests cooperative action. Whereas SNc nucleases localize to the plasma membrane, S1/P1 nucleases are nuclear, indicating distinct yet complementary nucleolytic pathways. We further demonstrate that SNc nucleases are specifically expressed in organs not undergoing PCD but characterized by endoreduplication, implicating them in an unexplored mechanism for redistributing polyploid DNA components.

Keywords

Staphylococcal-like nuclease, DNA degradation, programmed cell death, polyploidy, GUS assay, *Arabidopsis thaliana*

Background

The development of multicellular eukaryotic organisms is inextricably linked to degradative processes that occur both in living cells, as autophagy, and in cells destined for elimination through various forms of programmed cell death (PCD). At the molecular level, these processes primarily target membrane structures, proteins, and nucleic acids. In plants, a wide range of PCD processes has been documented. Model examples include developmental events such as xylem differentiation, root cap growth, tapetum degradation associated with pollen development, and senescence, which has been studied most extensively in withering leaves [1]. Degradative processes are also observed in environmentally induced PCD. The best-characterized case is the hypersensitive response (HR), a classical defence mechanism in which plant cells undergo localized death upon pathogen attack [2].

In all studied cases of PCD, intense nucleolytic activity has been observed, leading to the degradation of both genomic DNA and cellular RNA. Although DNA elimination occurs during PCD in both plants and animals, the underlying biological function differs substantially. In animals, DNA degradation primarily serves to protect surrounding tissues from inflammation. Such inflammation could otherwise be triggered by the release of nucleic acids from disintegrating cells into the intercellular space [3]. In contrast, this explanation does not apply to plants. Instead, it is generally assumed that nucleic acid degradation in plant tissues undergoing PCD facilitates the redistribution of their constituent building blocks. This role is particularly important because nucleic acids are rich in reduced nitrogen and readily available phosphorus - two essential mineral nutrients that frequently limit plant growth due to their scarcity in the natural environment. Consequently, the efficient recycling and redistribution of these elements is considered crucial for sustaining plant development [4].

The problem of DNA component redistribution is also connected with the phenomenon of DNA endoreduplication. Endoreduplication occurs in various plant tissues, but its biological significance is still unclear. However, considering how many valuable building blocks polyploid DNA contains, the question arises about their final fate, i.e., whether they can be recycled. In storage tissues such as endosperm and cotyledons, where endoreduplication occurs [5] and where, during the mobilization of accumulated reserves, strong nucleolytic activity has also been reported [6, 7], it has been proposed that the accumulated DNA may serve as a source of elements for the synthesis of nucleic acids in developing organs [8]. However, it remains unclear whether polyploid DNA in tissues that do not serve storage functions can also be utilized for a similar purpose.

A group of hydrolytic enzymes, commonly referred to as degradative nucleases, is responsible for the nonspecific breakdown of nucleic acids. Unlike nucleases involved in DNA repair or recombination, degradative nucleases exhibit high catalytic activity, low substrate specificity, and tissue-specific expression. Plants and animals use enzymes from distinct protein families to perform this function. In plants, BFN1, a member of the S1/P1 endonuclease family, is the nuclease with the most compelling evidence supporting its role in PCD [9].To establish this function, studies employing the GUS reporter gene under the control of the BFN1 promoter proved crucial [10]. In addition, analysis of BFN1 gene expression profiles based on available microarray data confirmed that the expression of this nuclease correlates with known instances of PCD [11].

However, analysis of degradative nucleolytic activities detected in various plant tissues undergoing PCD suggests that a larger number of nucleases are likely to be involved in these processes [6]. These enzymes differ considerably in their molecular weight and catalytic requirements. As we have established, this diversity arises primarily from the fact that individual proteins of the S1/P1 family are catalytically active under different pH conditions - mainly acidic or neutral - and also from the fact that they utilize distinct divalent cations, most commonly Zn^{2+}, Ca^{2+}, or Mn^{2+}, as cofactors. Moreover, members of this family are further distinguished by their sensitivity to inhibition by various factors [11]. Such extensive diversity among degradative nucleases can be attributed to their participation in different forms of PCD, which occur in tissues exhibiting substantial morphological and physiological variation. Furthermore, because DNA degradation appears to be a multistep

process, even a single type of PCD may involve the coordinated action of several nucleases, each operating under conditions specific to distinct stages of the process. Taken together, these observations indicate that plants require a broad repertoire of nucleases capable of hydrolyzing nucleic acids under diverse conditions.

According to our hypothesis, another family of DNases that, alongside the S1/P1 nucleases, may also participate in PCD-dependent DNA degradation in plants is the Staphylococcal-like nuclease family (SNc). Our previous studies on two plant members of this family, encoded by *Arabidopsis thaliana* – AtCAN1 and AtCAN2 – have demonstrated that these enzymes, similar to their bacterial homologs, are catalytically active at neutral pH in the presence of Ca^{2+} ions and display pronounced DNA-degrading activity. Moreover, expression analysis based on available microarray data suggested that tissue-specific expression of at least one of these genes, *AtCAN1*, may correlate with the progression of PCD. Notably, proteins of this family exhibit several unusual and intriguing features for degradative nucleases, including membrane localization and the presence of an ABC transporter motif, which points to their potential involvement in intercellular transport [12].

Therefore, we conducted studies to characterize the tissue-specific expression profiles of SNc family nucleases, employing the GUS reporter gene under the control of the *AtCAN1* and *AtCAN2* promoters to assess whether their activity overlaps with known degradative processes occurring at distinct stages of plant development, using *Arabidopsis thaliana* plants transformed with the aforementioned transgenes.

Methods

Generation of *AtCAN1/AtCAN2* Promoter*::GUS* gene fusion constructs and preparation of transgenic plants

To generate the At*CAN1* and At*CAN2* promoter::GUS gene fusions, the binary vector pCAMBIA3301 (CAMBIA, Black Mountain, Australia) was modified by removing the CaMV 35S sequence. This was achieved by excision of the promoter with the restriction enzymes PstI and NcoI, followed by end blunting and plasmid recircularization, resulting in a construct retaining only the multiple cloning site and the GUS gene.

To obtain the promoter sequences of the *AtCAN1* and *AtCAN2* genes from *A.thaliana* genomic DNA, nested PCR was performed. In the first stage, primers anchored in genomic regions flanking the target sequences were used. A 2.9 kb DNA fragment encompassing the *AtCAN1* promoter [At3g56170] was PCR-amplified with forward primer 5'-CACAGCAAGAGCAAGAGCAG-3' and reverse primer 5'-AGACGCCGTGAGAATTCAAG-3'. Similarly, a 1.8 kb DNA fragment containing the At*CAN2* promoter [At2g40410] was amplified with forward primer 5'-GGTGAGCAAGCAAAGAGGTC-3' and reverse primer 5'-GCACAAATGAAAGCAGCAGA-3'. In the second stage, the promoter sequences were amplified from purified first-round PCR products using primers carrying restriction enzyme recognition sites to facilitate cloning into the modified pCAMBIA3301 plasmid. The At*CAN1* promoter was amplified with *Eco*RI-forward primer 5'-AAAgaattcCTGAATTGGCAATATGATAAG-3' and *Xba*I-reverse primer 5'-CCCtctagaTTCTTAGATTTGATTTTCAAC-3' primers, while the At*CAN2* promoter was amplified with *Sac*I-forward primer 5'-AATgagctcGTATGGTGATGCGCGGTGC-3' and *Xba*I-reverse primer 5'-CCCtctagaCTTTCACCCAATTTCAGGGAATC-3'.

Following purification, the PCR products were digested with the corresponding restriction enzymes and ligated into the modified pCAMBIA3301 vector treated with the same enzymes. The ligation mixtures were used to transform E. coli DH5α. After verification of positive colonies by Sanger sequencing, the resulting plasmids were introduced into *Agrobacterium tumefaciens* GV3101 by the freeze-thaw method for subsequent stable transformation of *A. thaliana*.

Stable transformation of *A. thaliana* plants with the At*CAN1*/At*CAN2* promoter::GUS constructs was performed using *Agrobacterium tumefaciens*-mediated transformation according to the floral dip method [13].

Transformants were selected and analyzed to establish stable transgenic lines containing the integrated GUS gene under the control of the respective nuclease promoter sequences. Homozygous lines were established, and T3 lines were used for the experiments.

Plant growth and selection

For in vitro growth and selection of transformed plants, half-strength Murashige and Skoog (MS) solid medium was used. The pH of the medium was adjusted to 5.6-5.7 using KOH before adding Phytoagar, and the medium was sterilized by autoclaving. After that, BASTA (glufosinate-ammonium) was added at a final concentration of 15µg/ml as a selection agent. Sterilized seeds were sown on plates and PhytoCon in vitro plant culture vessels, subjected to vernalization for 48 h at 4°C in darkness, then transferred to a phytotron and grown at 22°C under a 16 h/8 h light/dark cycle, with a light intensity 120µmol/m²/s, and 70% relative humidity. Ten days after germination, *Arabidopsis* seedlings from selection plates were transferred to peat pellets (Jiffy Products) and, together with plants in PhytoCon, continued growing under the same conditions.

GUS histochemical staining

Reporter gene expression was visualized by *in situ* histochemical staining. Whole seedlings, fragments, or organs (roots, stems, leaves, flowers, and siliques) were collected, placed in chilled 90% acetone, and kept until all samples were collected. Samples were then transferred to a 12-well plate (3.8 cm² surface area per well) filled with staining buffer (50 mM phosphate buffer, pH 7.2, 0.2% (w/v) Triton X-100, 0.25 mM potassium ferricyanide ($K_3[Fe(CN)_6]$), 0.25 mM potassium ferrocyanide $K_4[Fe(CN)_6]$, and 2 mM X-Gluc in dimethylformamide).

The plate was placed in a desiccator connected to a vacuum pump, operated in 5 cycles consisting of 10 min of vacuum generation and 3 min of incubation without vacuum. This procedure was repeated until the plant material or individual organs freely settled to the bottom during incubation without vacuum. The tightly sealed plate was then incubated overnight at 37°C in the dark with shaking at 150 rpm. The following day, the staining buffer was removed, and samples were placed in absolute ethanol and incubated at 37°C with shaking, replacing the solution every 30 minutes until chlorophyll was completely removed and tissues became transparent. Stained samples were observed using a Zeiss SteREO Lumar.V12 and Zeiss AxioScope 2 Plus with ZEN 2.6 software.

For the sectioning, samples were embedded in LR White Acrylic Resin. Samples were incubated for 24 h in solutions with gradually increasing resin concentrations. The process began with a solution of 3 parts resin and 1 part absolute ethanol. After 12 h, samples were transferred to a 1:1 resin/absolute ethanol solution, and after another 12 h, to a 3:1 resin/ absolute ethanol solution. Subsequently, samples were incubated in resin containing a catalyst (1.88 g/100 g resin) for 24 h, replacing the resin solution halfway through. Finally, samples were placed in gelatin capsules, covered with resin and catalyst solution, sealed, and polymerised at 55°C for 24 h. Embedded samples were sectioned using a microtome with a glass knife and observed under a Zeiss AxioScope 2 Plus microscope.

Results

Production of *AtCAN1* promoter-GUS and *AtCAN2* promoter-GUS transgenic plants

The analysis of the expression profiles is one of the basic approaches to understanding biological function. In the studies presented below, we assumed that identifying the expression of SNc family genes in tissues undergoing well-defined nucleic acid degradation processes would support the hypothesis that these genes participate in such processes. This strategy was successfully applied in previous research, where analysis of plants transformed with a chimeric construct (*BFN1* promoter-GUS) demonstrated the involvement of the BFN1 (ENDO1) nuclease from the S1/P1 family in plant PCD [10]. In this work, we used a similar methodology. We selected potential

promoter sequences of the two *A. thaliana* SNc nucleases (2899 bp for *AtCAN1* and 1814 bp for *AtCAN2*) and introduced them into the modified pCAMBIA3301 genetic vector upstream of the GUS reporter gene. These constructs were introduced into *Arabidopsis* plants using the floral dip method, and the resultant transgenic lines were analysed for SNc promoter activity during plant development. The activity of the GUS reporter gene under the control of the selected promoters was examined comprehensively across all organs and developmental phases; however, in this paper, we present only the results demonstrating detectable GUS protein activity.

Expression pattern of *AtCAN1* during early stages of plant development

We began our study of the expression profile of *AtCAN1* nuclease in *A. thaliana* by detecting GUS signals at early stages of plant development, from seed germination through the third week of growth (**Fig. 1**). The first appearance of the GUS signal was observed in two-day-old seedlings. At this stage, the strongest signal was detected in the root cap. In addition, a pronounced GUS signal was visible in the transition zone between the hypocotyl and the root, with a particularly high density in the root hairs. Furthermore, in this part of the plant, the GUS signal was also present along the developing axial cylinder of the root. A weaker signal was also detected in the cotyledon area; however, it disappeared during subsequent stages of seedling development.

As shown in **Fig. 1**, which presents the staining results of a 7-day-old seedling, in the following days, the GUS signal extended along the entire length of the root, except for the youngest sections, where it remained confined to the root cap. In the above-ground part of the seedling, a specific GUS signal appeared in the stipules and along the vascular bundles of the stem and leaf petioles.

Expression pattern of *AtCAN1* in vegetative organs of mature plants

In the vegetative organs of mature plants, the activity of the *AtCAN1* promoter was detected in several locations (**Fig. 2**). In 14-day-old plants, intense GUS signals were observed in the petioles of rosette leaves. Furthermore, GUS activity was also detected in the inflorescence stem and the leaf blade area. In the stem, the GUS signal persisted in the vascular bundles and on its surface, where distinct and highly specific GUS signals were observed in stomata and trichomes. We also observed *AtCAN1* promoter activity in several leaf structures. To further characterize these signals, we analyzed cotyledons, rosette leaves, and stem leaves. When analyzing rosette leaves, we included both juvenile rosette leaves with smooth margins and adult leaves with serrated margins. Analysis of all these cases showed that the GUS signal in leaves was detected in the vascular bundles, trichomes, and hydathodes. However, the intensity of individual signals varied depending on the leaf category and growth conditions–for example, whether the plants were grown in soil or *in vitro* under relatively high humidity exceeding 95%. Signals in hydathodes were observed both along the smooth leaf margin and at the tips of the leaf serrations, which are tooth-like protrusions that also contain hydathodes in *Arabidopsis* [14]. Interestingly, while the GUS signal was present in both stem and leaf trichomes, it was detected only in stem stomata and not in those of leaves.

Expression of *AtCAN1* during leaf senescence

The spatial distribution of the GUS signal in leaves exhibited clear alterations associated with the progression of senescence. In fully developed, mature leaves, GUS activity was detected only in the previously mentioned, narrowly defined leaf structures and was not detectable in the green tissues, including the mesophyll and epidermis (Fig. 2). As illustrated in Fig. 3, however, the pattern of GUS expression underwent a pronounced shift upon the onset of leaf wilting. The signal initially emerged in the tissues displaying chlorosis on the leaf margins and subsequently expanded as senescence advanced, thereby providing clear evidence of *AtCAN1* promoter activation in response to senescence-associated factors.

Spatial pattern of *AtCAN1* expression in floral tissue and developing siliques

Expression of the *AtCAN1* promoter was also observed in flowers (**Fig. 4**). Between stages 10 and 16 of flower development (according to the classification proposed by [15]the GUS signal was detected exclusively in the anthers. At these stages, expression was observed both in the tapetum and the developing pollen. As flower development progressed, the distribution of the signal changed: it disappeared from the tapetum as this tissue degraded and became localized in the pollen grains. This expression pattern is characteristic of tapetum–derived proteins, which are known to be incorporated into pollen during its maturation.

At later stages, GUS activity was also detected during the transformation of the pistil into seed pods. Initially, a highly specific signal was observed in the transmitting tissue of the pistil style, whereas at later stages of silique maturation, the signal became visible along the entire surface of the valves (**Fig. 4**).

Expression of the *AtCAN2* promoter in plant tissues

Observations similar to those obtained for the gene encoding AtCAN1 were also made for the second member of the SNc family, *AtCAN2*. Despite the considerable similarity in the catalytic properties of these two enzymes, the expression profiles of their genes differed markedly. Expression of the *AtCAN2 promoter::GUS* construct was detected in only two cases. In the seedling, *AtCAN2*, similarly to *AtCAN1*, showed strong and highly specific expression in stipules. In mature plants, we detected the GUS signal only in the leaf hydathodes (**Fig. 5**). No GUS signal was observed in other tissues in which *AtCAN1 promoter::GUS* activity had previously been detected.

Discussion

Plant development is tightly linked to programmed cell death, with DNA degradation as a key hallmark. To date, research in this area has focused primarily on the role of S1/P1 family nucleases. However, given the complexity of PCD at both the molecular and histological levels, it is likely that a larger number of nucleases are involved. While reviewing the *A. thaliana* genome for genes encoding degradative nucleases, we found that, in addition to the S1/P1 family, *A. thaliana* also encodes two nucleases belonging to the SNc family. Previous studies have shown that in bacteria, members of this family participate in various degradative processes [16, 17]. Our own studies on plant members of this family confirmed the degradative nature of these enzymes, and an analysis of publicly available microarray data suggested that they may be involved in some specific types of plant PCD [12]. To confirm this hypothesis, we carried out histochemical analyses, considering GUS reporter assays to be the most informative in this context.

The results presented here demonstrate that *AtCAN1* and *AtCAN2* gene expression are highly tissue-specific. All observed cases of their induction, as revealed by promoter-driven GUS activity, can be grouped into three main categories.

The first of these categories includes plant structures that are clear examples of organs undergoing PCD, such as the root cap, vascular bundle elements, senescent leaves, tapetum, and maturing seed pod. Among vegetative plant organs, the root cap is a classic example of a structure undergoing PCD. As the root grows and pushes through the soil, the outermost layer of the root cap, the lateral root cap cells (LRC), is subject to mechanical damage. Therefore, to protect the delicate apical meristem, these cells regularly undergo programmed cell death [18]. Because the GUS signal we observed closely correlates with the LRC, we conclude that the AtCAN1 nuclease is involved in PCD in this tissue. The correlation of GUS activity with the vascular bundles of the root, stem, and leaves also suggests a link between AtCAN1 and PCD, as xylogenesis in these cells represents another well-established example of programmed cell death.

Our research also demonstrated that the GUS signal correlates with the best characterized example of PCD in flowers, namely, tapetal cell death. During PCD, the tapetum releases enzymes, lipids, and nutrients that contribute to pollen grain formation. Our observations further revealed that the GUS marker driven by the *AtCAN1* promoter also follows this pattern. At later stages, as flower development progresses into seed formation, the GUS signal appeared two additional times. The first occurrence was in the transmitting tissue of the pistil style. Programmed cell death takes place in the transmitting tract around the time of pollination and facilitates the penetration of the pollen tube into the ovary chamber. Moreover, it is believed that components of this tissue provide nutritional support to the pollen tube [19]. The final stage associated with seed formation, during which *AtCAN1* is expressed, occurred in the silique coats. As in the previous cases, the presence of PCD in the siliques was experimentally confirmed. This process is linked to silique valve dehiscence, which enables seed release. Silique cell death is commonly classified as a form of PCD-related senescence and is accompanied by the expression of the SAG13 gene, widely used as a marker for diverse processes associated with PCD [20].

The final and most striking evidence for the involvement of AtCAN1 nuclease in PCD is the detection of a GUS signal that emerges and spreads as leaf senescence progresses. Leaf senescence is one of the best-characterised forms of programmed cell death. During this process, gene expression shifts from pathways promoting photosynthesis and growth to those associated with degradation and recycling. This enables the plant to recover valuable nutrients (e.g., nitrogen and phosphorus from nucleic acids) and redistribute them to newly developing tissues or reproductive organs [21].

The second category of cases in which we identified *AtCAN1* promoter activity involves cells whose primary biological function is plant communication with the external environment. This group includes root hairs, stomatal guard cells, and hydathodes. Because these cells are particularly vulnerable to pathogens under natural conditions, they have evolved various protective mechanisms. Studies have shown that one of the main mechanisms employed by these structures is hypersensitive response (HR)-associated cell death [22–27]. In cells capable of HR, proteins involved in pathogen defence are pre-synthesized and maintained in a state ready for rapid activation, ensuring an immediate response to pathogen attack. Since the HR reaction is also accompanied by nucleolytic activity and host DNA degradation [28], we assume that *AtCAN1* expression may indicate the participation of this nuclease in such a process. Moreover, it cannot be excluded that AtCAN1 nuclease activity in infection-exposed cells is directed against pathogen genetic material, similarly to what has been demonstrated for PR-10 ribonuclease [29, 30].

A significant and unexpected finding of our study was the intense and highly specific expression of the *AtCAN1* promoter in a third group of plant structures, including stipules, trichomes, and the basal parts of the hypocotyl in early seedlings, as well as *AtCAN2* promoter activity in stipules. According to current knowledge, these structures, unlike the previously mentioned ones, are not subject to developmentally or environmentally induced programmed cell death in plants. Therefore, the presence of SNc nucleases in these structures cannot be explained by these processes. Instead, their expression might be associated with a shared feature of these structures – the occurrence of endoreduplication in their cells [31–33].

Endoreduplication is a process that has long been observed in various tissues of higher plants. It results in endopolyploidy through repeated rounds of DNA synthesis uncoupled from cell division and is restricted to specific, terminally differentiated cells. The primary biological consequence of endoreduplication is considered to be cell enlargement and enhanced metabolic capacity, as the presence of multiple gene copies enables increased transcriptional activity. This functional interpretation is consistent with the frequent occurrence of endoreduplication in storage tissues, such as the endosperm of monocotyledons and the cotyledons of dicotyledonous plants, as well as in various secretory cells, including glandular trichomes. Although *Arabidopsis* trichomes are not classified as glandular, they also produce and accumulate specific secondary metabolites, mainly flavonoids, phenolics, and stress-related proteins [34]. By contrast, the presence of polyploidy in seedling

stipules remains less readily explained. In *A.thaliana*, stipules are small, leaf-like structures located at the base of the leaf petiole and, unlike in many other species, do not play significant protective or mechanical roles. It has been suggested that polyploidy in these organs results from an endoreduplication-dependent mechanism that constrains tissue size [35].

However, regardless of the biological role of polyploidy, the question arises of what ultimately happens to the DNA accumulated in polyploid cells. Nucleic acids contain considerable amounts of nitrogen and phosphorus, which are well known to be limiting factors for plant growth due to their restricted availability in the environment. It is therefore widely accepted that the nucleolytic activity accompanying PCD in plants serves to recycle these essential elements from dying cells. This raises the question of whether mechanisms exist that allow the recovery of DNA components from polyploid cells once they have fulfilled their role as carriers of genetic information. Such a mechanism is likely to operate in the endosperm of monocot seeds, where strong nucleolytic activity has been observed during the mobilization of stored reserves [5, 36]. Nevertheless, no evidence of DNA recycling from living polyploid cells has yet been reported. In this context, our observation of SNc nuclease promoters activity in *Arabidopsis* polyploid cells is consistent with the hypothesis that plants may perform this type of recycling. Notably, because stipules, trichomes, and lower parts of the hypocotyl are not classical storage organs such as endosperm or cotyledons, the presumed degradation of polyploid DNA in these structures may represent a hitherto unidentified form of nutrient recycling. At this point, it is conceivable that polyploidy serves as a mechanism for storing components required for nucleic acid synthesis in growing leaves. Such a role could help to explain SNc nucleases activation observed in structures such as seedling stipules at the base of developing leaves.

The relationship presented in this paper between SNc nuclease expression and programmed cell death indicates that at least two nuclease families, SNc and S1/P1, participate in genomic DNA degradation during this process. It is instructive to compare the tissue expression profiles of the AtCAN nucleases with that of the BFN1 protein, the best-studied representative of the S1/P1 family [10]. This comparison shows that nucleases of both families are expressed in senescent leaves, differentiating xylem, degrading tapetum, siliques, valves, and the transmitting tract. Interestingly, *AtCAN1* is also expressed in the root cap, a classic example of a tissue undergoing PCD, in which BFN1 expression has not been reported despite comprehensive studies of its promoter. Conversely, BFN1 expression was detected in several tissues where *AtCAN1* gene expression was not observed, such as the abscission zone, developing seed, and senescing petals. These analyses indicate that the expression of nucleases from both gene families overlaps in some classical PCD tissues, while retaining individual specificity in others. Notably, BFN1 expression was not detected in cells we considered potentially susceptible to HR-PCD due to their high exposure to pathogens, such as root hairs, stomata, and hydathodes. Moreover, a specific feature of AtCAN1 and AtCAN2 is their occurrence in polyploid cells, as *BFN1* expression has likewise not been observed in these elements to date.

Comparison of the expression patterns of SNc family proteins with those of BFN1 raises the question of whether any functional interdependence exists between these nucleases. Analysis of the mechanisms by which they operate within the cell may help address this issue. In young leaves, BFN1 was localized to filamentous reticulum-derived structures surrounding the nuclei, whereas in senescing cells it was detected within fragmented nuclei. This localization pattern supports the proposed role of BFN1 as a genomic DNA-degrading enzyme involved in senescence[10]. By contrast, our previous studies have shown that the nucleases described in this work, AtCAN1 and AtCAN2, are localized to the plasma membrane and exhibit partial homology to certain periplasmic components of bacterial ABC-type transporters. It is worth noting that membrane localization of nucleases involved in PCD in plants has not previously been reported, although bacterial homologues of these nucleases have also been found to be anchored in the bacterial cell membrane [17]. Taking these observations into account, it can be concluded that S1/P1 nucleases are responsible for nuclear DNA degradation, while SNc nucleases

couple the DNA degradation process with the extracellular transport of their breakdown products. Thus, nucleases from these two families appear to represent distinct mechanisms in the cell. Their coexpression can therefore be interpreted as a form of cooperation, in which both mechanisms complement each other. However, the question of whether SNc nucleases can function independently of S1/P1 remains unresolved, since among the known S1/P1 nucleases, only the expression profile of *BFN1* has been characterized, while this family in *A. thaliana* comprises four additional genes [11].

Conclusions

The results presented here demonstrate that the expression of AtCAN1 and AtCAN2 genes is highly tissue-specific. All observed cases of expression, revealed by promoter-driven GUS activity, fall into three main categories: (i) plant structures that clearly represent organs undergoing PCD, (ii) elements whose primary biological function is communication with the external environment and are therefore susceptible to hypersensitive response-associated cell death, and (iii) in polyploid cells. These results show that the involvement of SNc family nucleases in DNA degradation during various forms of plant PCD is as widespread as the previously reported involvement of S1/P1 proteins. In many cases, the expression profiles of nucleases from these two families overlap, suggesting cooperative action during PCD. Since SNc nucleases are plasma membrane proteins, whereas S1/P1 nucleases have been identified within nuclei, they appear to represent distinct yet complementary mechanisms of nucleolytic activity in the cell. Furthermore, we demonstrate that SNc nucleases are also specifically expressed in organs that do not undergo PCD but are characterized by endoreduplication. This suggests that SNc nucleases participate in a previously unexplored mechanism enabling the redistribution of polyploid DNA building blocks.

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Figures

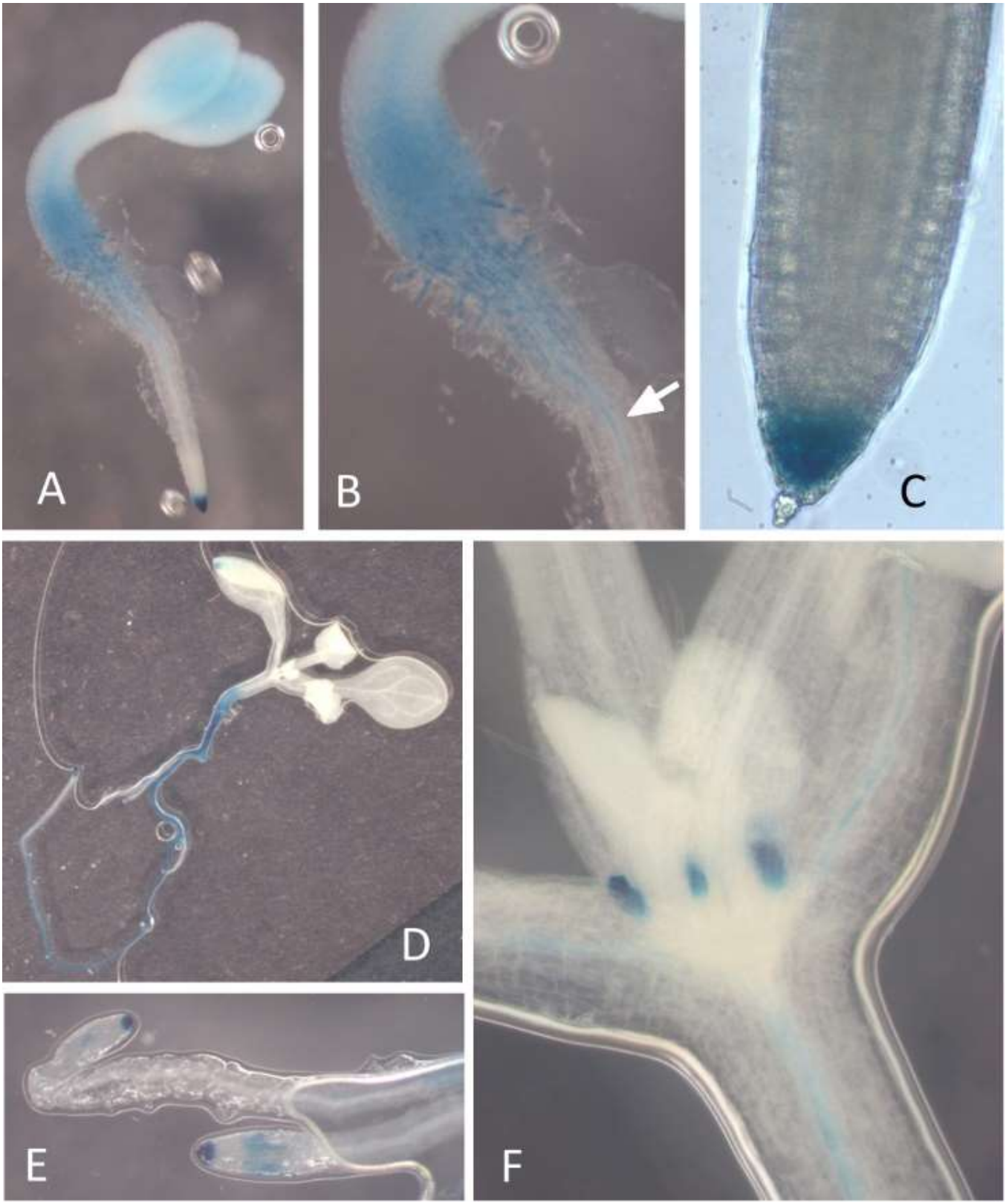

Fig. 1 Histochemical staining of GUS activity driven by the *AtCAN1* promoter in *A.thaliana* seedlings. (**A**) Overall view of a two-day-old seedling. (**B**) Enlargement of the transition zone between the hypocotyl and the root, showing GUS signal in the root hairs and in the developing axial cylinder (arrow). (**C**) Enlargement of the root cap. (**D**) Overall view of a 7-day-old seedling. (**E**) Enlargement of the terminal, younger parts of a 7-day-old seedling root. (**F**) Enlargement of the floral shoot apex region, with GUS signals in the stipules and in the developing vascular bundles of stem and leaf petioles.

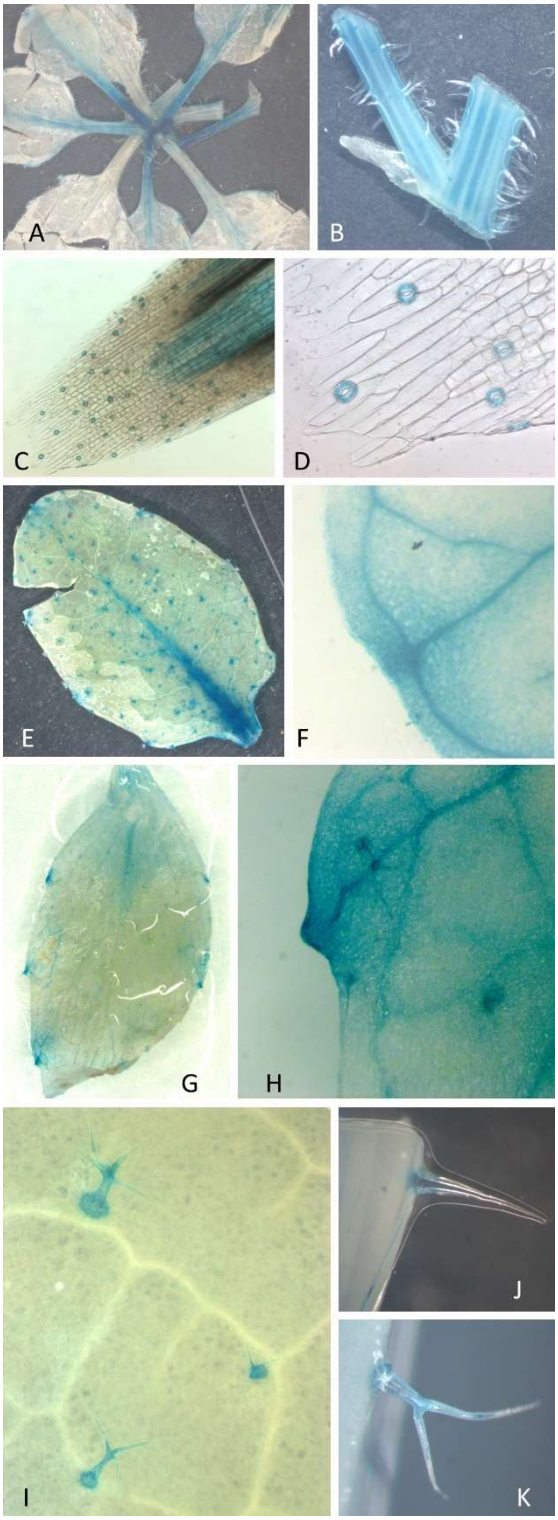

Fig. 2 Histochemical staining of GUS activity driven by the *AtCAN1* promoter in vegetative organs of mature plants (**A**) 21-day-old *A. thaliana* plant with dominant GUS signal in leaf petioles. (**B**) Cross-section of the lower part of the flower stem with an attached leaf petiole, showing GUS signals in the vascular bundles. (**C**) microscopic image of an oblique stem section, showing GUS signal in stomata and vascular bundles. (**D**) Enlargement of the stem epidermis with GUS signal in stomata. (**E**) Overall view of a rosette leaf. (**F**) Enlargement of a leaf region showing GUS signal in a hydathode located at the smooth leaf margin. (**G**) Overall view of a rosette leaf with serrated margins. (**H**) Enlargement of a GUS signal in a hydathode located at the tooth-like protrusion. (**I**) Trichomes on the leaf surface. (**J**) Enlargement of the stem trichome. (**K**) Enlargement of a single trichome at the edge of the leaf.

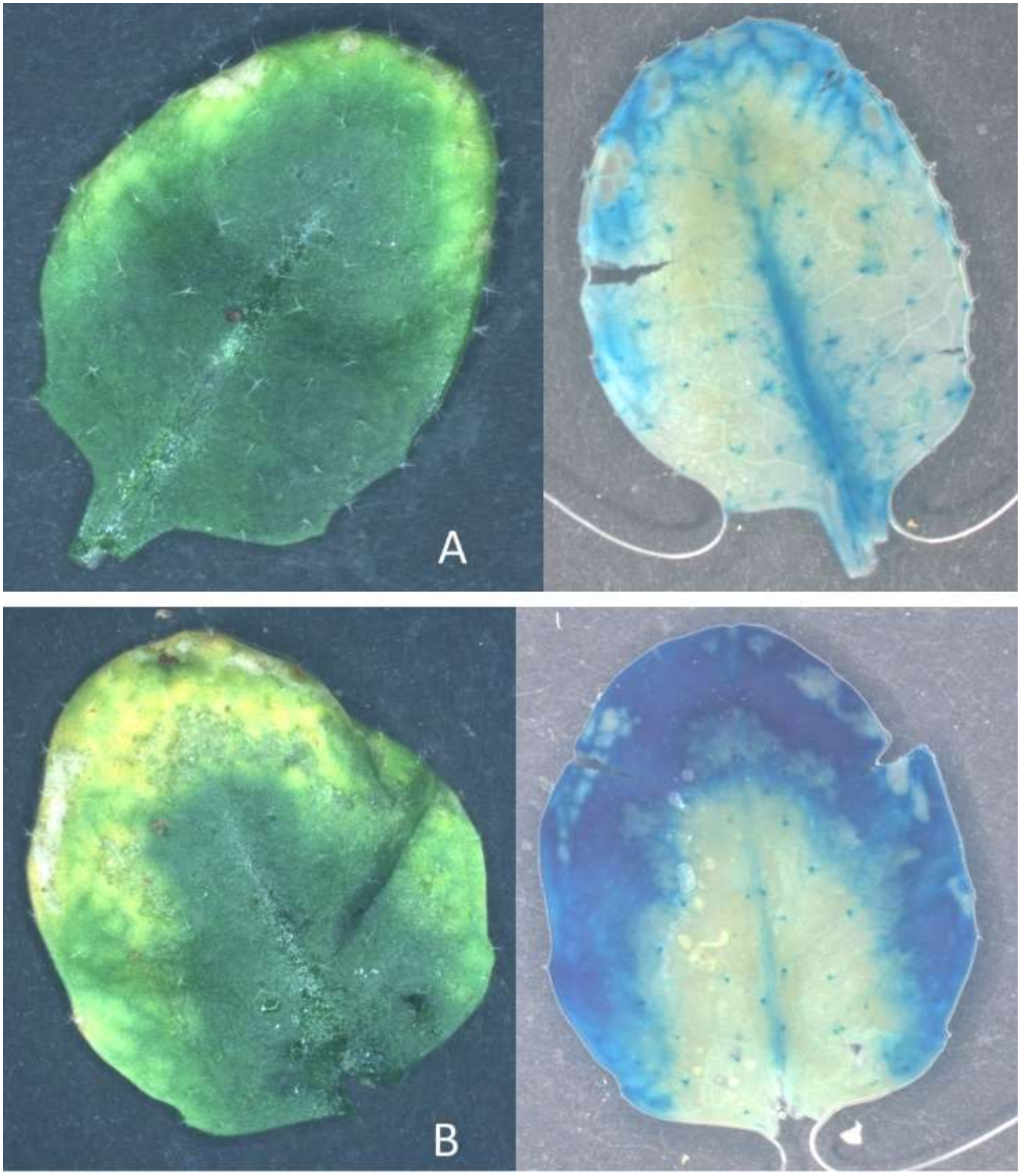

Fig. 3 Histochemical staining of GUS activity driven by the *AtCAN1* promoter in senescing leaves. (**A**) A leaf at an early stage of senescence, before (left) and after (right) GUS staining. (**B**) A leaf at a later stage of senescence, before (left) and after (right) GUS staining.

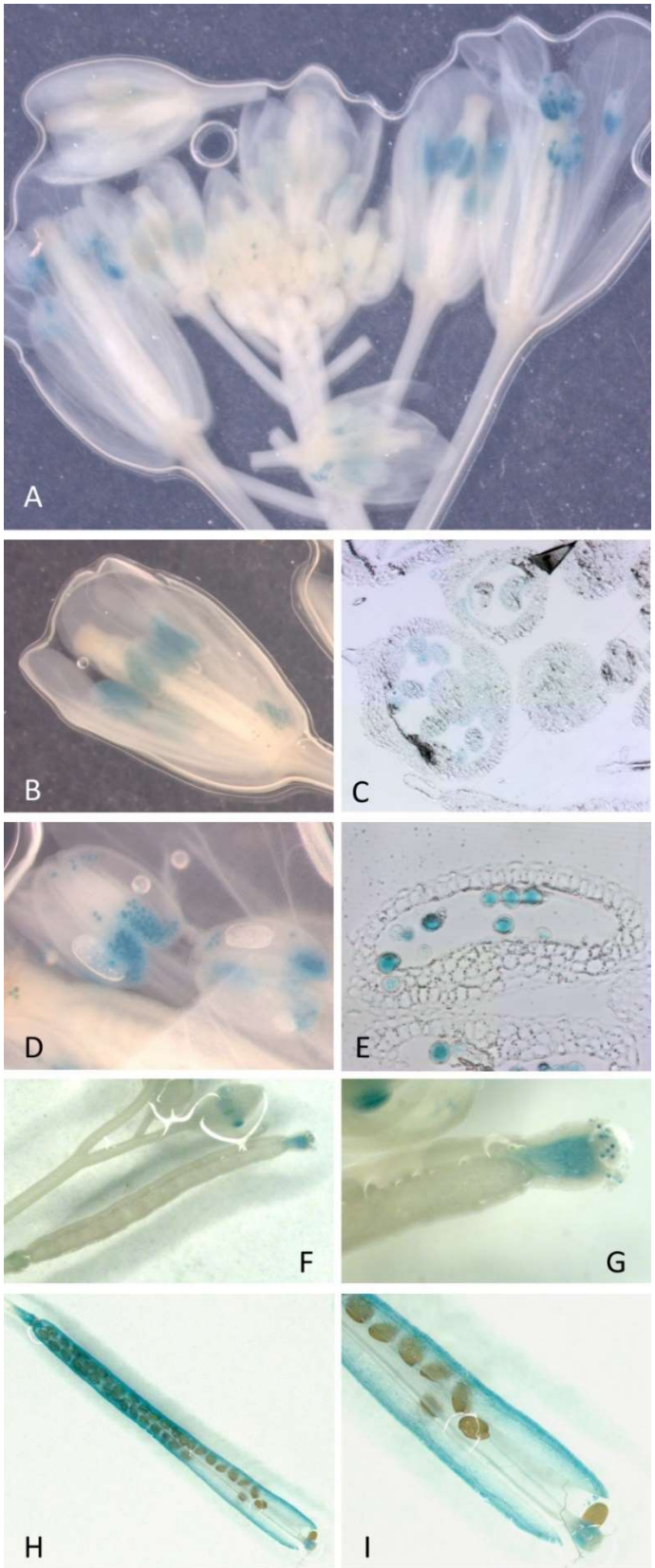

Fig. 4 Histochemical staining of GUS activity driven by the *AtCAN1* promoter in *Arabidopsis* flowers and developing siliques. (**A**) Overall view of the inflorescence. (**B**) Enlargement of a flower at 14 stage of development. (**C**) Microscopic image of a cross-section of anthers at 14 stage of flower development. (**D**) Enlargement of the flower at 15 stage of development. (**E**) Microscopic image of a cross-section of anthers at 15 stage of flower development. (**F**) Overall view of an early silique. (**G**) Enlargement of the transmitting tissue of the pistil style. (**H**) Overall view of a late silique. (**I**) Enlargement of the late silique showing GUS signal in the silique valves.

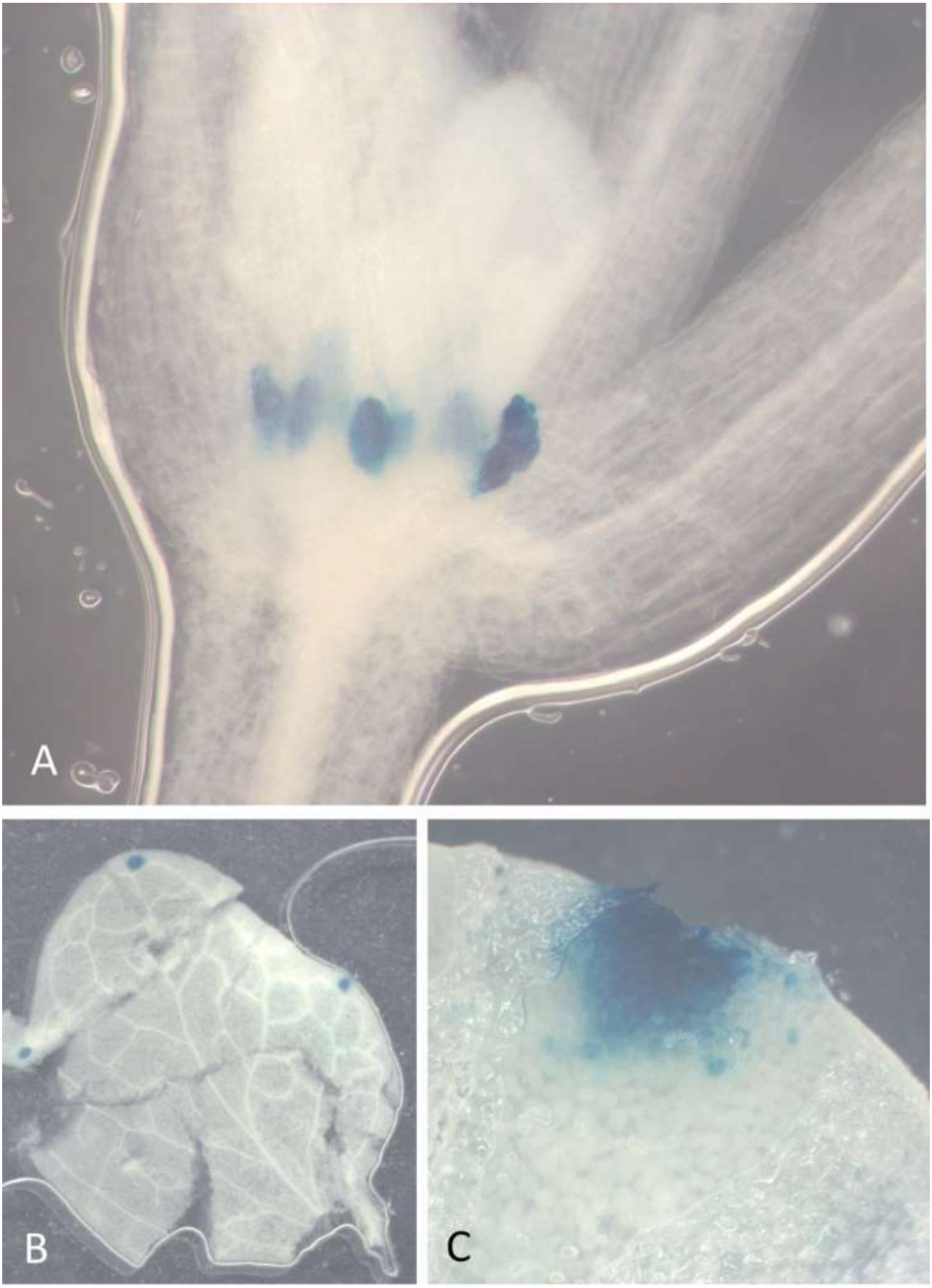

Fig. 5 Histochemical staining of GUS activity driven by the *AtCAN2* promoter in an *Arabidopsis* plant. (**A**) The floral shoot apex region of a 7-day-old seedling, showing GUS signal in the stipules. (**B**) Overall view of a mature leaf. (**C**) Enlargement of a leaf region showing GUS signal in the hydathodes.