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## **Molecular Cloning and Developmental Expression of AtGR1, a New Growth-Related Arabidopsis Gene Strongly Induced by Ionizing Radiation**

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# Molecular Cloning and Developmental Expression of *AtGR1*, a New Growth-Related Arabidopsis Gene Strongly Induced by Ionizing Radiation

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Deveaux, Y., Alonso, B., Pierrugues, O., Godon, C. and Kazmaier, M. Molecular Cloning and Developmental Expression of *AtGR1*, a New Growth-Related Arabidopsis Gene Strongly Induced by Ionizing Radiation. *Radiat. Res.* **154**, 355–364 (2000).

Screening for mRNAs that accumulate after DNA damage induced by ionizing radiation, we have isolated a 2.0-kb cDNA coding for a new Arabidopsis PEST-box protein named *AtGR1* (*A. thaliana* gamma response 1) with an expression profile similar to that observed for several plant cell cycle-related proteins. Using an anti-*AtGR1* antibody, we have shown that the *AtGR1* protein is expressed at basal levels in mitotically dividing cells (meristematic tissues and organ primordia) and at a strongly enhanced level in endoreduplicating cells (stipules, trichomes). Using transgenic Arabidopsis plants that express the *GUS* reporter gene under the control of the *AtGR1* promoter, we have demonstrated that the observed *AtGR1* protein distribution is due to the promoter activity. Our results suggest that basal *AtGR1* levels are associated with progression through mitosis, whereas elevated intracellular levels of *AtGR1* seem to induce changes between the S and M phases of the cell cycle that trigger somatic cells to enter the endoreduplication cycle. Ionizing radiation-induced rapid and dose-dependent accumulation of *AtGR1* mRNA in cell cultures and plant tissues leads to tissue-specific accumulation of *AtGR1* protein, best observed in ovules, which never undergo an endoreduplication cycle. It therefore appears that the radiation-induced transient *AtGR1* accumulation reflects DNA damage-dependent transient cell cycle arrest before mitosis, which is necessary to accomplish DNA repair prior to chromosome segregation and cytokinesis.

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## INTRODUCTION

Ionizing radiation causes a multitude of DNA lesions, including double-strand breaks, single-strand breaks, mod-

ified bases, and apurinic and apyrimidinic sites (1). This damage, if left unrepaired, can lead to mutation induction, neoplastic transformation, or cell death; alternatively, depending on the level of damage, cells can recover from the radiation insult. After exposure to ionizing radiation, eukaryotic cells respond by arresting cell cycle progression, inducing transcription of specific genes, and activating DNA repair machinery. The early events leading to the initiation of signaling cascades and the activation of the transcription of specific groups of genes after irradiation are under the control of a DNA damage surveillance network (2–4).

In mammalian cells, the protein TP53 plays a central role in the response to genotoxic stress (5–7). The upstream events or signals that flow to TP53 are mediated by several types of DNA damage, including double-strand breaks produced by  $\gamma$  radiation and the presence of DNA repair intermediates after ultraviolet irradiation or chemical damage to DNA. This results in a rapid and dose-dependent increase in the intracellular level of TP53 and activation of TP53 as a transcription factor (8). The activated TP53 protein enhances the rate of transcription of a few known genes that carry out the major TP53-dependent downstream events, namely cell cycle arrest and apoptosis. DNA damage-induced cell cycle arrest by TP53 is mediated mainly by transcriptional activation of the gene coding for CDKN1A (also known as p21), an inhibitor of cyclin-dependent kinases (9). This leads to the repression of growth-related genes and transient G<sub>1</sub>-phase arrest, to provide time for repair of damaged DNA (10).

The knowledge of the general response pathways to genotoxic stress in plants is less developed than the knowledge of these pathways in bacteria, yeast or mammals, especially the activation of specific signaling cascades leading to cell cycle arrest and DNA repair (11, 12). Also, plant development differs from that of animals in that the differentiation of germline tissue (gametogenesis) occurs very late in development; during the prominent vegetative phase of plant development, there is no strictly defined germline tissue. Thus the maintenance of genome integrity during plant development seems especially important in view of

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the transmission of an intact genome from the somatic cells to the germline. This is in line with the observation that plant cells are highly resistant to DNA-damaging treatments such as X rays, mitomycin C and extreme heat shock, since they are able to respond to such stress by the induction of significantly higher levels of induced recombination events than mammalian cells (13, 14). Progress in the identification of plant genes involved in the response to genotoxic stress is based on homology cloning (15–18) or stems from the identification of altered gene functions in mutants that are hypersensitive to ionizing radiation (19–21). Using differential display, a sensitive approach for evaluating changes in gene expression after exposure of plant cells to ionizing radiation, we anticipated that we would be able to characterize new plant genes involved in DNA damage-induced cell cycle arrest, DNA repair, and/or programmed cell death.

Here we describe a new gene, *AtGR1*, that is expressed in mitotically active tissues and endoreduplicating tissues from unstressed plants and that is similar in some respects to the expression profile of the Arabidopsis cell cycle-related gene *CKS1At* (22). Enhancement of constitutive levels of AtGR1 protein observed in specific tissues undergoing somatic endoreduplication suggests that the decision between the mitotic cell cycle and endoreduplication could be dependent at least in part on the intracellular content of AtGR1 protein. On the other hand, radiation-induced DNA damage leads to transient and dose-dependent transcriptional activation and protein accumulation, particularly in germline tissue that never undergoes endoreduplication. This is consistent with a potential role of AtGR1 in the response to genotoxic stress by blocking mitotic cell division in irradiated cells and thus preventing premature entry into mitosis before completion of DNA repair.

## MATERIALS AND METHODS

### Plant Materials and Treatments

*A. thaliana* cell suspensions were grown with constant shaking at 25°C and continuous white light as described earlier (23). For maintenance, cell cultures were split twice per month, and all experiments were carried out with 1-week-old cell cultures, corresponding to exponentially growing cells. *Arabidopsis thaliana* (L) Heynh (Columbia ecotype) plants were cultivated in a growth chamber on a 16 h light:8 h dark cycle. For microscopy and histology, young seedlings at the 2- to 4-leaf stage were grown on agar medium.

Chemical or physical treatments of cell suspensions were applied at the beginning of exponential growth phase, corresponding to 8–12% packed cell volume. To induce oxidative stress, 5 mM H<sub>2</sub>O<sub>2</sub> was added directly to the culture medium. For  $\gamma$  irradiation (<sup>60</sup>Co  $\gamma$  irradiator, 2200 TBq; CIS Bio International, France), cells were filtered to avoid side effects due to products of the radiolysis of the culture medium. After  $\gamma$  irradiation (25–100 Gy at a dose rate of 35 Gy/min), cells were resuspended in the culture medium, harvested at the indicated times, and stored at –80°C. Whole plants were irradiated individually, and the tissues were harvested and stored at –80°C.

### RNA Techniques

PolyA<sup>+</sup> RNA from plant tissues or cell cultures was prepared as described by Montané *et al.* (24). The DD-RT-PCR reactions were carried out with purified mRNA preparations from untreated and  $\gamma$ -irradiated cells as described previously (25). For Northern blot analyses, aliquots of 5  $\mu$ g of polyA<sup>+</sup> RNA were transferred to Hybond-N nylon membranes (Amersham) and hybridized with specific cDNA probes according to standard procedures (26). Unless otherwise stated, hybridizations were accomplished with two labeled probes, the cDNA of interest and an internal control, e.g. the *A. thaliana* 25S cDNA. Autoradiographs were scanned and quantified using a Storm 840 PhosphorImager (Molecular Dynamics, SA, Paris, France). For quantitative RT-PCR experiments, total RNA was extracted from plant tissues (27), and the RT-PCRs were run as described (28) with two *AtGR1* mRNA-specific primers, AATTCCTTCAGGAGGAGG and GTGTTTCCTTTCAAGATAACTCGAG. The specific primers GCTGAGAGATTCAGGTGCC and GAGATCCACATCTGCTGG of the constitutively expressed actin 8 gene (29) were used as an internal control. The PCR reactions were in the linear range, as determined by comparisons to the results of preliminary calibration experiments.

### Isolation of *AtGR1* cDNA

An *A. thaliana* cDNA library (30) was screened with the DD-RT-PCR *AtGR1* fragment as probe using standard protocols (26). Clones that gave strong positive hybridization signals and had plant cDNA inserts of approximately the size expected from Northern blot analysis were chosen for sequencing.

### Protein Extraction, SDS-PAGE and Western Blotting

To prepare total protein extracts from cells and plant tissues, frozen samples were ground in liquid nitrogen and dissolved in 1 ml g<sup>-1</sup> of a denaturing buffer [4 % SDS (w/v),  $\beta$ -mercaptoethanol (w/v), 5 % sucrose (w/v), and 120 mg of polyvinylpyrrolidone per gram of tissue]. The mixture was boiled for 3 min and centrifuged for 30 min at 20,000g and the supernatant was saved. Proteins were quantified by the method of Lowry *et al.* (26). Extracted proteins were diluted in Laemmli buffer (31), denatured, and loaded onto a 10% SDS acrylamide gel. The gels were stained using Coomassie blue or immunoblotted as described by Towbin *et al.* (32).

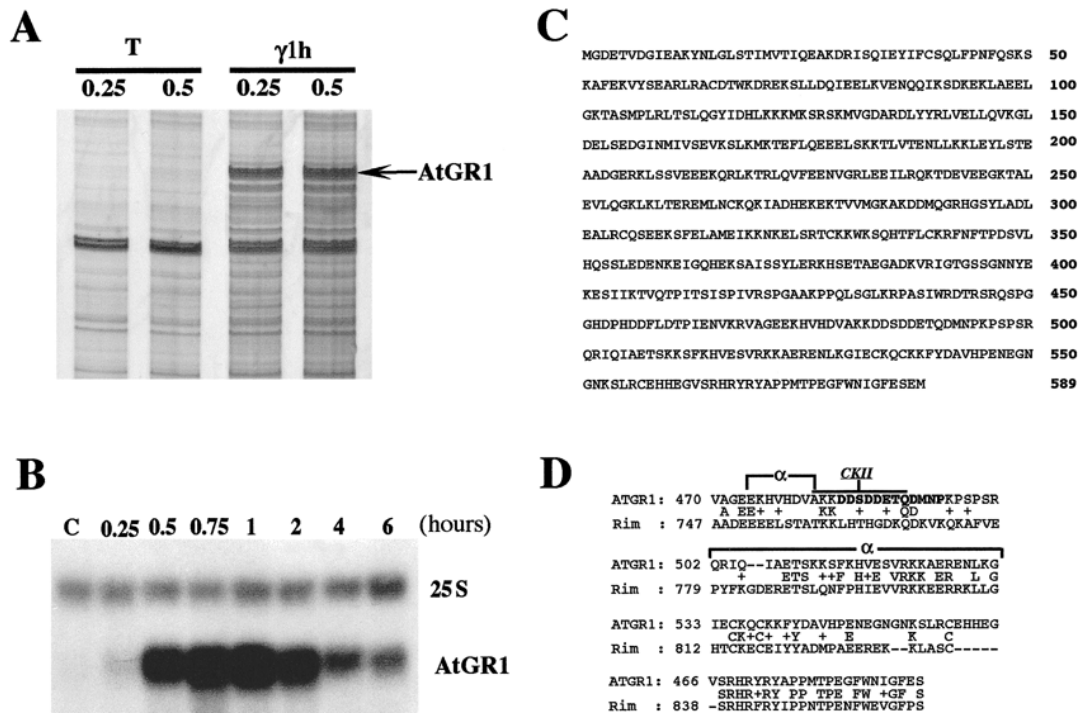
### Immunological Techniques

A C-terminal oligopeptide (SRHRYRYAPPMTPEGFWNIG), deduced from the *AtGR1* cDNA, was used to prepare a specific antibody (Neosystem, Département Peptides d'Isochem SA, France). The specificity of antibody recognition was demonstrated by Western blotting of bacterial extracts overexpressing a recombinant His-tag/*AtGR1* fusion protein with the anti-*AtGR1* antibody and a commercial anti-His tag antibody, as well as the recognition of a single protein band of the expected size in plant protein extracts (data not shown). Protein-antibody interaction was monitored using the alkaline phosphatase method according to Blake *et al.* (33). Immunolocalization experiments with histological sections were performed as described by Weiss *et al.* (34).

### Construction of Transgenic Plants and Histochemical Analysis

A 4.2-kb fragment of the Arabidopsis genomic DNA clone corresponding to the 5' end of the *AtGR1* gene (Deveaux and Kazmaier, in preparation) was cloned into the pBSK<sup>+</sup> vector. An *Nco*I site was introduced at the translation start by site-directed mutagenesis (35), and a 2.2-kb promoter fragment was excised as an *Eco*RI/*Nco*I fragment. The *Eco*RI/*Hind*III fragment of the pSLJ4D4 vector (36) was excised and cloned into a pBSK<sup>+</sup> vector. This fragment contained the *GUS* gene cloned under the control of the promoter 35S. The new vector pBSK<sup>+</sup>/*GUS* was digested with *Eco*RI and *Nco*I to eliminate the initial 35S promoter and





**FIG. 1.** Cloning of *AtGR1*, a new Arabidopsis cDNA that accumulates after irradiation. Panel A: Area of a differential display gel showing the high-molecular-weight amplified products obtained with the primers T11GC and AGCCAGCGAA using as templates the indicated amounts of reverse-transcribed mRNA derived from untreated (T) or irradiated (γ1h; i.e. harvested 1 h after γ irradiation) cell suspensions. The dose used was 25 Gy. The amplification product *AtGR1* corresponds to a putative mRNA induced by ionizing radiation (arrow). Panel B: Northern blot analysis of *AtGR1* mRNA accumulation in *A. thaliana* cell suspensions. Messenger RNA prepared from untreated or irradiated cells (25 Gy) at the indicated times was used for Northern blot hybridization with a mix of the 422-bp *AtGR1* differential display cDNA and the constitutive *A. thaliana* ribosomal 25S cDNA as an internal control. Panel C: The *AtGR1* amino acid sequence derived from the isolated cDNA. The *AtGR1* cDNA sequence has been deposited in the EMBL database and was assigned the accession number AJ131705. Panel D: Amino acid sequence alignment of C-terminal conserved domain between *AtGR1* and human protein RIM (now known as RBBP). Identical amino acids are indicated by their symbols, similar amino acids by (+). Bold symbols specify the PEST-box domain. The black line above residues 481–491 indicates the casein kinase II phosphorylation site consensus. Alpha helices predicted by the NNSSP (next-neighbor secondary structure prediction) program are indicated by boxes.

was ligated with the 2.2-kb *AtGR1* promoter. The promoter–GUS construct was digested by *EcoRI* and *XhoI* restriction enzymes and ligated into the transformation vector SLJ7555I6 (gift from J. Jones) to generate the plasmid p*AtGR1*/GUS.

Plasmids were incorporated into *Agrobacterium* using a triparental mating system with tetracyclin and rifampycin selection, and Arabidopsis (Columbia ecotype) plants were transformed by vacuum infiltration (37). Primary transformants were screened through BASTA resistance. Homozygous transformant lines were isolated in generation T2. RT-PCR was performed on total RNA to measure the presence of *GUS* mRNA (28) using the gene-specific primers GTCACGCCGTATGTTATTGCCGGG and CGAGGTACGGTAGGAGTTGGCCCC to generate a 600-bp fragment and actin 8 as an internal control. For *in situ* histochemical staining, plantlets or organs were incubated in acetone for 30 min to increase the diffusion of GUS substrate, and samples were prepared as described by Yokoyama *et al.* (38). Examination of plant tissue samples was carried out using a Leica Dialux 20 microscope. The determination of GUS activity by fluorescence spectrometry was carried out according to standard protocols (39).

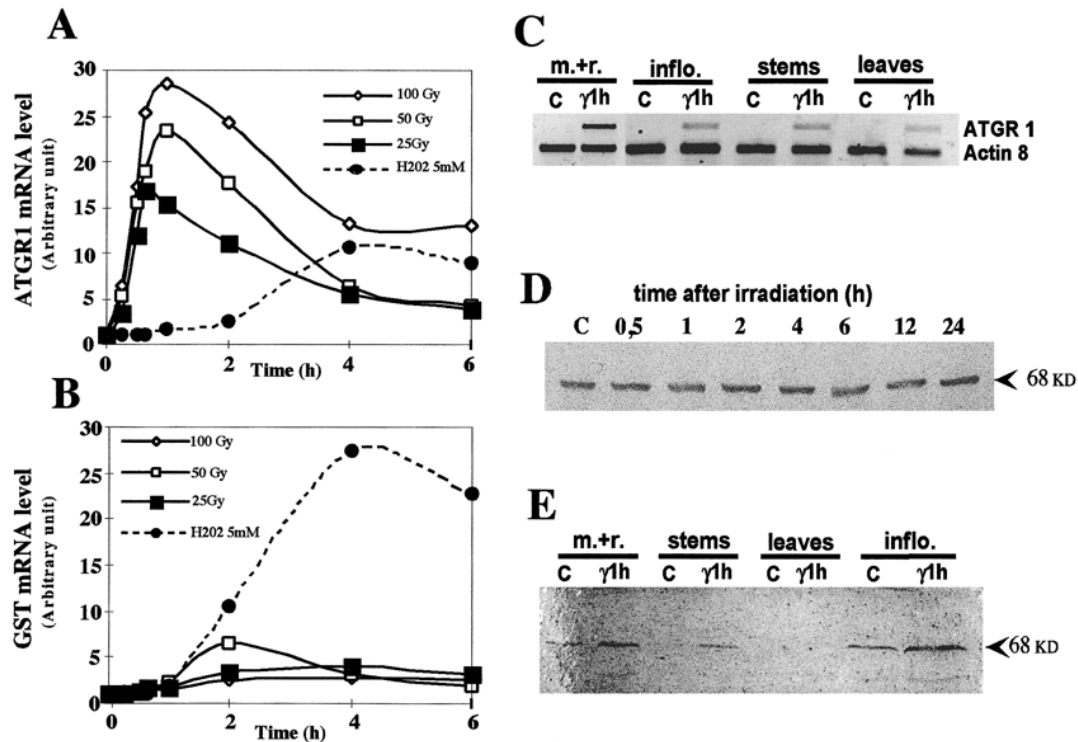
## RESULTS

### Cloning and Characterization of the *AtGR1* Gene

We employed differential display (25) to screen for mRNAs that are rapidly induced by ionizing radiation in

Arabidopsis cell cultures. On the display gels, among the candidate bands corresponding to differentially expressed mRNA species, one appeared to be strikingly accumulated in cells 1 h after irradiation with 25 Gy (indicated by an arrowhead in Fig. 1A). For further analysis, the corresponding cDNA fragment was reamplified by PCR and used as a probe in an RNA blot hybridization experiment to confirm the observation of the display gel. As shown in Fig. 1B, the cDNA fragment hybridized to a single RNA species of about 2 kb that rapidly and strongly accumulates after irradiation with 25 Gy in nonsynchronous Arabidopsis cell cultures. To identify the gene product, one clone containing a 1971-bp cDNA insert, which closely corresponds to the size of the transcript observed in RNA blot analysis, was isolated from an Arabidopsis cDNA library (30). Sequence analysis revealed an open reading frame of 1767 nucleotides, sharing identity at the 3' part with the PCR fragment as expected, and the gene was subsequently named *AtGR1*.

The protein encoded by the *AtGR1* cDNA (EMBL database accession no. AJ131708) is composed of 589 amino acids with a predicted size of 68 kDa (Fig. 1C). A BLAST similarity search in the available databases revealed that



**FIG. 2.** Expression studies at the level of mRNA and protein in *Arabidopsis* cell cultures or plant tissues. Panels A and B: Differential effect of ionizing radiation and hydrogen peroxide on *AtGR1* and *AtGST* mRNA accumulation. mRNA prepared from untreated cell suspensions, cell suspensions that had been irradiated with 25, 50 or 100 Gy, or cell suspensions that had been treated with 5 mM H<sub>2</sub>O<sub>2</sub> were used for Northern blot hybridization with two pairs of probes: *AtGR1/At25S* cDNAs and *AtGST/25S* cDNAs. Autoradiographs were scanned and quantified. Panel C: Analysis of *AtGR1* mRNA accumulation in plant tissues by quantitative RT-PCR. Irradiated plants (25 Gy) were harvested 1 h after treatment ( $\gamma$ 1h), and total RNA was prepared from several tissues: shoot meristem and roots (m.+r.), inflorescence (inflo.), stems and mature leaves. Reverse transcription (RT) and gene-specific PCR amplification were carried out with primers chosen to avoid genomic DNA amplification. Panel D: Time course of relative *AtGR1* protein level in *A. thaliana* cell suspension after  $\gamma$  irradiation. Western blot analysis using a specific anti-*AtGR1* antibody was carried out with protein extracts (50 mg) prepared at different times up to 24 h after irradiation (the dose was 100 Gy for results shown in panels D and E). Untreated cells were used as a control. Panel E: Immunodetection of relative *AtGR1* protein levels in the different plant tissues, such as shoot meristem and roots (m.+r.), stems, leaves and inflorescences (inflo.), was carried out with 50  $\mu$ g of total protein extracted from untreated plant tissues (C) and irradiated plant tissues ( $\gamma$ 1h).

*AtGR1* is a new plant protein of unknown function with no structural homologue in bacteria, yeast or animals. The C-terminal part of *AtGR1* produces one significant alignment of sequence conservation with a human retinoblastoma-binding protein, RIM (now known as RBBP) (40), encompassing 80 amino acid residues (Fig. 1D), but the *AtGR1* protein does not contain the highly conserved LxCxE motif for direct protein-protein interaction with RB. Using the PESTFIND program (41), a very strong consensus for a PEST destruction box has been predicted at positions 485 to 496 in the *AtGR1* protein, adjacent to the domain of sequence conservation, and in the center of this predicted PEST box is a strong consensus sequence for a casein kinase II phosphorylation site, which may indicate the phosphorylation-dependent regulation of the stability and/or activity of *AtGR1* (Fig. 1D).

#### DNA Damage is Responsible for Rapid Accumulation of *AtGR1* mRNA and Protein

To establish whether induction of *AtGR1* mRNA is the direct consequence of radiation-induced DNA damage or is

the consequence of radiation-induced hydrogen peroxide formed through water radiolysis, we compared the time course of RNA accumulation in cell cultures after different doses of radiation or application of H<sub>2</sub>O<sub>2</sub> with two probes, the *AtGR1* cDNA and the *Arabidopsis* glutathione-S-transferase cDNA (*AtGST*), a marker gene commonly used for oxidative stress (42). Figure 2A shows that the peak levels of *AtGR1* mRNA accumulation between 45 min and 1 h after irradiation are dependent on dose. Furthermore, a dose of 100 Gy, which corresponds to a severe radiation stress, results in a prolonged persistence of elevated *AtGR1* mRNA levels. In contrast, basic *AtGST* gene expression is not affected by ionizing radiation, regardless of the radiation dose (Fig. 2A). Severe oxidative stress induces, as anticipated, a significant increase in *AtGST* mRNA levels (Fig. 2B). Induction of *AtGR1* mRNA by hydrogen peroxide occurs over similar periods; however, the relative levels of maximal accumulation are less important (about 30%). Essentially, increased levels of *AtGR1* mRNA are first detected 4 h after application of 5 mM H<sub>2</sub>O<sub>2</sub>, a time at which radiation-induced *AtGR1* mRNA levels have almost

returned to basal levels. This observation can be interpreted on the basis that extracellular  $\text{H}_2\text{O}_2$  will diffuse to the nucleus and give rise to DNA damage when the concentration is sufficient to saturate intracellular antioxidant systems. This would then account for the delay and attenuation of induction of the *AtGR1* gene after treatment with  $\text{H}_2\text{O}_2$  compared to the response to ionizing radiation, which penetrates the nucleus instantaneously.

To follow the radiation-induced accumulation of *AtGR1* transcript in whole plants, we performed quantitative RT-PCR (28) on RNA preparations from different plant tissues. As shown in Fig. 2C, within 1 h, ionizing radiation (25 Gy) induces an increase in *AtGR1* mRNA from undetectable basal levels in all major plant tissues tested. *AtGR1* mRNAs accumulate to high levels in the root/meristem fraction and to a lesser extent in leaves, stems and inflorescences. Taken together, our results demonstrate a direct correlation between the radiation dose and *AtGR1* mRNA accumulation, suggesting that radiation-induced DNA damage is the primary signal leading to dose-dependent accumulation of *AtGR1* transcripts in all plant tissues. Similar dose-effect relationships between ionizing radiation and DNA-damaging agents have been reported in mammalian cells (43).

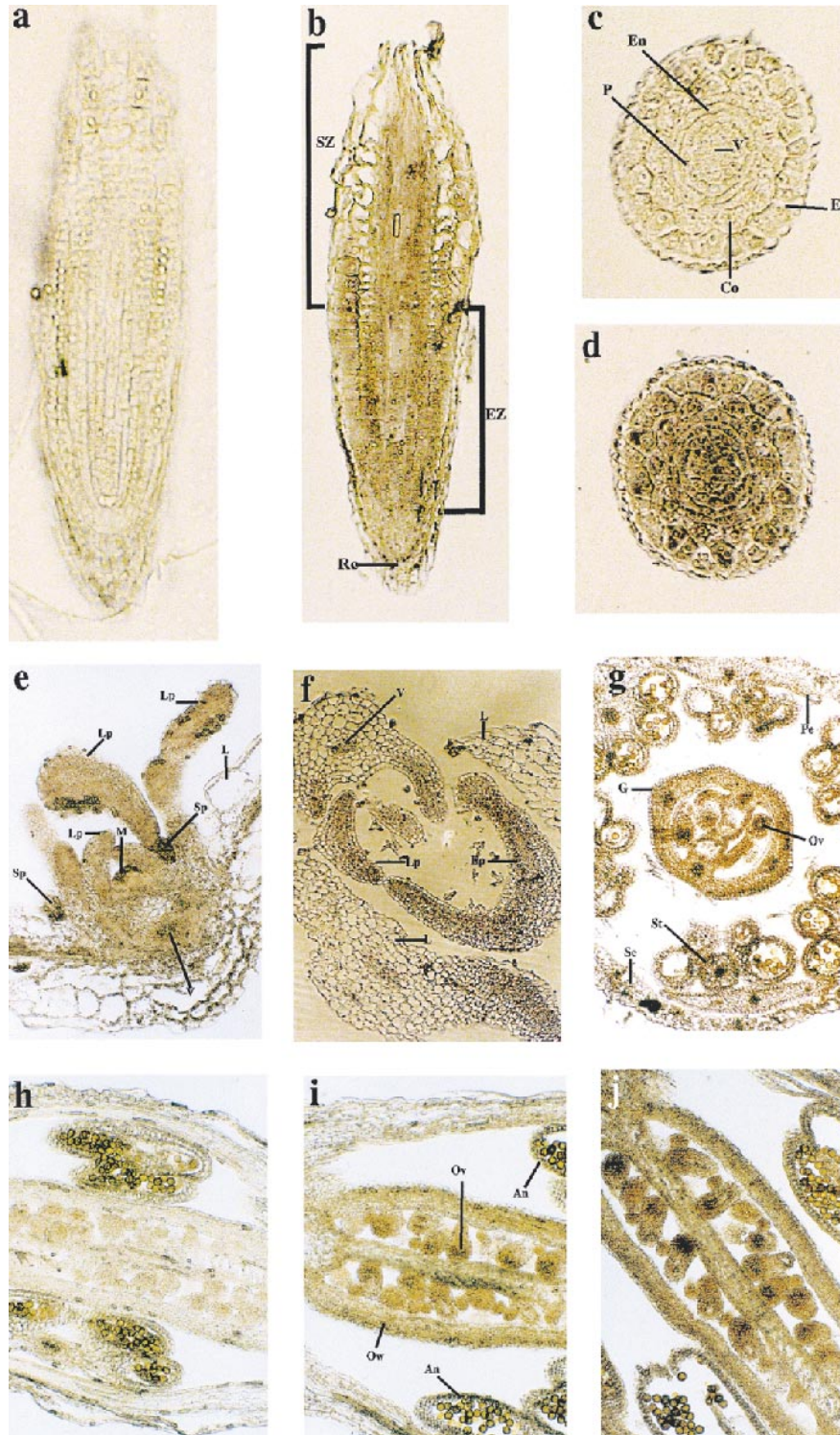
The corresponding relative levels of AtGR1 protein before and after irradiation were revealed by Western blot analysis with a specific anti-AtGR1 antibody. The anti-AtGR1 antibody reveals a protein band of the expected molecular weight (68 kDa) that is present at significant levels in untreated cell cultures, although *AtGR1* transcripts are barely detectable. This may be because the antibody is very efficient in recognizing the minute amounts of AtGR1 protein that are synthesized when mRNA levels are below the limits of detection. Massive *AtGR1* transcript accumulation in these unsynchronized suspensions does not lead to a measurable increase (prompt or delayed) of the 68-kDa protein detected by the anti-AtGR1 antibody (Fig. 2C, D). One possible explanation for this observation is that cell cycle-related events cannot be measured in unsynchronized cell suspensions. We therefore decided to analyze the relative content of AtGR1 protein in extracts prepared from tissues of unstressed and irradiated plants (Fig. 2E). Western blot analysis of protein extracts prepared from the same plant material used for RNA preparations reveals detectable amounts of immunoreactive 68-kDa protein in inflorescences or the root and shoot meristem fraction from untreated plants, but the protein is not present in detectable amounts in stems and leaves from untreated plants. In contrast to the observations made in cell cultures, the basal level of the putative AtGR1 protein increases upon irradiation, except in leaves, where induced protein levels may be below the limit of detection. Thus the 68-kDa protein band as recognized by the anti-AtGR1 antibody is present at detectable levels in nonsynchronously dividing cell cultures and plant tissues that contain large numbers of dividing cells, such as immature flowers, shoot meristem and roots, although the *AtGR1* mRNA is below the level of

detection in those tissues. Mature tissues that are essentially composed of differentiated and nondividing cells (adult leaves and stems) do not contain significant amounts of AtGR1 protein. Ionizing radiation-induced DNA damage leads to accumulation of AtGR1 protein that is observable in plant tissues but not in unsynchronized cell suspensions.

#### *AtGR1 Protein Expression Profile and Ionizing Radiation-Induced Protein Accumulation are Mediated by Promoter Activity*

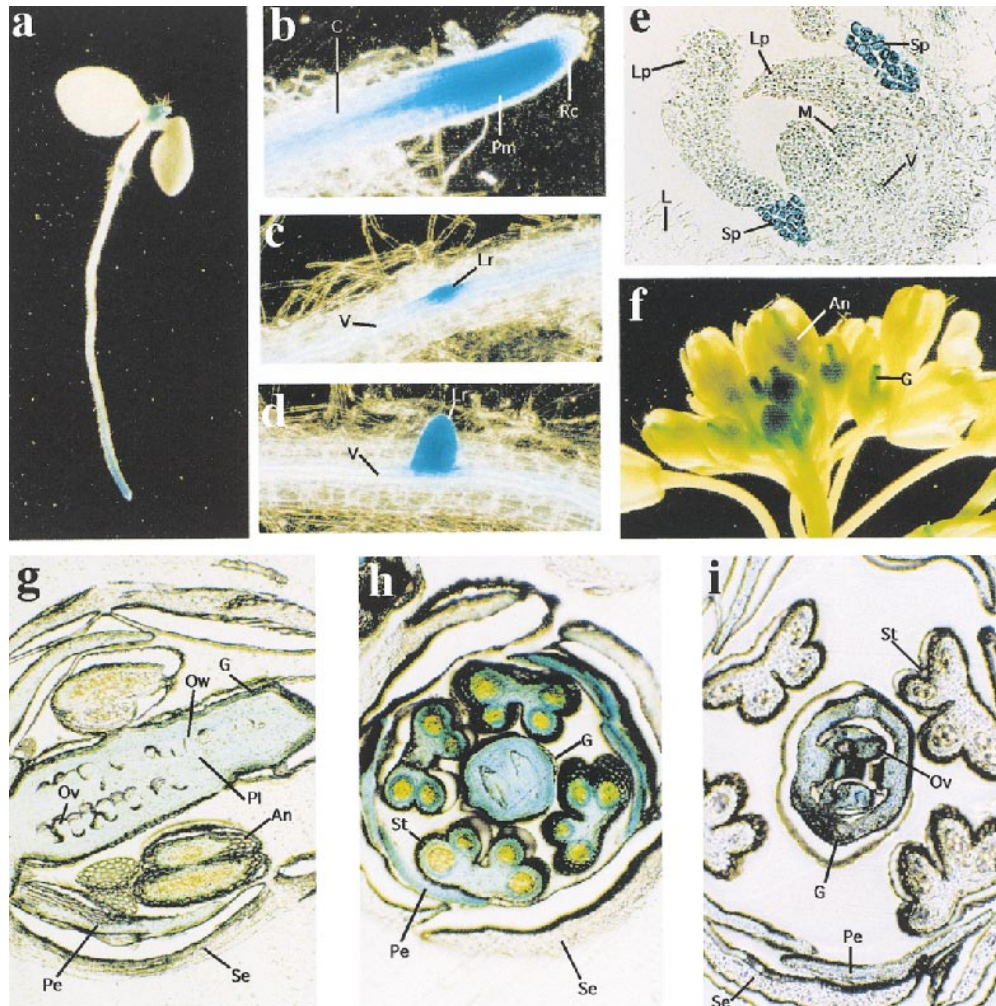
To investigate the spatial and developmental distribution of AtGR1 in more detail, we performed immunolocalization studies on sections of various Arabidopsis organs at different stages of development. Examination of root tips from 10-day-old seedlings revealed homogeneous staining of all distinct cell layers in the entire root elongation zone, in which cells divide and expand. This stain was progressively internalized and was limited to vascular tissue in the specialization zone, in which cells attain their final differentiated state (Fig. 3b, d). No staining was observed in the root cap. AtGR1 protein expression in the aerial parts during early stages of vegetative growth was investigated in Arabidopsis plantlets at the 2–4-leaf stage (14 days after sowing). At that stage, the anti-AtGR1 antibody stains immature tissues undergoing intense division and differentiation activity such as the central meristematic dome and early leaf primordia, where staining is homogeneous in cotyledons. Antibody staining is found only in the vascular tissue (Fig. 3e). We have repeatedly observed much stronger staining of particular organs such as the stipules (Fig. 3e), which are composed of endoreduplicating cells (22). Such cells have acquired the ability to undergo cell division but are blocked before mitosis, leading to consecutive doublings of genomic DNA in the absence of chromosome segregation and cytokinesis. In cross-sections through leaf primordia and the base of young developing leaves (Fig. 3f), AtGR1-specific staining is restricted to regions of small, largely undifferentiated and dividing cells that will give rise to the spongy and palisade mesophyll. In cells at the base of cotyledons that are vacuolated, are undergoing expansion, and have stopped dividing, no staining is observed. During flower development, the developing ovules are stained most markedly by the anti-AtGR1 antibody. Staining of the ovules involves several tissues including the funiculus, the integuments, and particularly the embryo sac (Fig. 3g, i). In stage 10 flowers, the false septum and ovary wall of the gynoecium as well as the anthers and petals are weakly stained above background levels. In stage 10 flowers prepared from irradiated plants and fixed 2 h after irradiation with 100 Gy  $\gamma$  rays (Fig. 3j), an increase of the antibody staining intensity is observed most markedly in the developing ovules. Taken together, these results demonstrate that basal AtGR1 expression is associated with mitotic activity in rapidly growing organs, whereas constitutive strong AtGR1 expression is restricted to endoredupli-





**FIG. 3.** Distribution of AtGR1 protein during plant development and ionizing radiation-induced accumulation in germline tissue. Panels b, d, e, f, g, i and j: Immunolocalization on histological sections with anti-ATGR1 antibody. Panels a, c and h: Control sections incubated with the preimmune antibodies. Protein-antibody interaction is revealed by alkaline phosphatase secondary antibody. Specific coloration is seen in the root elongation zone, in the apical shoot meristem, in leaf primordia, in stipules, in developing floral organs, and particularly in the developing ovules. Panels a and b: Longitudinal section through a primary root. Panels c and d: Cross-section through a primary root in the upper zone of the procambium. Panel e: Longitudinal section through a vegetative meristem. Panel f: Cross-section through basal area early leaf primordia. Panel g: Cross-section through a stage 12 flower. Panels h and i: Longitudinal section through stage 10 flower. Panel j: Longitudinal section through stage 10 flower 2 h after irradiation with 100 Gy. an, Anthers; Ci, cortex inner layer; Co, cortex outer layer; En, endodermis; Ep, epidermis; EZ, elongation zone; G, gynoecium; L, leaf; Lp, leaf primordium; M, meristem; Ov, ovules; ow, ovary wall; P, pericycle; Pe, petal; pl, placenta; Rc, root cap; Se, sepal; Sp, stipule; St, stamen; SZ, specialization zone; V, vasculature.





**FIG. 4.** Histochemical localization of the *pAtGRI*-driven *GUS* gene expression in transgenic *Arabidopsis* plants. *GUS* activity is monitored by histochemical staining of plantlets, plant organs, or histological sections. Strong blue coloration is seen in primary root tips, the lateral root initials, the apical shoot meristem, and leaf primordia including stipules. Strong *GUS* activity in young inflorescences is progressively switched off as floral organs mature. Panel a: Dark-field microscopy view of 10-day plantlet. Panel b: Detailed view of panel a. Panels c and d: Dark-field microscopy view of lateral root initiation. Panel e: Longitudinal section through a vegetative meristem. Panel f: Dark-field microscopy view of an inflorescence. Note the differential coloration of inflorescences during flower maturation. Panel g: Longitudinal section through an early stage 10 flower. Panel h: Cross-section through an early stage 10 flower. Panel i: Cross-section through an early stage 12 flower. Annotations are identical to those for Fig. 3, except: C, cambium; Lr, lateral root meristem; Pm, procambium; Rc, root cap.

cating structures. Radiation-induced *AtGRI* protein accumulation observed in ovules, which never undergo endoreduplication, suggests a potential role in the decision for growth arrest in both irradiated cells and endoreduplicating cells, which may be mediated by enhanced intracellular levels of *AtGRI* protein.

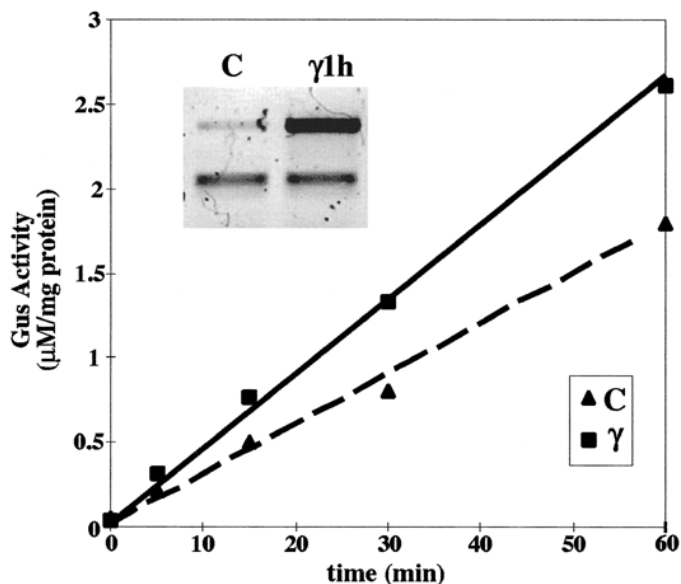
To correlate the protein expression profile with promoter activity, we have analyzed transgenic plants bearing a fusion construction between genomic *AtGRI* DNA, which contains a 2-kb 5'-flanking region including the initiator Met codon, with the *GUS* reporter gene. High *GUS* activity is revealed in the root and shoot meristems of 4-day-old seedlings (Fig. 4a). As shown before with the anti-*AtGRI* antibody, *GUS* staining of the root tip is present in the elongation zone, which is progressively internalized to the vascular tissue in the specialization zone, where cells reach

maturity and stop dividing. The root cap is not stained (Fig. 4b). Strong homogeneous *GUS* staining is also apparent during the lateral root initiation from the very early stages, including ordered cell divisions and cell differentiation to generate a highly organized lateral root primordia (Fig. 4c) and activation of the lateral root meristem to allow continued growth of the organized lateral root (Fig. 4d). Staining is not confined to specific cell layers or tissues in the developing root, indicating that the promoter activity is associated with cell proliferation, cell differentiation, and meristematic activity. In the aerial parts of young plantlets, basic *GUS* activity is detected in the apical meristem and leaf primordia, while stipules show particularly strong staining (Fig. 4e). Microscopic observation of whole transgenic plantlets also reveals similar strong staining of the trichomes (data not shown), which, like the stipules, are

composed of endoreduplicating structures. In adult plants, the *AtGR1* promoter-driven GUS activity is found in immature, developing flowers. As shown in Fig. 4f, there is a clear inverse correlation between the strength of GUS staining and the degree of maturation. Closer examination of immature flowers reveals GUS expression in petals, the gynoecium, and the anthers. The pollen grains remain completely unstained, suggesting either that the *AtGR1* promoter is not active in this tissue or that the chromogenic substrate X-Gluc does not diffuse into pollen grains (Fig. 4g, h). At later stages of flower development (Fig. 4i), staining is limited to the carpel wall and ovules. Taken together, the observed GUS expression profile under the control of the *AtGR1* promoter matches the results obtained by immunolocalization, which supports the specificity of antibody recognition. These results also suggest that the promoter fusion construction used contains all the signals necessary for developmental and tissue-specific regulation of the *AtGR1* gene. The GUS expression profile and a similar level of staining are observed in at least 10 independent transformant lines, thereby proving that the promoter is active independent of the chromosomal locus of insertion. To measure *AtGR1* promoter-dependent *GUS* mRNA induction upon irradiation, we compared 10-day-old untreated and irradiated seedlings. In these plantlets, *GUS* mRNA is detected in total RNA preparations, in contrast to the *AtGR1* mRNA, suggesting that the *GUS* mRNA expressed in the root tip and the apical meristem under the control of the *AtGR1* promoter has a slower turnover rate than the *AtGR1* mRNA (Fig. 5). Ionizing radiation induces strong *GUS* mRNA accumulation followed by an increase in GUS activity, providing evidence that developmental expression and radiation-induced *AtGR1* mRNA accumulation are both controlled at the level of transcription. Thus histochemical localization of GUS activity is identical to the spatial pattern of AtGR1 protein expression detected by immunolocalization. This is a strong argument in favor of the 68-kDa protein recognized by the antibody in meristematic tissues, organ primordia and endoreduplicating cells being effectively encoded by the *AtGR1* gene. Furthermore, we can conclude that the *AtGR1* genomic fragment used in our experiments contains all the information necessary for tissue-specific and developmental regulation and the response of the *AtGR1* gene to genotoxic stress.

## DISCUSSION

In animal cells, ionizing radiation and DNA-damaging agents induce the activation of genes associated with DNA repair and growth control. Screening for genes induced by ionizing radiation, we expected to identify new plant genes with functions similar to those of their animal counterparts and to shed light on how plant cells respond to the perturbation of cell division caused by DNA damage. In this paper, we report the isolation of *AtGR1*, a member of the emerging group of Arabidopsis genes whose transcript lev-



**FIG. 5.** Ionizing radiation-induced *GUS* mRNA accumulation and GUS activity in young transgenic plantlets. Native protein extracts from unstressed (C) or irradiated transgenic plantlets at the 2- to 4-leaf stage were used for determination of GUS activity by fluorescence spectrometry. Inset: RNA analysis by quantitative RT-PCR with internal gene-specific primers for *GUS* and *Act8* was carried out with total RNA prepared from 10-day-old *pAtGR1-GUS* plantlets that had been irradiated with 100 Gy ( $\gamma$ ) or not (C).

els are increased after treatment with DNA-damaging agents, namely *AtRAD51* (15), which is involved in the double-strand break repair pathway, and the *AtPARP1* and *AtPARP2* genes (Doucet-Chabeaud and Kazmaier, unpublished results), which are involved in recognition of DNA strand interruptions.

Although the biochemical function of the AtGR1 protein remains unknown, we have assembled several arguments that point to a potential role of AtGR1 in the G<sub>2</sub>/M-phase transition of mitotic cell division during plant development. Stipules and trichomes are both highly endoreduplicative tissues that are characterized by the absence of mitotic activity, active DNA synthesis, and polyploid nuclei (22). Since DNA synthesis occurs but not cytokinesis, these observations are consistent with a cell cycle arrest at the G<sub>2</sub>/M-phase transition. Stipules and trichomes are also characterized by very high levels of AtGR1 protein when compared to actively dividing tissues, suggesting that the block in late G<sub>2</sub>-phase could be achieved at least in part by elevation of the intracellular AtGR1 protein level. Indeed, in yeast and *Drosophila*, it has been shown that alterations between the S and M phases depend on the presence of mitotic cyclins and that the down-regulation of M-phase-associated CDK activities is sufficient to drive cells into the endoreduplication cycle (44, 45). Similar systems might be operational in plants, implicating elevated intracellular amounts of AtGR1 protein.

In line with a possible functional link between the AtGR1 protein level and the progression from G<sub>2</sub> to M



phase in mitotic cells is the presence of a very strong consensus to the PEST destruction motif in the C-terminal domain of the deduced amino acid sequence. The regulation of protein turnover by PEST sequences during cell cycle progression is highly conserved in eukaryotes (46). A general feature of cyclins is that they are destroyed rapidly at certain points in the cell cycle, and the ability to activate this turnover at the correct time is central to the cell cycle control. As for yeast and animal cells, the plant B-type cyclins are expressed in G<sub>2</sub> phase and mitosis and have to be actively degraded upon entry into mitosis (47). Therefore, the *AtGR1* protein may well be a new cell cycle-related protein that has to be destroyed rapidly in late G<sub>2</sub> phase to allow progression to mitosis; permanent or transient elevation of *AtGR1* would be implicated in cell cycle arrest in G<sub>2</sub> phase.

The presence of damaged DNA, particularly DNA strand interruptions that can be introduced experimentally by exposure to sublethal doses of ionizing radiation, is a stress situation that temporarily precludes entry into mitosis to ensure the transmission of an intact genome to the daughter cells. In yeast and animal cells, DNA damage-dependent growth arrest operates at the G<sub>1</sub>/S- and G<sub>2</sub>/M-phase transitions of the mitotic cycle (7). Radiation-induced *AtGR1* protein accumulation is best observed in developing germline tissue, which is composed exclusively of euploid and mitotic cells. By analogy with the permanently elevated *AtGR1* levels that are observed in endoreduplicating cells, we propose that similar processes implicating transiently elevated *AtGR1* levels would be induced by genotoxic stress in mitotically cycling cells to block them in late G<sub>2</sub> phase and prevent premature progression through mitosis prior to the repair of DNA lesions.

An additional argument to support the potential involvement of *AtGR1* in cell proliferation and DNA damage-induced growth arrest is the pattern of *AtGR1* protein expression, which strikingly overlaps that of the Arabidopsis *aMAG* DNA repair gene, which catalyzes the excision of 3-methyladenine from the sugar-phosphate backbone (48). This gene is preferentially expressed in meristematic cells and organ primordia, which led the authors to propose that *aMAG* gene function is linked to the maintenance of an intact genome as a template for DNA replication. On the other hand, the pattern of *AtGR1* expression is similar in some ways to the expression of the plant *CDC2* homologues, which are required for exit from G<sub>1</sub> phase and entry into S phase. The Arabidopsis *CDC2aAt* and *CDC2bAt* transcripts are found in both meristematic cells and cells with a high proliferative capacity (49–51). Fobert and co-workers (52) studied the transcriptional regulation of five cell cycle-related genes in *Antirrhinum majus* and found that these transcripts accumulate only in dividing tissues, and in this case only at specific stages of the cell cycle.

Taken together, the fact that the pattern of *AtGR1* expression overlaps that of genes required for DNA repair and cell cycle control and that dose-dependent *AtGR1* biosyn-

thesis is induced after treatment with genotoxic agents suggests that modifications in the intracellular levels of this novel plant protein may be implicated in DNA damage-induced growth arrest. Such growth arrest after DNA damage, which is well described in yeast and animal cells, occurs at the major checkpoints of the mitotic cell cycle, G<sub>1</sub>/S phase and G<sub>2</sub>/M phase. Although considerable progress has been made recently in the characterization of the molecular mechanisms underlying the regulation of the cell cycle in plants, DNA damage-dependent checkpoints have not been studied in plants. Additional studies which are under way to elucidate *AtGR1* function will contribute to an understanding not only of how plant cells respond to the perturbation of cell division caused by DNA damage, but also of how plant cells regulate cell division during growth and development.

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