Mutation in the *Arabidopsis* PASTICCINO1 Gene, Which Encodes a New FK506-Binding Protein-Like Protein, Has a Dramatic Effect on Plant Development

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In flowering plants, morphogenesis depends on the control of the pattern and numbers of cell divisions and on the control of cell elongation. Although there are many examples of controlled patterns of cell division, we still know very little about how local patterns of cell division are established and maintained (30). In *Arabidopsis thaliana*, the roles of cell division control in the development of the embryo, the shoot, and the root have been extensively studied (reviewed in references 29 and 30). In the last few years, much progress has been made in this field by the isolation of mutants in which single-gene mutations affect specific modes of cell division control. Some of the corresponding genes have been cloned from *Arabidopsis* ([SHOOT MERISTEMLESS](STM) and [SCARECROW](SCR)) maize ([KNOTTED1](Kn1)), and petunia ([NO APICAL MERISTEM](NAM)) (reviewed in reference 30). These genes do not seem to specify components of the cell division machinery, but they are thought to act upstream in the control of cell division. The elements at the interface between genes like *STM* and *SCR* and cell cycle regulators, such as cyclins and the CDC genes, are still unknown.

The growth and differentiation of higher plants is also greatly dependent on environmental stimuli, such as light and temperature, and on endogenous factors, such as phytohormones. Cytokinins (CKs) were originally discovered because of their ability to promote, along with auxins, plant cell division. They are thought to act upstream in the control of cell division. The elements at the interface between genes like *STM* and *SCR* and cell cycle regulators, such as cyclins and the CDC genes, are still unknown.

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The growth and differentiation of higher plants is also greatly dependent on environmental stimuli, such as light and temperature, and on endogenous factors, such as phytohormones. Cytokinins (CKs) were originally discovered because of their ability to promote, along with auxins, plant cell division and organogenesis (reviewed in reference 9). Although this discovery initiated a vast amount of fundamental and applied research on the hormonal control of cell proliferation and regeneration, the mechanisms by which auxins and CKs act and interact at the molecular level are unknown. Steroid-like plant growth factors termed brassinosteroids (BR) were first characterized as inducing cell elongation in synergy with auxin, but recently these hormones have also been found to control plant cell divisions and morphogenesis (15; reviewed in reference 11).

The genetic and molecular analysis of hormonal mutants is proving to be a powerful tool for unraveling the mode of action of these molecules. In an attempt to understand the mode of action of CKs and their molecular relationships with auxins in promoting plant cell division, we looked for *Arabidopsis* mutants with phenotypes which were affected by exogenously applied CKs. We have previously reported the isolation of the *pasticcino* mutants (*pas1*, *pas2*, and *pas3*) which are affected in both embryonic and vegetative development. Their phenotypes are similar to that of wild-type shoots which have been regenerated in vitro from explants, in the presence of an unbalanced auxin/CK ratio in the medium (12).

The *pas1-I* mutant was isolated from the transfer DNA (T-DNA) mutant collection of INRA-Centre de Versailles (2, 12). Here we describe the cloning of the *PASI* gene from the T-DNA-tagged *pas-I* allele. *PASI* codes for an immunophilin-like protein similar to the FK506-binding proteins (FKBP). We also demonstrate that the *PASI* mRNA steady-state level is increased in the presence of CK and that *PASI* gene expression is affected in the other *pas* mutants.

**MATERIALS AND METHODS**

*Arabidopsis* lines and growth conditions. Seeds from *Arabidopsis* Heynh., ecotype Columbia (Col0) and ecotype Wassilewskija (WS), were kindly provided by P. Giraudat (CNRS, Gif sur Yvette, France) and by K. Feldman (University of Arizona, Tucson, Ariz.), respectively. The mutated lines were produced as already described (2, 12). For growth in the greenhouse, seeds were sown on soil and seedlings were transferred into individual pots 10 days after germination. Plants were grown under the following conditions: 16 h of light, 20 to 25°C day temperature, and 10 to 15°C night temperature. For in vitro growth, seeds were sterilized and grown as already described (12). Benzyladenine (BA), zeatin, 1-(2-chloropyrid-4-yl)-3-phenylurea, and picloram were applied to the medium as described (12).
Cytological analysis. For light microscopy, seedlings were fixed in 4% formaldehyde-0.2% glutaraldehyde and then embedded in Historesin (Leica, Rueil Malmaison, France) in accordance with the manufacturer's instructions. Ultrathin sections (3 to 5 μm thick) were cut on a Jung RM microtome, stained with 0.05% methylene blue, and examined with a Nikon Microphot FXA microscope.

Isolation of the pas1-1 mutant. The pas1-1 (pas-1) mutant was identified in the progeny of T-DNA-mutagenized Arabidopsis ecotype WS lines produced at the Station de Génétique et Amélioration des Plantes (Versailles, France) and had been previously screened for resistance to the Basta herbicide (2, 12). Screening for mutants was based on the early phenotype of 9-day-old plants that were both in the light and in the dark. It was determined that pas1-1 contained a single T-DNA insert, and linkage of the T-DNA insert to the PAS1 gene was tested. More than 100 putative heterozygotes (kanamycin-resistant Arabidopsis plants) had been previously screened for resistance to the Basta herbicide. A pas1-1 allelic mutant (pas1-2) and several other pas mutants representing three complementation groups were isolated from an ethyl methane sulfonate (EMS) mutant collection based on their abnormal responses to exogenous CKs (12). Mutants grown in the dark had short and wide hypocotyls and lacked apical hooks (Fig. 1A). For pas1 mutants grown in the light, the phenotype was characterized by a very short and thick hypocotyl and an altered cotyledon shape (Fig. 1B). The pas1 mutants could not survive under normal growth conditions but could be maintained in vitro. They often had fused leaves with a vitreous appearance. Both pas1 allelic mutants were characterized by the absence of secondary roots and a primary root shorter than that of the wild type (Fig. 1B and C). After 3 months, pas mutants developed abnormal compact and vitreous rosettes (Fig. 1D). Several mutants produced finger-like structures between the right and left borders of the seedling (data not shown).

While the growth of the wild type was severely inhibited by the presence of 5 μM BA (Fig. 1E and F), the mutant displayed a hypertrophy of the apical part (Fig. 1G). We have previously shown that the response to CK of the pasticcino mutants was characterized by an increase in cell divisions, specifically in the apical part. No significant difference between the pas mutants and the wild type with regard to root growth inhibition in the presence of BA was observed (12). The same results were obtained with other active CK molecules, such as zeatin and 1-(2-chlorophenyl)-3-benzyloxyurea (data not shown). The growth responses of the pas mutants in the presence of other plant hormones were analyzed in both light and dark growth conditions, but none of the hormones tested (auxin, ethylene, gibberellic acid, abscisic acid, and BR) was able to induce a hypertrophy of the apical parts of pas mutants, indicating that this particular response was specific to CKs (12).

Cytological analysis showed that pas1-1 hypocotyls have extra disorganized cell layers, irregular numbers of cortex cells, a loss of cell adhesion, and ectopic periclinal divisions in the epidermis (Fig. 2A and B). The different meristematic cell layers were never clearly distinguishable. The pas1-1 mutants had meristems with a highly variable structure, ranging from plants with almost no meristem to those with a very large meristem, which filled the entire apical region (Fig. 2C, D, and E).

After three crosses of the pas1-1 mutant with the wild type, segregation was found to be consistent with pas1-1 being a nuclear, recessive, and monogenic mutation. In the progeny of plants heterozygous for pas1-1, the pas1-1 mutation was shown to cosegregate with a single T-DNA insertion carrying the kanamycin resistance gene (see Materials and Methods).
the T-DNA insert and the PAS1 gene, we performed a molecular characterization of the pas1-1 mutation. Southern analysis of DNA extracted from pas1-1 mutants and probed with both the T-DNA right border (data not shown) and left border (Fig. 3A) revealed that the pas1-1 mutation was caused by the insertion of a single T-DNA unit. We isolated different genomic DNA fragments adjacent to the right and left borders of the T-DNA. BamHI (4 kb) and XbaI (0.4 kb) genomic fragments were cloned as sequences flanking the right border of the T-DNA by using the kanamycin plasmid rescue strategy (6) (see Materials and Methods). A 1.6-kb XbaI genomic fragment adjacent to the T-DNA left border was isolated by an inverse PCR strategy (see Materials and Methods). Synthetic oligonucleotides corresponding to the genomic XbaI fragments, flanking both the T-DNA right and left borders, were used in a PCR on a wild-type genomic DNA template to verify whether the T-DNA insertion had caused any deletion or rearrangement (data not shown). A sequence analysis of the T-DNA insertion site in the pas1-1 mutant revealed a deletion of 14 bp in the PAS1 gene.

In the pGBK5 binary vector used to generate the T-DNA collection (5), the ATG of the promoterless uidA gene is 40 bp downstream of the T-DNA right border, with no in-frame stop codon. An analysis of the genomic sequence flanking the T-DNA insert revealed an open reading frame (ORF) adjacent to the right border insertion sequence, in frame with the methionine initiation codon of the promoterless uidA (GUS) gene (Fig. 3B). This indicated that a translational fusion of the pas1-1-tagged allele with the uidA coding sequence had occurred, an event which is consistent with the GUS staining of the pas1-1 mutant (Fig. 3C). In further agreement with this, Northern blot analysis of pas1-1 RNA identified a 3.4-kb transcript that hybridized with both a uidA and a PAS1 probe (data not shown).
The two XbaI fragments adjacent to the right and left T-DNA borders were used as probes to screen both a wild-type Arabidopsis genomic library (EEC-BRIDGE Arabidopsis DNA Stock Center) and a cDNA Arabidopsis library (31). The genomic sequence of the PAS1 gene was obtained by sequencing both the BamHI and the XbaI clones. Synthetic oligonucleotides corresponding to the genomic sequence allowed us to amplify and sequence the same region from a wild-type (WS) template. The analysis of the genomic sequence and its comparison with the cDNA sequence revealed that the PAS1 gene was interrupted by 18 introns and that the entire gene was 4.2 kb long (Fig. 5A). The T-DNA was inserted before the last intron, 1,747 nucleotides from 5′ end of the cDNA. All the introns identified in the PAS1 gene showed the plant canonical acceptor and donor splice sites (NetPlantGene mail server: www.cbs.dtu.dk/NetPlantGene.html). Several independent cDNA clones were isolated. Among them, cDNA-D was chosen as containing the full-length PAS1 transcript, because Northern blot analysis of RNA from 9-day-old wild-type seedlings showed a transcript of the expected size (data not shown). A sequence analysis of cDNA-D revealed an ORF of 1,902 bp, corresponding to a protein of 634 amino acids (69.7 kDa). The first ATG in the ORF was preceded by several in-frame stop codons, suggesting that this is indeed the start codon. The full-length cDNA and the partial cDNA clones had poly(A) tails, although they differed slightly in the length of the 3′ untranslated region, perhaps due to the presence of different polyadenylation sites (Fig. 4 and 5A). Another class of PAS1 cDNA (cDNA-A), which contained an insert 70 bp longer than that of cDNA-D, was identified. A sequence comparison of the two cDNA classes (A and D) revealed that this difference was due to the lack of splicing of the second intron in the cDNA-A class (Fig. 4). As the possibility exists that the cDNA-A represents an aberrant PAS1 unspliced transcript, we focused our attention on cDNA-D. However, it is possible that the cDNA-A class results from a differential splicing, as more than one independent clone of the cDNA-A type was found in the library. Human FKBP12 is encoded by different mRNAs, varying in abundance and 3′ untranslated region, deriving from the differential splicing of five exons (1, 33).

The PAS1 cDNA of the pas1-2 allelic mutant was sequenced. A comparison with the wild-type gene sequence revealed a G-to-A point mutation at nucleotide 1317 (starting from the 5′ end), which creates a translational stop codon (W397STOP),
FIG. 3. Molecular characterization of the pas1-1 mutation. (A) Southern analysis of pas1-1 and wild-type genomic DNA probed with the left T-DNA border (left) and with the genomic fragment adjacent to the left T-DNA border (right). (B) Schematic map of the T-DNA-tagged pas1-1 allele. The arrows with the dashed lines show the two mRNAs transcribed from the pas1-1 allele. The first transcript is transcribed from the PAS1 promoter through the right T-DNA border giving rise to the translational fusion between the 5′ part of the PAS1 gene and the GUS gene. The second transcript arises due to transcription from the cauliflower mosaic virus 35S promoter (35S2) through the BglII gene (which confers resistance to Basta) and the 3′ end of the PAS1 gene. The thin black line represents the genomic DNA, and the T-DNA is shown by the black box. B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; N, NcoI; P, PstI; S, SacI; V, EcoRV; X, XbaI. a and b denote the probes used for the Southern analysis. (C) GUS staining of pas1-1/+ plants (a) and pas1-1 mutants (b), and in a pas1-1/+ plant root (c) and in a pas1-1 plant root (d). The plants were grown for 9 days in standard conditions in the light.

and a G-to-C point mutation at nucleotide 417, which causes a lysine-to-asparagine substitution (K87N) within the first FKBP domain (Fig. 4 and 5B).

The PAS1 gene has been mapped by RFLP analysis with the genomic-DNA fragment adjacent to the T-DNA right border as a probe (Fig. 3B). The PAS1 gene is located on chromosome 3 at 106.6 centimorgans (marker v042) on the recombinant inbred map (25).

**Complementation of the pas1-2 mutant.** We wished to determine whether the mutation in the PAS1 gene was responsible for the Pas1-1 phenotype. The coding region of the full-length PAS1 cDNA-D was cloned into plant binary vector pKYLV71 (28) under the control of the cauliflower mosaic virus promoter (35S2). *A. tumefaciens* C58C1 (pMP90) transformants were selected on kanamycin and used to transform plants, which were heterozygous for the pas1-2 mutation, by the in planta *Agrobacterium*-mediated transformation method (2). Kanamycin-resistant T1 plants were allowed to self-pollinate. If the cDNA was able to complement the pas1-2 mutation in the progeny of the heterozygous transformed plant, we expected to obtain only 1 kanamycin-sensitive mutant plant for every 15 wild-type plants (12 kanamycin-resistant and 3 kanamycin-sensitive plants). The segregation analysis was performed in vitro on a kanamycin-supplemented medium and revealed that several independent transformants, heterozygous for the *pas1* mutation, segregated with the expected ratio. The segregation analysis of four such transformants is shown in Table 1. Some of the transformants segregating as wild-type kanamycin-resistant plants showed abnormal developmental phenotypes. Whether this is due to the overexpression of *PAS1* or to a phenomenon of cosuppression of the *PAS1* gene in a wild-type background has yet to be determined. However, we never found any *pas1* mutant plant resistant to kanamycin in the progeny of the transgenic plants analyzed.

**PAS1 has similarities to the immunophilin proteins.** The predicted amino acid sequence of PAS1-D was compared with sequences in current databases (National Center for Biotechnology Information; BLAST network server). The PAS1 protein has significant similarity to FKBP5s belonging to the family of immunophilins (reviewed in reference 20). The term FKBP refers to any protein which binds both FK506 and rapamycin (23, 34, 40). The overexpression of PAS1 in a study of *Pas1*-1 homozygous and heterozygous plants. The *pas1*-1 mutant showed strong GUS staining in all of the apical part, in the vascular cylinder of the root, and in the root tip (Fig. 3C, panels b and d). This expression was not affected by light or dark growth conditions or by the seedling developmental stage. GUS staining in the *pas1*-1/+ plants showed a strong staining of the apical and root meristematic regions and no detectable staining in the cotyledons, leaves, hypocotyls, or roots under all conditions tested (Fig. 3C, panels a and c).

We also investigated CK regulation of *PAS1* gene expression in 9-day-old wild-type plants. The steady-state level of *PAS1*...
transcripts was higher in wild-type (ecotype Columbia) plants grown on BA concentrations up to 5 mM than in untreated controls (Fig. 7B). We have previously shown that the pas mutants display a normal response to auxin (12). We analyzed total RNA extracted from 9-day-old wild-type plants grown on 5 mM picloram, an auxin analog. The results revealed that the steady-state level of PAS1 mRNA was weakly increased by auxin, but to a much lesser extent than by CK (data not shown).

The pas mutants show altered expression of the PAS1 gene. PAS1 gene expression in the two allelic pas1 mutants and in the pas2 and pas3 mutants (12), either in the presence or absence of exogenous CKs (BA) was analyzed. In pas1-1 mutants two transcripts can be detected due to the insertion of the T-DNA (see above), but the transcript of the wild-type size is absent (Fig. 7C). In heterozygous pas1-1 plants the wild-type transcript is present with the two other transcripts (data not shown). PAS1 mRNA levels were still increased by CK in the pas1-1 mutant, but the response was shifted towards lower BA concentrations compared to the response of the corresponding wild type (ecotype WS) (Fig. 7C). This result was confirmed by a fluorimetric GUS quantitative analysis. PAS1 expression was still induced by BA, both in pas1-1 homozygous and heterozygous plants, but the peak of BA induction of GUS activity was reached at 0.1 mM BA (data not shown). In pas1-2 plants, a transcript of the wild-type size is present and is constitutively expressed (Fig. 7D). PAS1 expression was affected in pas2-1 and pas3-1 mutants. For pas2-1 mutants, the PAS1 mRNA could be detected in untreated mutants and in mutants grown in the presence of 0.1 mM BA but not in presence of 5 mM BA (Fig. 7C). For pas3-1 mutants, PAS1 mRNA could not be detected unless the mutants were grown at a low BA concentration (0.1 mM) (Fig. 7D). These results suggest that the CK sensitivity of PAS1 expression is modulated by the other two PAS genes, which may be required for its controlled expression.

**DISCUSSION**

**PAS1 is involved in the control of cell proliferation.** We have previously reported the isolation of three classes of mutants with very similar pleiotropic phenotypes characterized by the presence of large, abnormal meristems leading to disorganized...
rosettes made of fused, vitreous leaves (12). The pas mutants were shown to be particularly altered in their response to CKs (12). The three PAS genes are also involved in the control of embryogenesis. Both pas1-1 and pas1-2 mutants contain mutations (T-DNA insertion and point mutations, respectively) in the coding sequence of the PAS1 gene. The fact that pas1-2 can be complemented with the wild-type PAS1 cDNA confirms that the gene corresponding to the mutant phenotype has indeed been cloned. The expression of the PAS1 gene, which is impaired in pas1 mutants, is also affected in pas2 and pas3 mutants, suggesting a possible molecular basis for the similarities of their phenotypes. These results tend to confirm the previous biochemical analysis of the pas mutants, which demonstrated that pas mutants were biochemically closely linked (12).

The pas mutants are an example of plant mutants which have a general deregulation of the control of cell proliferation. We hypothesize that this deregulation is due to the absence of a functional PAS1 protein, which in the wild type would antagonize cell proliferation. It is proposed that the PAS1 protein accumulates and functions in dividing tissues, such as the meristems, to prevent uncontrolled cell division. In pas1-1/+ plants, the PAS1-GUS protein accumulates preferentially in the meristematic area. In the wild type, posttranscriptional regulation possibly occurs to prevent the production of the protein in all the organs (the PAS1 mRNA is not organ specific). However, in pas1-1 plants, the chimeric PAS1-GUS protein is overproduced not only in the meristematic zones but also in all the tissues undergoing cell division. Possibly the lack of functional PAS1 in the pas1 mutant causes ectopic cell proliferation, which in turn induces ectopic PAS1 expression in a regulatory feedback loop.

The cellular proliferation in pas1-1 plants is enhanced specifically by CKs. In wild-type plants PAS1 expression is upregulated by CKs, and in pas1-1, pas1-2, and pas2-1 plants, CK regulation of PAS1 expression is altered. Although we previously showed that only CKs have an effect on the pas phenotype (12), the possibility exists that the action of CKs is mediated by other hormones, such as auxins and BR, which are known to interact with CKs in plant development. Therefore, PAS1 may function directly in a CK pathway controlling cell division or in a pathway controlling similar downstream events.

PAS1 is an FKBP-like protein with TPR domains. The PAS1 protein has significant sequence similarity with the immu-
nophilin family of FKBP. Two immunophilin families can be distinguished: cyclophilins and FKBP. The FKBP, represented by the well-studied FKBP12, bind the immunosuppressants FK506 and rapamycin, whereas the cyclophilins bind cyclosporin A (42). Although the two families do not have structural or sequence homology, all immunophilins identified to date exhibit peptidyl-prolyl cis-trans isomerase (rotamase) activity. Immunophilins are housekeeping proteins, highly conserved throughout evolution, which may mediate critical cellular functions (reviewed in reference 20). Despite the widespread occurrence and strong sequence conservation of immunophilin genes, their inactivation has been shown to have little or no effect on cell viability. This work shows that when the expression of an FKBP-like gene is impaired, as in pas1 mutants, the control of cell proliferation is affected.

In conclusion, we believe that the pas1 mutants represent the first inactivation of a gene encoding an FKBP-like protein in higher eukaryotes. Genetic studies on immunophilin gene disruption in microorganisms have been performed (reviewed in reference 10). Despite the widespread occurrence and strong sequence conservation of immunophilin genes, their inactivation has been shown to have little or no effect on cell viability. This work shows that when the expression of an FKBP-like gene is impaired, as in pas1 mutants, the control of cell proliferation is affected.

In conclusion, we believe that the pas1 mutants represent a useful tool to understand the role of immunophilin-like proteins in plant development and to unravel their potential functions in plant hormonal signalling pathways.
REFERENCES


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