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The *PASTICCINO* genes of *Arabidopsis thaliana* are involved in the control of cell division and differentiation

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SUMMARY

The control of cell division by growth regulators is critical to proper plant development. The isolation of single-gene mutants altered in the response to plant hormones should permit the identification of essential genes controlling the growth and development of plants. We have isolated mutants *pasticcino* belonging to 3 complementation groups (*pas1, pas2, pas3*) in the progeny of independent ethyl methane sulfonate and T-DNA mutagenized *Arabidopsis thaliana* plants. The screen was performed in the presence or absence of cytokinin. The mutants isolated were those that showed a significant hypertrophy of their apical parts when grown on cytokinin-containing medium. The *pas* mutants have altered embryo, leaf and root development. They display uncoordinated cell divisions which are enhanced by cytokinin. Physiological and biochemical analyses show that cytokinins are probably involved in *pas* phenotypes. The *PAS* genes have been mapped respectively to chromosomes 3, 5 and 1 and represent new plant genes involved in the control of cell division and plant development.

Key words: *Arabidopsis thaliana*, Cytokinin, Cell division, *PASTICCINO* (*pas*)

INTRODUCTION

Plant developmental pathways are induced and controlled by both external stimuli and endogenous factors, such as phytohormones, through the activation of specific genetic programs. For instance, cell division and proliferation, the control of which are essential for proper plant development are tightly regulated by cytokinin and auxin. Cytokinins were originally identified by their ability to stimulate division and sustained tobacco pith cell growth when added in combination with auxin (Miller et al., 1955; Skoog et al., 1965). Since their discovery, numerous reports have demonstrated that cytokinins play a role throughout development, from seed germination to leaf senescence. Cytokinins delay tissue senescence (Noodén et al., 1988) and promote shoot organogenesis (Skoog and Miller, 1957; Estruch et al., 1991), lateral bud release, chloroplast development, leaf expansion (Devlin and Witham, 1983; Horgan, 1994), tumors (Akiyoshi et al., 1984) and fasciations (Kenneth et al., 1966; Crespi et al., 1992).

Although cytokinins have an important role in various physiological processes and were one of the first plant hormones to be isolated, there are only scattered data concerning their mode of action. One of the main problems in studying the mode of action of cytokinin is the lack of fast and specific responses. Moreover, other plant hormones, such as auxin, ethylene or abscisic acid, or environmental stimuli, such as light or temperature, can act synergistically or antagonistically on cytokinin responses. Several cytokinin binding proteins were recently purified following photoaffinity labelling, but their function as receptors has not yet been proved. Changes in gene expression may mediate the response of plant cells to cytokinins. Several genes which are up or down-regulated by cytokinins have been isolated but they do not provide direct information on the mode of action of cytokinins (for review, see Binns, 1994).

The genetic and molecular analysis of cytokinin mutants should serve as a powerful tool to unravel the mode of action of these molecules. However, lack of knowledge on the physiology and biochemistry of cytokinins has made the isolation of such mutants difficult. *Arabidopsis thaliana* mutants that are cytokinin-overproducing or insensitive have been described. The *amp1* mutant exhibits a higher endogenous cytokinin level than the wild type, and shows a mild light-grown morphology when grown in the dark. The increased cytokinin level in this mutant was also correlated with an altered embryonic and vegetative development (Chaudhury et al., 1993). The mutants *det1* and *det2* were isolated for constitutive light responses in the dark and seem to be affected in their sensitivity to cytokinins (Chory et al., 1991, 1994). The *DET2* gene was cloned and the

corresponding protein was found to be involved in the brassinosteroid biosynthesis pathway (Li et al., 1996). Other *Arabidopsis* mutants have been isolated on the basis of root elongation on inhibitory cytokinin concentrations, including the mutants *cyr1* (cytokinin response), *stp1* (stunted plant 1) and *ckr1* (cytokinin resistance) (Su and Howell, 1995; Deikman and Ulrich, 1995; Baskin et al., 1995). While *cyr1* and *stp1* seemed to be specific to cytokinin, *ckr1* was shown to be resistant to ethylene and allelic to the ethylene insensitive mutant *ein2* (Cary et al., 1995). These results highlight the difficulty in isolating specific cytokinin mutants due to interaction with other hormones. Recently, Kakimoto (1996) was able to isolate activation tagged mutants, called *cytokinin independent* (*cki*), which regenerate shoots in the absence of cytokinin. The gene *CKI1*, corresponding to one of these mutants, was cloned and found to code for a protein with homology to both the receiver domain and the histidine kinase domain of the prokaryotic two component system.

In *Nicotiana plumbaginifolia*, the *zea* mutants, belonging to three complementation groups, were identified on the basis of their cotyledon and hypocotyl hypertrophy, due to an increase of cell proliferation, in the presence of added zeatin (Jullien et al., 1992; Faure et al. 1994). It has been shown that this hypertrophy was highly specific to the active cytokinin isomers (Nogué et al., 1995). Analysis of one *zea* mutant, *zea3.1*, demonstrated that it was hypersensitive to cytokinin leading to increased root inhibition, hypocotyl swelling and cell proliferation in the cotyledons in the presence of cytokinin (Martin et al., 1997). These results suggest that *zea3.1* could encode a negative modulator of cytokinin responses. The isolation of *zea* mutants in *N. plumbaginifolia* led us to seek similar classes of mutants in *A. thaliana* that would be more amenable to molecular studies. Here, we describe the characterization of the *pasticcino* mutants of *Arabidopsis*, which show a similar response to exogenous cytokinins. In contrast to *zea* mutants, the *pas* mutants display several developmental abnormalities beginning at the embryo stage, even in the absence of cytokinin, suggesting that they represent a new class of developmental mutants, affected also in cytokinin response.

MATERIALS AND METHODS

Plant material and tissue culture

Seeds of *Arabidopsis thaliana* Heynh, ecotype Columbia (Col0) were used for ethyl methane sulfonate (EMS) mutagenesis. Seeds of the ecotype Wassilewskija (WS) were used for the generation of T-DNA insertional mutants.

In the greenhouse

Seeds were sown on soil and seedlings were transferred into individual pots 10 days after germination (d.a.g.). Plants were grown under 16 hours light, 20-25°C day temperature, 10-15°C night temperature. They were watered with a nutrient solution (Coïc and Lesaint, 1971) by subirrigation.

In vitro

Seeds were sterilized and grown as described by Santoni et al. (1994). Seed germination was induced by 8 hours of light (200 $\mu E/m^2$/second). Light grown plants were kept in a controlled environment chamber (irradiance 200 $\mu E/m^2$/second, 16 hours light, 60% humidity, 20°C day temperature, 15°C night temperature). To maintain plants in dark-growth conditions, Petri dishes were wrapped in four layers of aluminium foil.

Benzyladenine (BA), naphthaleneacetic acid (NAA) and indol acetic acid (IAA) were dissolved in ethanol. Piclorame (analog of auxin), aminoethoxyvinyl glycine (AVG) and 1-amino-cyclopropane-1-carboxylic acid (ACC) were dissolved in dimethyl sulfoxide (DMSO). Gibberellic acid (GA4+7) was dissolved in distilled water and filter-sterilized prior to storage.

Isolation of the mutants

The *pasticcino* mutants *pas1-2*, *pas2*, *pas3-1*, *pas3-2*, *pas3-3* and *pas3-4* were isolated from an EMS mutagenized seed stock. The screen was performed in vitro, either in the light or in the dark, in the presence or absence of 5 μM BA. The mutant *pas1-1* was identified in the progeny of T-DNA-mutagenized *Arabidopsis* lines currently produced in the Station de Génétique et Amélioration des Plantes (Versailles, France) (Bechtold et al., 1993). Plants heterozygous for the mutations were self-fertilized, and the transmission of the phenotypes was confirmed in the M_3 generation.

Complementation and genetic segregation analysis

Identification of plants heterozygous for *pas1*, *pas2* or *pas3* was by examination of immature seeds cleared with a buffer described by Herr (1971). The abnormal embryo phenotype was scored using a Nikon microphot FXA microscope, with Nomarsky optics. Plants used as female parents were emasculated and cross-pollinated. Progenies were harvested, vernalized and germinated. The phenotype of seedlings was scored 9 d.a.g.

Mapping of the *PAS* loci

Heterozygous *pas2* and *pas3* plants (Col0 background) were crossed with the wild type (WT) Landsberg *erecta*. Progenies segregating *pas2* or *pas3* homozygous mutants upon selfing were used for mapping. Fifty seedlings homozygous for *pas2* or *pas3* mutations were selected in the F_2 populations. The localization of the mutations was determined using cleaved amplified polymorphic sequences (CAPS) markers (Konieczny and Ausubel, 1993) and microsatellite markers (Callum et al., 1994). The oligonucleotides were purchased from Genset (Paris, France).

Cytological analysis

For light microscopy, seedlings were fixed in 4% formaldehyde/0.2% gluteraldehyde then embedded in Historesin^{TM} (Leica, France) following the manufacturer's instructions. Semithin sections (3-5 μm) were made with a Jung RM microtome, stained with 0.05% methylene blue, and examined with a Nikon microphot FXA microscope.

Hormone quantification

Cytokinins and auxins were measured as described previously (Prinsen et al., 1995). Free IAA was extracted from 0.1-0.3 g frozen tissue in 80% methanol followed by solid phase extraction using RP-C18 (Varian) and DEAR-Sephadex A25 (Pharmacia) cartridges. Phenyl-[$^{13}C_6$]IAA (100 ng, CIL, USA) and ^{15}N1-indol-3-acetonitrile (150 ng), gift from N. Ilic, University, Maryland, USA) were initially added as internal tracers for recovery and analytical purposes. After derivatization, IAA-Me-HFB was analysed by (+)EI GC-SIM-MS. Cytokinins were extracted overnight from 0.1-0.3 g frozen tissue and purified combining solid phase extraction and immunoaffinity chromatrography using a broad spectrum anti-cytokinin antibody. The stable isotopes $^2H_5$-Z, $^2H_5$-[9R]Z, $^2H_5$-[9G]Z, $^2H_5$-[7G]Z, $^2H_5$-[9G]Z, $^2H_5$-[0G], [9R]Z, $^2H_5$- [9R]Z-phosphate, $^2H_6$-iP, $^2H_6$-[9R]iP, $^2H_6$-[9G]iP (50 ng each, APEX Int., UK) were initially added as internal tracers for recovery and analytical purposes. The different cytokinin fractions obtained after purification were analysed by (+)ES LC-MRM-MS/MS.

2-D proteins analysis

Protein extraction and two-dimensional gel electrophoresis

Protein extraction and 2-D gel electrophoresis were performed according to Santoni et al. (1994). IEF was performed with a preformed immobilized pH gradient (pH gradient from 4 to 7) supplied by Pharmacia. The second dimension was performed with a preformed SDS-PAGE gel (acrylamide gradient between 8% and 18%, Pharmacia). The gel was silver-stained according to Santoni et al. (1994). An average of 570 spots were analyzed in each 2-D gel.

Computer analysis of 2-D gels

Three independent protein extractions and independent electrophoretic separations were performed for each sample. Data were normalized as follows: one gel, randomly selected as a reference gel, was matched to all the other 2-D gels in order to calculate a quantitative ratio. Each quantitative ratio was then applied to its respective gel in order to normalize all the gels to the reference one. All the data have been computerized and analyzed as described by Santoni et al. (1997).

Detection of qualitative and quantitative variations:

We compared gels derived from wild-type or mutant plants from the same genetic background (Columbia or WS) and cultivated in the same conditions. Included in this analysis were gels derived from light-grown wild-type plants cultivated in the presence or in the absence of 5 μM benzyladenine (BA) or 5 μM piclorame (PC), and gels of mutants previously characterized in our laboratory: the mutants *sur1-2*, *sur1-3* and *cri1* (Boerjan et al., 1995; Delarue et al., 1997).

Spots that showed qualitative or quantitative variations in a studied genotype when compared to the wild-type control genotype were called “variable spots” (Santoni et al., 1997). Qualitative variation

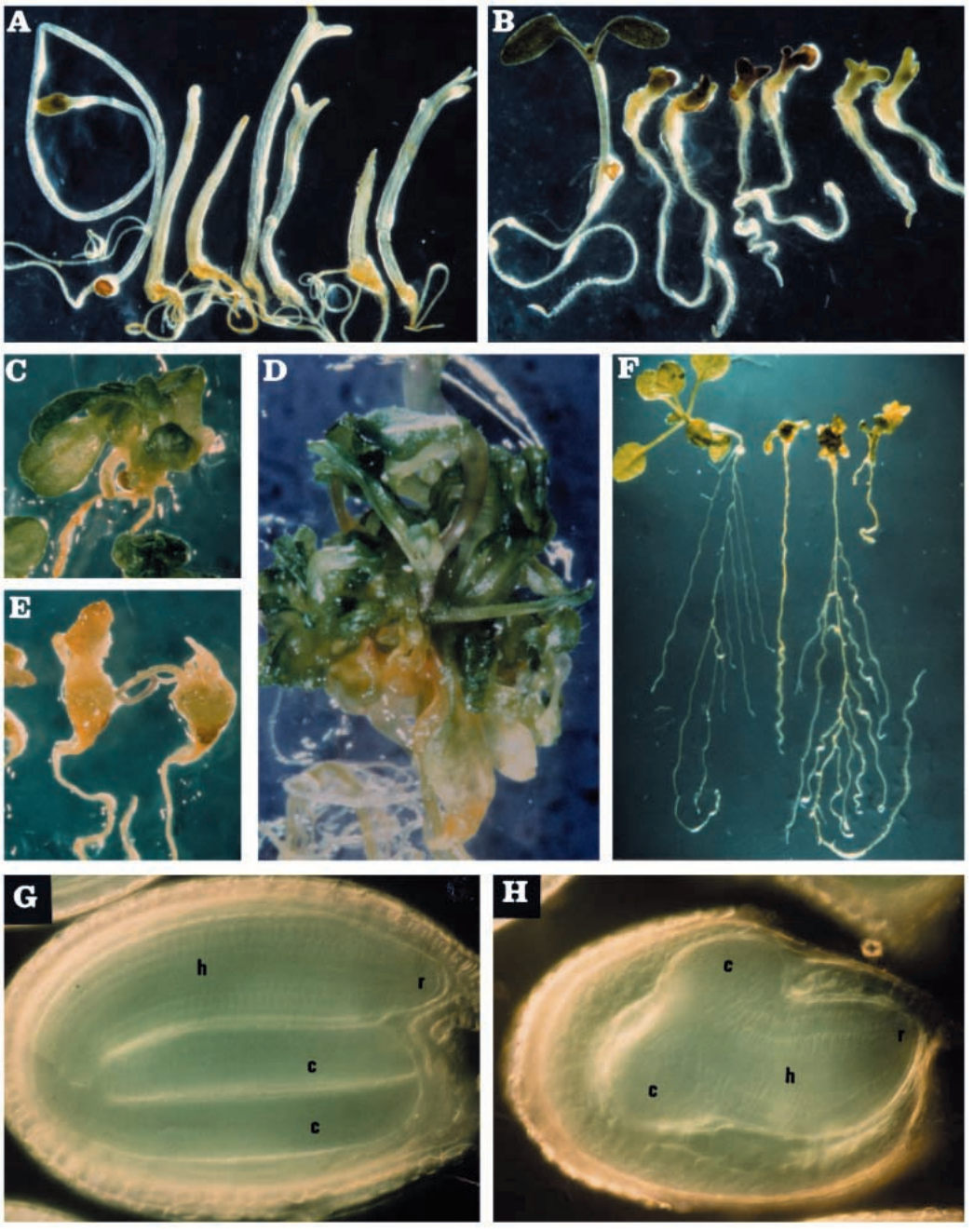

Fig. 1. Phenotypes of *pasticcino* mutants. (A) Wild type and 2 seedlings of each mutant (*pas1*, *pas2* and *pas3*, from left to right) grown in the dark, and (B) in the light, 7 d.a.g. (C) *pas* mutants show fused and vitreous leaves 15 d.a.g. (D) Compact, vitreous rosettes with multiple shoots, 3 months after germination. (E) More severe phenotypes of *pas1* and *pas3* with finger-like structures. (F) Three-week-old seedlings (WT, *pas1*, *pas2*, *pas3* from left to right). (G) Wild-type embryo, (H) *pasticcino* embryo; h, hypocotyl; c, cotyledons; r, root.

refers to a spot which is either new or has disappeared in the analyzed genotype when compared to the control. Quantitative variation refers to an increased or decreased optical density in the studied genotype when compared to the control. Variation in the optical density of each spot was assessed with Bioimage software (Bioimage Corporation, Ann Arbor, Michigan, USA) and compared between the different genotypes using analysis of variance (proc GLM of a SAS procedure, SAS/STAT 1988); the integrated optical density of each spot and the genotype were respectively taken as the dependent variable and the factor (Santoni et al., 1997).

Determination of distance indices

We have taken into account the variable spots common between mutants. A first matrix described the number of variable spots in common between two mutants for every combination of mutants. This matrix was then treated with the Jaccard index in order to calculate the biochemical distance between the mutants (Santoni et al., 1997). The distance matrix was then treated with agglomerative hierarchical clustering methods (UPGMA).

RESULTS

Isolation of pleiotropic mutants altered in their development and response to benzyladenine

Cary et al. (1995) demonstrated that cytokinin-induced inhibition of root and hypocotyl elongation was mediated by ethylene, while cotyledon and leaf inhibition was more specific to cytokinin. Thus, altered cotyledon and leaf growth reponses to benzyladenine were used as a criterion for the screen in order to reduce the chance of selecting ethylene mutants. Light and cytokinins have been found to have additive effects on seedling growth (Su and Howell, 1995) so the screen was performed in the dark or in the light, in the presence or in the absence of cytokinin in order to ensure an unsaturated cytokinin response. Finally, because cytokinin response could be lethal or could profoundly affect seedling development, the mutant screening was performed on the progeny of individual M_1 plants in order to be able to find heterozygous plants segregating the mutations.

Six mutants, called *pasticcino*, were isolated from 673 individually harvested M_2 EMS seed stocks. These mutants shared similar developmental traits such as stunted growth and deformed leaves. They all showed hypertophy of the apical parts in the presence of cytokinin. One mutant (*pas1-1*) with a very similar phenotype was isolated in the progeny of the 10,250 T-DNA mutagenized plants.

After outcrossing the mutants with the WT, allelic tests and segregation analysis were performed. Complementation analysis showed that the seven mutants belong to three independent groups named *pas1* (*pas1-1, pas1-2*); *pas2* ; *pas3* (*pas3-1, pas3-2, pas3-3, pas3-4*). To determine the genetic basis of the mutations, heterozygous plants segregating the different mutations were crossed with the WT, and the Pas^- phenotype was scored in the F_1 and F_2 generations. All the results were consistent with monogenic, recessive traits conferring the Pas^- phenotype (data not shown). Further characterization of the *pas* mutants was performed on one allele of each group, i.e., *pas1-2* , *pas 2* and *pas 3-1*, all of Col0 ecotype. In the progeny of plants heterozygous for *pas1-1* , the mutation *pas1-1* was shown to cosegregrate with a single T-DNA insertion carrying a kanamycin resistance gene (*NPTII*) (Vittorioso et al., unpublished data). For convenience only homozygous *pas* mutants are designated *pas* in the following text.

The *PAS2* gene was localized on the top of chromosome 5. Using the Kosambi function (Koorneef and Stam, 1992) it has been mapped between the markers nga249 and nga151, at 2 and 4.1 cM respectively. The *PAS3* gene showed linkage with the GAPB marker, on chromosome 1. The map position was calculated at 1 cM north of GAPB. The *PAS1* locus has been mapped to chromosome 3 (marker ve042 on the RI map) (Vittorioso et al., unpublished data).

Phenotype of *pasticcino* mutants

pasticcino mutants show altered leaf and root development

Fig. 1 shows dark (A) and light (B) grown plantlets of WT, *pas1-2*, *pas2* and *pas3-1*, 7 d.a.g.. All light grown mutants showed a very short and thick hypocotyl and misshaped cotyledons that never expanded. While *pas2* mutants always showed two small and rough cotyledons, *pas1-2* and *pas3-1* displayed variable phenotypes with plants with one, two or no cotyledons. Even when cotyledons were present, the apical hook was never observed in the *pas* mutants grown in the dark.

None of the mutants was able to grow directly in soil or in vitro on a medium free of sucrose. In vitro, on a medium containing 1% sucrose, all three mutants developed abnormal fused leaves, that were often vitrified (Fig. 1C). After several weeks, all mutants developed abnormal compact rosettes with multiple shoots similar to that observed in shooty teratomas (Fig. 1D). Several *pas1* and *pas3* plants did not develop leaves but only finger-like structures and rapidly stopped growing (Fig. 1E). Several *pas1* plants were able to develop very short stems with abnormal flowers (data not shown). A few *pas2* plants were able to make nearly normal leaves and could even produce sterile flowers (data not shown).

All *pasticcino* mutants showed abnormal root development. Seven days after germination, *pas3* already had a shorter primary root than the WT and the other mutants (Fig. 1B). The mutant *pas1* developed a primary root which was shorter than that of the WT, with no or very rare secondary roots; the mutant *pas2* showed a longer primary root than the WT as well as an increased number of secondary roots; the mutant *pas3* had a very short primary root with rare secondary roots (Fig. 1F).

pasticcino mutants have an altered embryo development

Optical sections of *pas* and WT embryos were performed to check whether the alterations observed in *pas* seedlings occurred during germination or were already present in the embryo. Before the heart stage, no obvious differences could be detected between WT and mutant embryos. At the heart stage where cotyledon primordia are initiated in the WT, the cotyledon primordia of the three mutants did not form correctly, leading to a flat apex. At later stages, mutant cotyledons failed to develop properly and the hypocotyl remained short and wide. This abnormal development of the hypocotyl in the *pas* mutants prevented the normal curvature of the embryo in the seed (Fig. 1G,H).

PAS mutants displayed uncoordinated cell divisions in the apical portion of the plant

During germination, cotyledons and hypocotyl failed to

elongate properly. Cytological analysis of the hypocotyl of the three mutants demonstrated that, in association with the cell elongation defect, the cell division pattern was also modified. Fig. 2A and B show that *pas* hypocotyls were disorganized with extra layers and variable number of cells in the cortex. Furthermore a loss of cell adhesion was observed which led to large intercellular spaces. In particular, Figure 2B shows the presence of ectopic periclinal divisions in the epidermis (L1 layer), which were never observed in the WT. Cotyledons of *pas* mutants also showed a similar disorganized structure with a loss of cell adhesion (Fig. 2C). The mutants' cells were generally more rounded than normal with larger intercellular spaces. This increased and disorganized cell proliferation, together with defects in cell adhesion, gave rise to a callus-like morphology of the tissues of *pas* mutants. In the case of *pas2* seedlings, the presence of clusters of dense, non-vacuolized cells, reminiscent of meristematic cells, were observed under the epidermis on the edges of cotyledons or leaves (Fig. 2D).

The apical meristem structure was highly modified and the different cell layers characteristic of a WT meristem were never distinguishable (Fig. 2E-G). This structure of the mutant meristem was found to be highly variable among siblings. In some of the mutants, the apical meristem was barely visible, and in others it was so large that it occupied all of the apical region. In many instances, the general pattern of the apical zone looked more like a teratoma than a true functional meristem flanked by cotyledons (Fig. 2G-H). This defect in the control of cell division seemed to be specific to the apical part of the plant since the radial pattern of the root was identical to the WT. Ectopic and anarchical divisions were never observed in the root of the mutants (data not shown).

pasticcino mutants have an altered response to exogenous cytokinin

Dose-response experiments in the presence of increasing concentrations of BA (from 0 to 10 μM) showed that the growth of the mutants' primary root was inhibited as was that of the WT (data not shown), indicating that the mutants are not resistant to cytokinin. On the contrary, the apical part of the three mutants at least doubled in size (Fig. 3A,B) while the WT had a short hypocotyl and small cotyledons. After 3 weeks in culture in the presence of cytokinin, the WT developed a few stunted chlorotic leaves (Fig. 3C,D), while mutants developed large, fused, green leaves (Fig. 3E). The mutant leaves were vitreous and sometimes were converted into a callus-like structure as a result of the increased cell proliferation. Quantification of fresh and dry weight of the mutants showed that this hypertrophy was not due to water accumulation resulting in cell expansion, but more likely was due to tissue proliferation (data not shown). This was also confirmed by longitudinal and transverse sections of mutants grown in the presence of

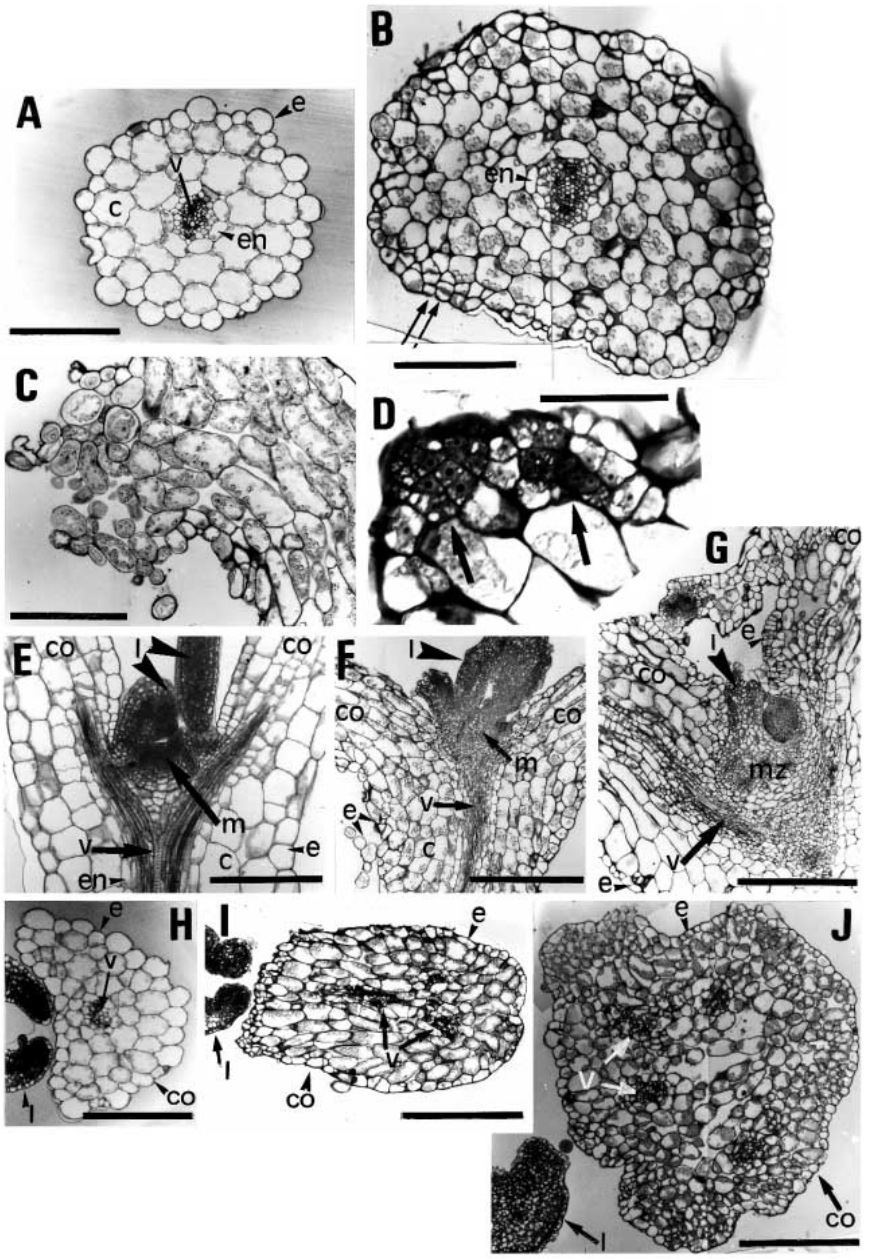

Fig. 2. Effects of *pas* mutations on cell division and cell adhesion in 12-day-old seedlings. Hypocotyl cross sections of WT (A) and of *pas2* mutant (B). In B, ectopic divisions (double arrows) and larger intercellular spaces are shown. (C) Longitudinal sections of mutant cotyledons clearly show the loss of cell adhesion. (D) Cross section of a *pas2* cotyledon, showing dense meristematic cells (arrows). Longitudinal sections of the shoot apical meristem of WT (E), and *pas* mutants (F,G). Cotyledon cross sections of WT grown on 5 μM BA (H), *pas* mutants (I), and *pas* mutants grown on 5 μM BA (J). c, cortex; co, cotyledons; e, epidermis; en, endodermis; l, leaf primordia; m, meristem; mz, meristematic zone; v, vessels. Scale bars represent 66 μm (D), 160 μm (A), 200 μm (B,C,E,H), 400 μm (F,G,I,J). Sections were made on 12 day-old seedlings.

cytokinin. Fig. 2H,J shows that cytokinin enhanced cell divisions and intercellular spaces in the mutants. Wild type seedlings treated with cytokinin never showed such structures. Several *pas2* plants in the presence of cytokinin even regenerated ectopic shoots on the edge of the leaves (Fig. 3F). Similar results were obtained with other types of cytokinins, such as zeatin or CPPU (results not shown).

None of the hormones tested (cytokinin, auxin, ethylene, gibberellic acid and brassinosteroides) was able to induce a *pasticcino*-like phenotype on wild-type plants (data not shown). At the same time, unsuccessful attempts were made to restore, completely or partially, a wild-type phenotype on the mutants by growing them in the presence of these hormones. This suggests that *pas* mutants are not deficient in the ability to synthesize these hormones. Neither AVG (an inhibitor of ethylene synthesis) or GA induced an elongation of the hypocotyl of *pas* mutants (data not shown). The mutants were not insensitive to these hormones as their root elongation was inhibited to the same extent as in the WT (data not shown).

An impaired response to cytokinin could also be a consequence of a defect in auxin responses. The effect of exogenous auxin application on the development of the mutants was analyzed by growing *pas* mutants in the presence or absence of 5 μM of piclorame. Conversely to what occurred in the presence of cytokinin, no apical hypertrophy of the mutants could be observed in the presence of auxin (Fig. 3G,H). Dose response experiments in the presence of increasing concentrations of piclorame showed that the percentage of root inhibition was the same for the WT and the mutants (data not shown). This indicates that the *pas* mutants are not resistant to auxin either. The subsequent response of the *pas* mutants to exogenous auxin was similar to that of the WT. The epidermal and cortical layer peeled off along the hypocotyl, and adventitious and secondary root primordia were stimulated (data not shown).

Hormone quantification

Because cytokinin enhanced cell proliferation in *pas* mutants, it was necessary to check whether *pas* phenotypes could be related to abnormal cytokinin production. Cytokinins of 3-week-old seedlings were quantified to assess whether the *pas* phenotype is related to a modification of endogenous cytokinin or auxin levels. No significant differences in cytokinin levels were observed in the mutants compared to the WT indicating that the *pas* mutations do not affect cytokinin biosynthesis (Table 1).

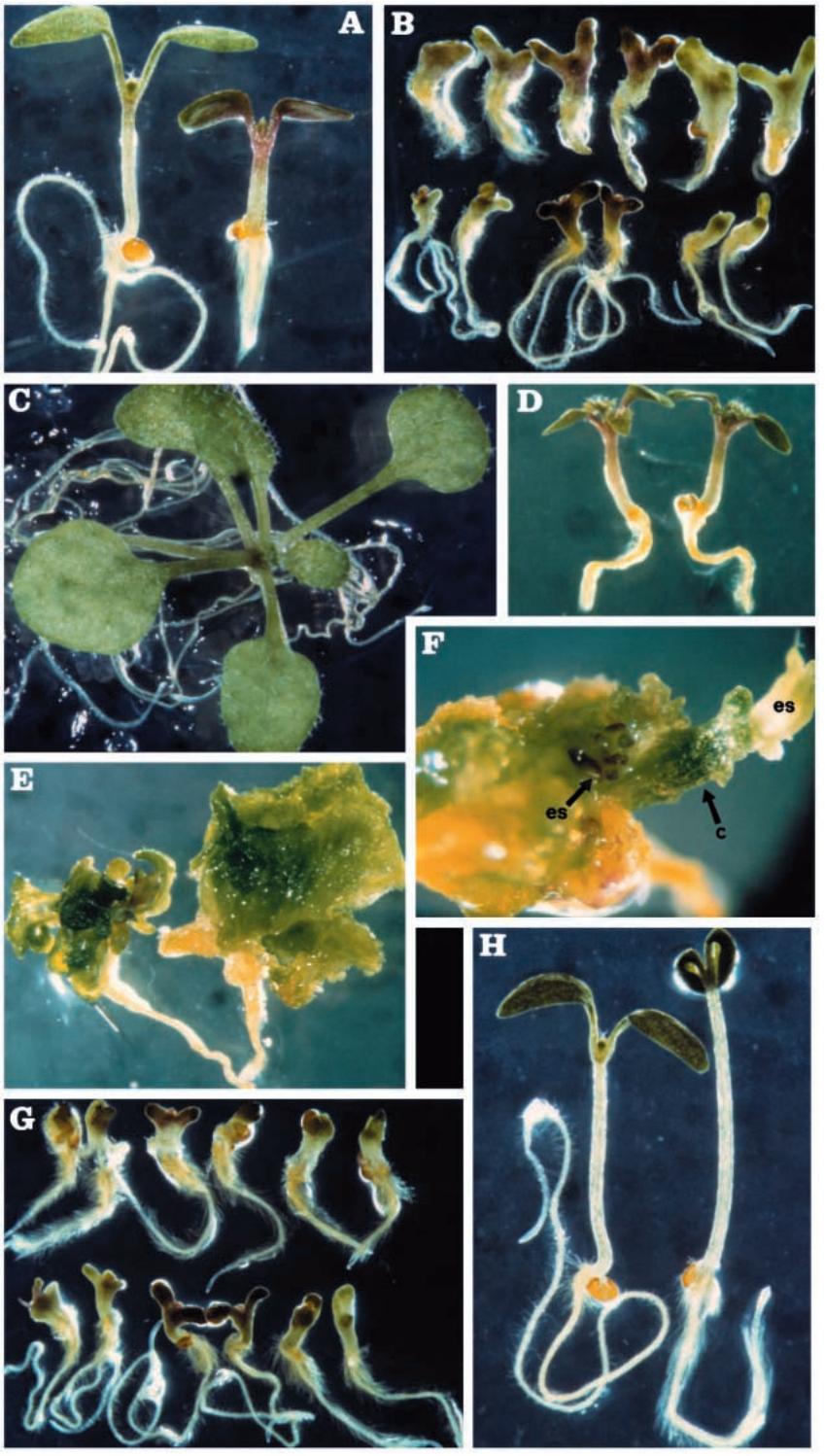

Fig. 3. Effect of cytokinin and auxin on *pas* mutants. (A) Wild type grown in the light with (right), and without (left) 5 μM BA, 7 d.a.g. (B) Mutant seedlings (*pas 1*, *pas2* and *pas3*, from left to right), grown in the light with (top), and without (bottom) 5 μM BA, 7 d.a.g. Two seedlings are shown for each mutant. (C) Wild-type plants 21 d.a.g. (D) Wild-type plants grown in the presence of 5 μM BA, 21 d.a.g. (E) *pas* mutants grown in the light, 21 d.a.g., in absence (left) or in the presence of 5 μM BA (right). (F) Ectopic shoots are regenerated in *pas2* mutants in the presence of BA. (G) Mutant seedlings (*pas 1*, *pas2* and *pas3* , from left to right), grown in the light with (top), and without (bottom) 5 μM piclorame, 7 d.a.g. (H) Wild type grown in the light without (left), and with (right) 5 μM piclorame.

Table 1. Hormone quantification

	WT		*pas1-2*		*pas2*		*pas3*	
	1	2	1	2	1	2	1	2
IAA	2,135.00	3,007.00	2,938.00	11,631.0	1,799.00	3,098.00	6,075.00	16,827.0
ZR	20.00	<100	17.00	41.00	25.00	29.00	15.00	25.00
ZNG	12.00	<100	42.00	65.00	58.00	48.00	17.00	42.00
Z	<10	<100	<9	49.00	<10	<10	<7	<7
IP	<9	<40	22.00	<9	<9	<9	<7	<7
IPA	–	<40	<10	<10	<10	<10	8.00	<7
IPG	16.00	<40	41.00	48.00	37.00	31.00	25.00	22.00

The amount of hormone is expressed in pmol/g dry weight.
<X means that the amount of hormone in the sample is under the detection threshold indicated in the table.
For each genotype,w the quantification has been repeated on two independant samples.
IAA, indole acetic acid; ZR, zeatine riboside; ZNG, zeatine Nglucoside; Z, zeatine; IP, isopenthenyl; IPA, isopenthenyladenine; IPG, isopenthenylglucoside.

Auxin content was also analyzed to ascertain that a modified auxin/cytokinin ratio was not responsible for the Pas⁻ phenotype. *pas2* mutants did not show any modification of the endogenous amount of auxin compared to the WT. Conversely, taking into account the variability due to experimental procedures, *pas1-2* and *pas3-1* seem to have a slight increase in endogenous free IAA concentration (Table 1).

Genetic analysis of double mutants

Crosses between heterozygous plants segregating the *pas* mutations have been performed. Double heterozygotes were selected in the F_1 progenies and allowed to self to produce F_2 seeds. The different mutant phenotypes were scored looking at the root phenotype which allows differentiation of the *pas* mutants. The results are reported in Table 2. In the case of all three crosses we could not detect new phenotypes, indicating that the three mutations are involved in the control of common processes. Because the mutants are all in the same ecotype background it is not possible to use a molecular marker to detect the double mutants. We observed that the proportion of one of the two mutants was always higher than the other for a same cross. This proportion (1/6 to 1/8) corresponds approximately to that expected if epistasis were involved (1/7 of total mutants). The results suggest that *pas3* is epistatic to *pas2* which in turn is epistatic to *pas1*. In the progeny of the crosses between *pas3* and *pas1* and between *pas2* and *pas1*, we obtained more wild-type plants than expected. This can be explained by the fact that, even after several back-crosses with the wild type, other mutations can still interact and induce bias in the segregation. Nevertheless, the genetic analysis confirms the subsequent biochemical analysis, suggesting that the *pas* mutations are closely related and presumably affect the same pathway.

Table 2. Genetic analysis of double mutants between the three *pas* mutants

Crosses	Total	WT	*pas1* phenotype	*pas2* phenotype	*pas3* phenotype
pas2 / + × *pas1* / +	1529	1045	202	282	
pas3 / + × *pas1* / +	1230	784	194		252
pas3 / + × *pas2* / +	1322	757		250	315

Crosses have been performed between heterozygous plants segregating one or the other *pas* mutation. The analysis of double mutants is done in the F2 generation of selfed double heterozygous plants.

Analysis of the 2-D protein patterns of the *pas* mutants

pas mutants showed altered cytokinin response in the apical portion without any major changes in endogenous cytokinin and auxin levels, suggesting that the *pas* mutations could affect the sensitivity to either hormone or both. Because a phenotype-based comparison of mutants can be misleading, we used a new approach to discriminate the role of cytokinin and auxin in the Pas⁻ phenotype. This method involves comparison of 2-D protein pattern of mutants and wild-type plants cultivated in the presence of various hormones. This technique has already proved its efficiency in the characterization of developmental mutants (Santoni et al., 1997; Delarue et al., 1997).

A statistical procedure was used to compare the amount of each spot in the 2-D pattern between the *pas* mutants and the wild type (see Materials and Methods). The spots that showed quantitative or qualitative variations were called “variable spots”. *pas* mutants showed a high number of variable spots compared to the WT (approx. 12%), among which one third are qualitative variations (Table 3). Some of variable spots in the *pas* mutants were also variable between the wild-type plants cultivated in the presence or absence of BA or the auxin analog piclorame (Table 4). Statistical analysis of these variations showed that a higher number of variable spots were found in common between the *pas* mutants and the wild-type cultivated in the presence of BA (from 31 to 47%) than between the *pas* mutants and the wild-type plant cultivated in the presence of piclorame (17 to 32%, Table 4).

A biochemical classification was undertaken by grouping the *pas* mutants with the mutant *sur1-3* (Boerjan et al., 1995)

Table 3. Quantity of ‘variable spots’ showing qualitative and quantitative variations in the mutants *pas* when compared to the wild-type plant

Genotype	Quantitative variations	Qualitative variations	Total variable spots (%)
pas2	45	19	10.5
pas3	47	18	11.5
pas1-2	48	17	11.2
pas1-1	59	18	13.4

The variable spots refers to the number of spots that showed quantitative and/or qualitative variations in the mutants when compared to wild-type plants. The percentage of total variable spots was calculated by dividing the number of spots that displayed qualitative and quantitative variations by the mean number of total spots contained in the patterns used for the comparison.

Table 4. Amount of variable spots in common between the mutants *pas* and the wild-type cultivated in the presence of of BA or PC

Genotype	BA	PC
pas2	24 (37%)	11 (17%)
pas3	20 (31%)	12 (18%)
pas1-2	23 (35%)	12 (18%)
pas1-1	36 (47%)	25 (32%)

The column BA indicates the number of variable spots in common between the mutants and the wild-type plant cultivated in the presence of BA and PC between the mutants and the wild-type plant cultivated in the presence of piclorame. We have indicated in brackets the percentage of the variable spots in the mutants that are also affected in the wild-type plant cultivated in the presence of the corresponding hormone.

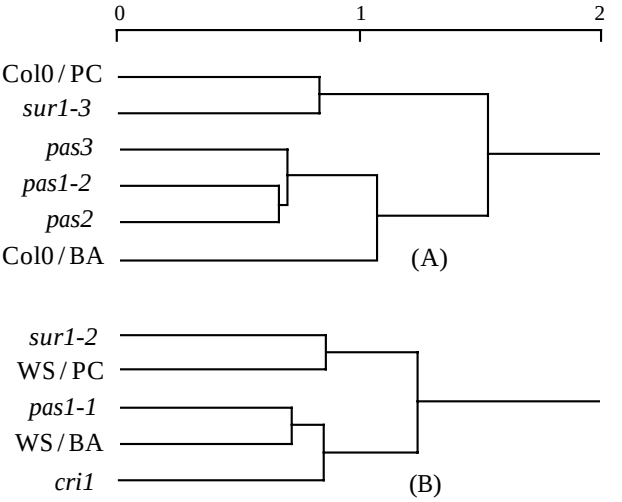

Fig. 4. Phenogram of biochemical distances between several genotypes and *pasticcino* mutants. WS/BA, Col0/BA and Col0/PC refer to WT ecotypes Wassilewskija (WS) and Columbia (Col0) grown in the presence of 5 μM benzyladenine (BA) or 5 μM piclorame (PC). The scale at the top of the phenogram shows the Jaccard index. A value of 0 means that all the protein spots are affected in the same way in two mutants and a value of 1 means that no spots are simultaneously affected in two mutants compared to the wild type. A Col0 background. 13 WS background.

which overproduces auxin and the wild-type Col0 treated either with piclorame or benzyladenine. This biochemical classification was based on the analysis of the variable spots common to the different genotypes (see Materials and Methods). Fig. 4A shows the results of the classification presented as a dendogram of the biochemical distances between the genotypes which demonstrates that the *pas* mutants are biochemically closely linked together. Furthermore, they make part of a cluster that includes the wild-type grown in the presence of BA. The *sur1-3* mutant which overproduces auxin makes an independant cluster together with the WT grown in the presence of auxin. These results suggest that the *PAS* genes are more involved in cytokinin-related functions than auxin-related functions. To support this hypothesis and because the use of exogenous cytokinin might induce a bias in the interpretation, the *pas* mutants were compared to the *cristal* mutant which has been shown to accumulate high endogenous levels of cytokinins (Delarue et al., 1997; Santoni et al, 1997). Since *cristal* was derived from the WS ecotype the experiment was performed with WS wild-type plants and the alleles *pas1-1* and *sur1-2* (Boerjan et al., 1995) which were all derived from WS to avoid any ecotype-related spot variation in the 2-D analysis. Figure 4B shows that *pas1-1* was biochemically closer to the *cristal* mutant and the cytokinin-treated WT than to the *sur1-2* mutant and the auxin-treated WT. Together these data indicate that the *pas* phenotype is biochemically more related to a cytokinin effect than an auxin effect.

DISCUSSION

Mutations in the three *PAS* genes induced a similar apical phenotype characterized by the presence of abnormal meristems leading to anarchic bushy rosettes made of fused vitrified leaves. The presence of clusters of dense non-vacuolated cells in the cotyledons and leaves of the mutants which are similar to meristematic cells, suggest that processes which control cell division are partially under the negative control of the *PAS* genes. These ectopic cell divisions have not been observed in the roots of the mutants and seem to be restricted to the apical portion of the plants. The restriction of cell division control by *PAS* genes to apical part of seedlings is already seen during embryogenesis. *pas* embryo defects appear at the begining of the heart stage when the cotyledons are initiated. Thus, the increase in cell division is probably not due to a mutation in house-keeping cell-cycle genes but is most likely a consequence of the loss of regulatory genes involved in the control of cell division by developmental stimuli, tissue specificity, or by growth regulators such as cytokinin and auxin. The phenotype of *pas* mutants is reminiscent of the abnormal shoots regenerated from explants *in vitro* when there is an unbalanced cytokinin/auxin ratio in the medium (Tran Thanh Van, 1981). In the presence of high concentrations of cytokinin, regenerated shoots often show abnormal fused vitreous leaves. The general phenotype of the *pas* mutants is also very similar to the “fasciation disease” caused by *Corynebacterium fascians* or *Rhodococcus fascians* which is directly correlated to an increase in the cytokinin concentration in the infected tissues (Kenneth et al., 1966; Crespi et al., 1992; Murai et al., 1980). Cytokinins were also found to contribute to cell divisions in apical meristems and to release the dormancy of secondary meristems. These meristematic cells were able to regenerate ectopic shoots after stimulation with cytokinin. This phenomenon was also observed in transgenic plants producing viviparous leaves after somatic activation of an *IPT* gene (Estruch et al., 1991). Vegetative adventitious buds arose from the subepidermal layer of leaf cells and formed viviparous leaves at the tip and in the midle of the leaf midrib. In *pasticcino* mutants, the clusters of meristematic cells were also always observed in the subepidermal layer.

The fact that exogenously applied cytokinin dramatically enhanced the effect of the *pas* mutations and the similarity between *pas* mutants and the various cytokinin-related phenotypes described above, suggest that cytokinin might be involved in the *pas* phenotype. None of the hormones exogenously applied to wild-type plants can mimic the *pasticcino* phenotype, indicating that the phenotype is not due

to hormone overproduction. When exogenous hormones are applied to the mutants the wild-type phenotype is not restored, indicating that the *pasticcino* mutants are not simply deficient in the production of any of the hormones tested. Quantification of cytokinin shows that the *pas* mutants are not cytokinin overproducers. Some differences in auxin concentrations between *pas* mutants and wild type have been found. It should be noticed that despite the variability observed in auxin measurements, it seems that *pas1-2* and *pas3* have a higher endogenous level of auxin than the wild type which might explain their altered root development. To further investigate whether cytokinins are involved in the *pas* phenotype we applied a new unbiased approach consisting of a 2-D biochemical analysis. The biochemical classification shows that the *pas* mutants are always in a cluster including the wild-type grown in the presence of cytokinin and the mutant *cristal* which has been shown to be a cytokinin overproducer (Delarue et al., 1997). The *pasticcino* mutants are never found associated with the wild-type grown in the presence of auxin or with an auxin-overproducing mutant. These results together with the absence of altered endogenous cytokinin levels would suggest that *pas* mutants may be altered in their sensitivity to cytokinin. *pas* mutants were always found to be tightly linked to one another in the dendogram of biochemical distances, indicating that they probably act in common functions. This has been confirmed by the genetic analysis of double mutants which showed a clear epistasis between the three *pas* mutants, and by the fact that the expression of the *PAS1* gene is altered in *pas2* and *pas3* mutants (Vittorioso et al., unpublished data). When compared to other mutants described by Santoni et al. (1997), the *pas* mutants were characterized by a higher number of variable spots when compared to the wild-type plant. The large number of proteins affected by *pas* mutations suggest that the corresponding genes play a central role in cell physiology.

The *pas* mutants represent a new class of mutants in which altered cell division leads to ectopic cell proliferation. The further genetic, physiological and molecular characterization of these mutants and the cloning of the corresponding genes will provide further information on the regulatory mechanisms involved in the control of cell division and differentiation during plant development. The results we describe here also suggest that cytokinins are possibly involved in the *pas* phenotype especially with regard to the control of cell division. The *PAS1* gene has been cloned and its characterization will provide further insights into the function of *PAS* genes in the control of plant development.

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