Hormonal Control of Cell Proliferation Requires *PASTICCINO* Genes

Yaël Harrar, Yannick Bellec, Catherine Bellini, and Jean-Denis Faure*

Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, 78026 Versailles cedex, France

PASTICCINO (*PAS*) genes are required for coordinated cell division and differentiation during plant development. In loss-of-function *pas* mutants, plant aerial tissues showed ectopic cell division that was specifically enhanced by cytokinins, leading to disorganized tumor-like tissue. To determine the role of the *PAS* genes in controlling cell proliferation, we first analyzed the expression profiles of several genes involved in cell division and meristem function. Differentiated and meristematic cells of the *pas* mutants were more competent for cell division as illustrated by the ectopic and enlarged expression profiles of *CYCLIN-DEPENDENT KINASE A* and *CYCLIN B1*. The expression of meristematic homeobox genes *KNOTTED-LIKE IN ARABIDOPSIS (KNAT2*, *KNAT6*), and *SHOOT MERISTEMLESS* was also increased in *pas* mutants. Moreover, the loss of meristem function caused by *shoot meristemless* mutation can be suppressed by *pas2.* The *KNAT2* expression pattern defines an enlarged meristematic zone in *pas* mutants that can be mimicked in wild type by cytokinin treatment. Cytokinin induction of the primary cytokinin response markers, *ARABIDOPSIS RESPONSE REGULATOR (ARR5* and *ARR6*), was enhanced and lasted longer in *pas* mutants, suggesting that *PAS* genes in wild type repress cytokinin responses. The expression of the cytokinin-regulated cyclin D, *cyclin D3.1*, was nonetheless not modified in *pas* mutants. However, primary auxin response genes were down-regulated in *pas* mutants, as shown by a lower auxin induction of *IAA4* and *IAA1* genes, demonstrating that the auxin response was also modified. Altogether, our results suggest that *PAS* genes are involved in the hormonal control of cell division and differentiation.

Plant cell proliferation and differentiation are controlled by many factors and, in particular, by the hormones auxin and the cytokinins. High auxin/cytokinin ratios usually induce root formation, whereas low auxin/cytokinin ratios induce shoot formation. Intermediate auxin/cytokinin ratios induce cell dedifferentiation and proliferation, leading to callus development. Several cytokinin signaling and early response genes were recently found to be involved in the control of cell proliferation and differentiation (for review, see Haberer and Kieber, 2002). Cytokinin perception involves three membrane-associated receptors (AtHK2, AtHK3, and AtHK4/CRE1/WOL) similar to the lower eukaryotes two-component system, which are constituted by a sensor His kinase (also termed sensor) and one or more response regulators. An additional gene called *CKI1*, which is structurally related to *CRE1*, is also able to activate the cytokinin signaling pathway, but does not bind cytokinins, at least at physiological concentrations (for review, see Hwang and Sheen, 2001). Cytokinins induce a multistep phosphorelay transfer from the receptor to small phosphotransmitter proteins called Arabidopsis His-phosphotransfer proteins (AHP). Upon phosphorylation by one of the cytokinin-activated His kinase receptors, AHP1 and AHP2, but not AHP5, migrate from the cytosol to the nucleus where they activate B-type response regulators (ARR2 or ARR1; Hwang and Sheen, 2001). B-type response regulators are composed of a receiver domain with a C terminus output domain related to those found in the DNA-binding motifs of basic helix-loop-helix and MYB transcription factors (Lohrmann and Harter, 2002). B-type *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*) are located in the nucleus where they activate the transcription of A-type *ARRs* such as *ARR5* or *ARR6*. The transcription of A-type *ARRs* is rapidly induced by cytokinins, whereas that of B-type *ARRs* has been reported to be unaffected by cytokinins (Imamura et al., 1999; Kiba et al., 1999; D'Agostino et al., 2000). Mutations in the *AtHK4/CRE1/WOL* gene decrease cell proliferation in response to cytokinins (Inoue et al., 2001). Overexpression of *ARR2* and *CKI1* leads to extensive cell proliferation and shoot regeneration in the absence of cytokinins (Kakimoto, 1996; Hwang and Sheen, 2001). Similarly, overexpression of *ARR1* results in increased sensitivity to cytokinins as illustrated in a cell proliferation assay (Sakai et al., 2001). Conversely, the *arr1* mutant is less sensitive to cytokinins (Sakai et al., 2001). Overexpression of the cytokinin-induced gene *Enhanced Shoot Regeneration1* also confers cytokinin-independent shoot regeneration (Banno et al., 2001). Mutations in other yet-uncharacterized genes are also responsible for the deregulation of cell proliferation and regeneration in vitro. Mutations in *POM1/ERH2* and *IRE1* induce shoot regeneration in the presence of

* Corresponding author; e-mail faure@versailles.inra.fr; fax 33–1–30–83–30–99.

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suboptimal concentration of cytokinins, but do not modify cytokinin endogenous levels (Cary et al., 2001). The analysis of cytokinin responses demonstrated that *pom1* and *ire1* mutants are cytokinin hyper-responsive rather than hypersensitive. In contrast, *ckh1* and *ckh2* mutants, isolated in similar screens, present typical cytokinin hypersensitivity (Kubo and Kakimoto, 2000).

Cytokinins were also involved in regulating cell division in the shoot apical meristem (Schmulling, 2002). Cell divisions in the apical meristem have been shown to be controlled by the homeobox-related factors like SHOOT MERISTEMLESS (*STM*; Weigel and Jurgens, 2002). *STM* was required for the maintenance of the meristematic stem cells. KNOTTED-like1 (KNAT1), which is related to *STM*, induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis (Chuck et al., 1996). Both genes were up-regulated by cytokinins (Rupp et al., 1999). The converse is also true because overexpression of the maize (*Zea mays*) *KNOTTED1* (*KN1*) gene in tobacco (*Nicotiana tabacum*) and overexpression of *KNOTTED-LIKE IN ARABIDOPSIS* (*KNAT1*) in lettuce (*Lactuca sativa*) lead to cytokinin accumulation (Hewelt et al., 2000; Frugis et al., 2001). Ectopic expression of maize KN1 resulted in cytokinin-autotrophic growth of cultured tobacco tissues (Hewelt et al., 2000).

Although a large set of mutants and genes have been isolated and characterized from in vitro cell proliferation and regeneration experiments, much less is known about the genetic control of cell proliferation and differentiation in planta. Several mutant lines of Arabidopsis were directly selected for their ability to grow as callus on hormone-free medium (Frank et al., 2000). These different mutant lines were altered in their cytokinin and/or auxin sensitivity and contents. Unfortunately, these lines could not be studied further because of their sterility. Recently, three different genes *TUMOROUS SHOOT DEVELOPMENT* (*TSD1, 2, 3*) were genetically identified because their loss of function resulting in disorganized tumorous tissues instead of leaves and stems (Frank et al., 2002). Inhibitory concentrations of cytokinins for wild type enhance cell proliferation in *tsd* seedlings. The *tsd* phenotype is reminiscent of the phenotype of the *pasticcino* (*pas*) mutants (Faure et al., 1998). The *pas* mutants were isolated in a screen for uncontrolled growth in presence of cytokinins, leading to cell proliferation and callus formation. *Pas* mutants had an altered development starting at the embryo heart stage, leading to abnormal leaf and root development (Faure et al., 1998). Seedlings presented an enlarged hypocotyl and small rounded cotyledons, which did not develop further after germination. The apical meristem structure was variable, often showing a large disorganized meristematic-like zone. Organs of the plant apices showed ectopic and anarchic cell divisions, which were not observed in the root. The *pas* mutants represent three complementation groups (*Pas1*, *Pas2*, and *Pas3*). Double-mutant analysis revealed epistatic relationships between them, suggesting genetic interactions between the *PAS* genes. The *PAS1* gene was cloned and found to encode an immunophillin-like protein of the FK506-binding protein family (Vittorioso et al., 1998; Harrar et al., 2001). FK506-binding proteins probably hold signaling proteins in "poised" states that can be triggered to active forms by protein modifications, such as phosphorylation (Rutherford and Zuker, 1994). *PAS2* gene was recently identified and found to encode a protein Tyr phosphatase-like structurally conserved among eukaryotes (Bellec et al., 2002).

As cytokinins are involved in cell differentiation and proliferation and have been shown to enhance proliferation in the *pas* mutants, we have analyzed the relationship between the *pas* phenotype and cytokinin responses. Here, we present evidence that *pas* mutants have a modified competence for cell division in meristematic and differentiated tissues that is associated with the deregulation of cytokinin but also auxin primary response genes.

RESULTS

The Expression Pattern of Cell Cycle Genes Is Deregulated in *pas* Mutants

To evaluate the differentiation state of the cells in different seedling tissues, we examined the expression of *CYCLIN-DEPENDENT KINASE A* (*CDKA*), a marker of cell division competency, which is weakly expressed in differentiated cells (Hemerly et al., 1993). *CDKA::β-glucuronidase* (*GUS*) expression was analyzed in 10-d-old *pas* and wild-type seedlings (Fig. 1A). In wild type, *CDKA* was mainly expressed in the root and apical meristems as well as in the leaf primordia, but was absent or weakly expressed in differentiated tissues like hypocotyls, roots, or cotyledons. In the three *pas* mutants, *CDKA* expression was enhanced in seedling apices, particularly in the meristematic area. *CDKA* expression was also ectopically expressed in the hypocotyl and the cotyledons, demonstrating that the cells of these normally differentiated tissues retained some competence to divide. To examine whether CDKA expression was correlated with the expression of other cell division markers, we monitored *CYCLIN B1* (*CYCB1* also named *Cyc1a*) expression in *pas* mutants (Fig. 1, B and C). *CYCB1* is a mitotic cyclin specifically expressed at the G2/M transition and provides a marker for cells undergoing mitotic divisions (Ferreira et al., 1994). We introduced *CYCB1::GUS* marker into the *pas* mutants to monitor the pattern of cell divisions in seedlings. Due to the stability of GUS and the absence of the cyclin "destruction box" (db) in the construct, *CYCB1::GUS* does not indicate the cells dividing at the time of observation, but rather the history of divisions that took place in the different tissues the

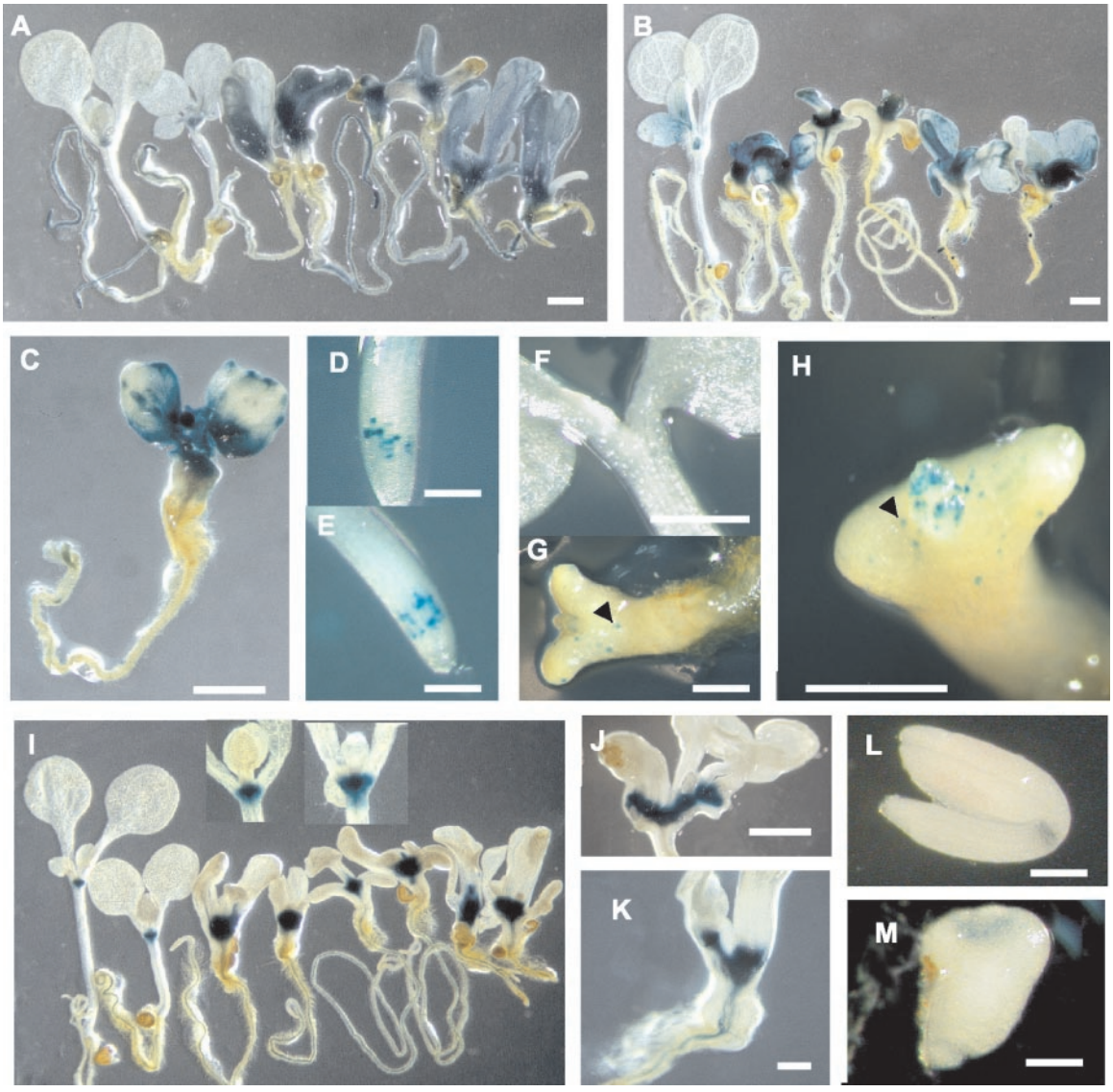

Figure 1. Expression of cell division and meristematic-associated markers in the *pas* mutants. (A) *CDKA::GUS* expression in wild-type, *pas1*, *pas2*, and *pas3* seedlings (from left to right, two seedlings each) 10 d after germination. Wild-type seedlings were grown in the absence (left) or presence of 0.1 μM 6-benzyladenine (BA; right). All of the *pas* mutants were grown in absence of cytokinins. (B) *CYCB1::GUS* expression in wild-type, *pas1*, *pas2*, and *pas3* seedlings (from left to right, two seedlings each) 10 d after germination. *CDKA* and *CYCB1* were strongly expressed in the meristems and were ectopically expressed in the leaves of the *pas* mutants. (C) *CYCB1::GUS* expression in *pas1* mutant 10 d after germination showing localized area of ectopic *CYCB1* expression in the leaves. (D) *CycB1::db::GUS* expression in 5 d after germination wild-type root. (E) *CycB1::db::GUS* expression 5 d after germination in *pas2-1* root. (F) *CycB1::db::GUS* expression 5 d after germination in wild-type seedling. (G and H) *CycB1::db::GUS* expression 5 d after germination in *pas2-1* seedling. (I) *KNAT2::GUS* expression in wild-type, *pas1*, *pas2*, and *pas3* seedlings (from left to right, two seedlings each) 10 d after germination. Wild-type seedlings were grown in the absence (left) or presence of 0.1 μM BA (right). The corresponding meristematic areas are enlarged, showing increased expression of *KNAT2* in the presence of cytokinins (right insert) compared with control (left insert). The *pas* mutants were grown in the absence of cytokinins. (J) *KNAT2::GUS* expression in the *pas2* mutant 18 d after germination. (K) *KNAT2::GUS* expression in the *pas3* mutant 10 d after germination. *KNAT2::GUS* staining shows the existence of enlarged or doubled meristem in the *pas* mutants. (L) *KNAT2::GUS* expression in mature wild-type embryo. (M) *KNAT2::GUS* expression in mature *pas2* embryo. Scale bar represents 1 mm except for D, E, L, and M, where it represents 200 μm.

days preceding the observation. Wild-type seedlings showed *CYCB1* expression in roots and apical meristems as well as in young leaf primordia. The three *pas* mutant seedlings showed enhanced expression of *CYCB1* in the meristems, but also ectopically in the hypocotyl and the cotyledons. *CYCB1* expression often appeared in localized areas of *pas* leaves, suggesting heterogeneous activation of cell division (Fig.

1C); this patchy pattern was never observed in wild type. To monitor cell division at the time of staining, *CYCB1::db::GUS* was introduced into *pas* mutants and was analyzed in 5-d-old seedlings to check whether ectopic cell division observed with *CYCB1::GUS* was occurring earlier in development. In wild type, 5 d after germination *CYCB1::db::GUS* staining was only observed in the root meristematic zone (Fig. 1D). No staining could be observed in the seedling apical part (Fig. 1F). In the *pas* mutants, *CYCB1::db::GUS* staining in the root is similar to wild type, indicating that *pas* mutations did not alter directly the cell cycle and thus the expression of *CYCB1*. Contrary to the root, the apical part of *pas* seedling showed ectopic expression of *CYCB1::db::GUS* in differentiated tissues such as the hypocotyl and the cotyledons (Fig. 1, G and H).

Several *KNAT* Genes are Up-Regulated in *pas* Mutants

Analysis of cell division markers showed an enlarged meristematic region in the three *pas* mutants. Enlargement of meristematic area was associated with disorganized meristem structure, as already described (Faure et al., 1998). Thus, we analyzed the expression pattern of several *KNOTTED-like in Arabidopsis* (*KNAT*) genes, which where found to be involved in meristem function. Expression levels of *KNAT1*, *KNAT2*, *KNAT6*, and *STM* were analyzed by reverse-transcriptase (RT)-PCR and real-time quantitative PCR (for *STM* only). *KNAT2*, *KNAT6*, and *STM* genes, but not *KNAT1*, showed increased expression levels in *pas* seedlings compared with wild type, suggesting that *PAS* genes act as negative regulators of the expression of several *KNAT* genes (Fig. 2, A and B).

KNAT expression was further analyzed in the case of *KNAT2*, which is specifically expressed in the inner part of the meristem that contains the founder cells that will be recruited by the peripheral and rib zones to initiate organs (Dockx et al., 1995). *KNAT2* provides a marker for the most undifferentiated cells of the meristem. *KNAT2::GUS* was observed in 10-d-old wild-type and mutant seedlings (Fig. 1I). Although *KNAT2::GUS* was restricted in wild type to a small internal zone of the meristem, the zone of its expression was enlarged in the three *pas* mutants. In some *pas2* and *pas3* mutant seedlings, *KNAT2::GUS* staining defined several distinct but connected regions in the meristematic zone, suggesting the occurrence of meristematic activity outside the usual bounds of the shoot apical meristem (Fig. 1, J and K). The ectopic formation of shoots in *pas2* and *pas3* suggests that multiple sites of shoot initiation could develop from these enlarged meristematic regions. *KNAT2* enlarged expression could be a consequence of the modified meristematic zone in the *pas* mutants. *KNAT2::GUS* expression was analyzed in mature embryo imbibated for 16 h (Fig. 1, L and M). The *pas* embryos still presented an enlarged *KNAT2::GUS* ex-

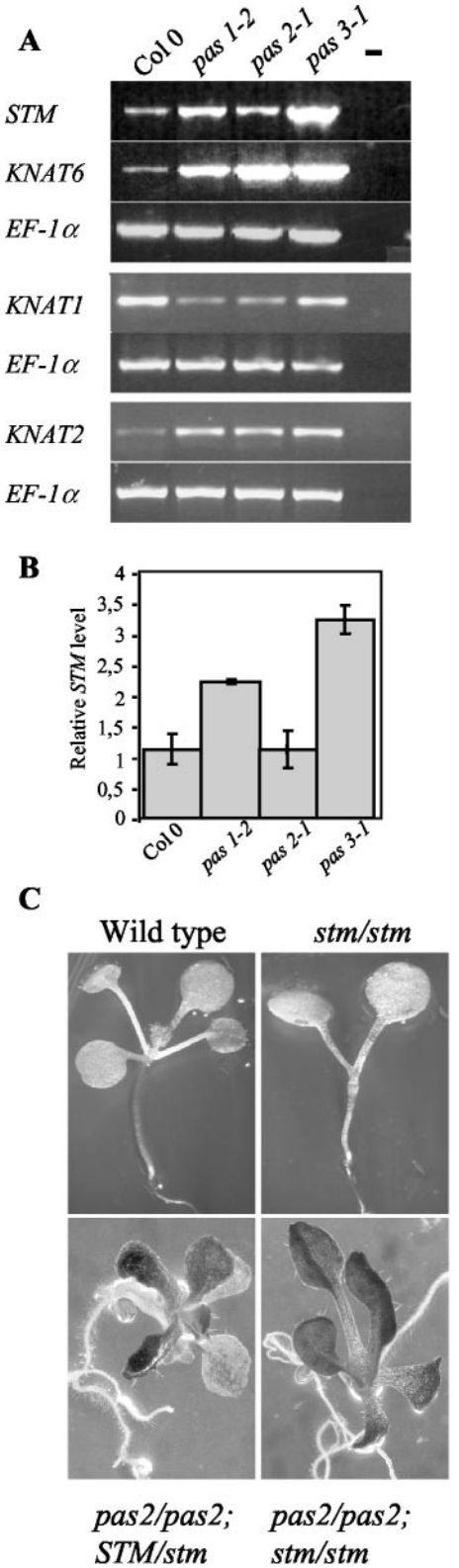

Figure 2. Interaction between *KNAT* genes and *pas* phenotype. (A) RT-PCR analysis of *KNAT* mRNA levels in wild-type and pas mutants. (B) Real-time quantitative PCR of *STM* mRNA levels in wild-type and pas mutants. (C) Phenotype of wild type, *stm/stm*, *pas2/pas2*, and the double-mutant *pas2/pas2;stm/stm*.

pression zone compared with wild type, even if the expression pattern was not as pronounced as in the seedlings. As previously shown (Hamant et al., 2002), *KNAT2* expression is enhanced in wild-type apical meristems of seedlings treated with exogenous cytokinins (Fig. 1I, inserts). Cytokinin treatment also induced ectopic *KNAT2* expression in the vascular tissue of wild-type roots (data not shown). The *KNAT2::GUS* pattern in *pas* mutants were similar to those observed in cytokinin-treated wild type, i.e. enlarged meristematic GUS staining and ectopic staining in root vascular bundles. In a wild-type background expressing *KNAT2::GUS*, GUS activity as quantified by fluorometry was 2 ± 0.2 nmol MU min^{-1} mg^{-1}. In *pas* seedlings, GUS activity was 7.2 ± 0.2 nmol MU min^{-1} mg^{-1} for *pas1*, 5.6 ± 1.5 for *pas2*, and 8.2 ± 0.2 for *pas3*. This result confirmed the enhanced expression of *KNAT2* in *pas* mutants.

To investigate how the increased cell division in *pas* meristems is relevant to meristem function, we analyzed the phenotype of double mutants between *pas2-1* and a strong *stm* allele (*stm dgh6*), which is unable to form a shoot apical meristem (Fig. 2C). We have chosen *pas2* because it has the less altered postembryonic phenotype among the *pas* mutants, in particular at the levels of leaves and stems (Bellec et al., 2002). Plants from the F_2 population were genotyped by PCR. All the double mutants *pas2/pas2; stm/stm* analyzed at the seedling stage showed fused and deformed leaves characteristic of *pas2* mutants. The presence of leaves in the double mutants indicated that the shoot apical meristem was functional and that *pas2* mutation was able to suppress strong *stm* mutation, restoring an active meristem.

Cytokinin Primary Response Genes Are Up-Regulated in *pas* Mutants

Because *KNAT* expression and function were associated with cytokinins in several studies (Rupp et al., 1999; Hamant et al., 2002), an attractive hypothesis could be that *pas* mutations affect directly cytokinin responses. Thus, cytokinin responses were analyzed in the *pas* mutants compared with wild type by quantifying the expression of two A-type *ARR* genes, *ARR5* and *ARR6* (Fig. 3). We used an experimental system that allowed rapid and reproducible treatment of seedlings by hormones. Seedlings were germinated on solid media and were grown in the light for about 12 d and were then transferred to the same liquid media for 2 d and finally were directly treated with cytokinins in liquid. Liquid culture allows homogeneous seedling treatment and rapid access of the hormone to all seedling tissues.

ARR5 and *ARR6* expression were first monitored in wild-type and *pas* mutants after 30 and 60 min, respectively, of induction with 10 μM BA (Fig. 3A). *Pas* mutants showed increased expression levels of both genes after cytokinin treatment compared with wild

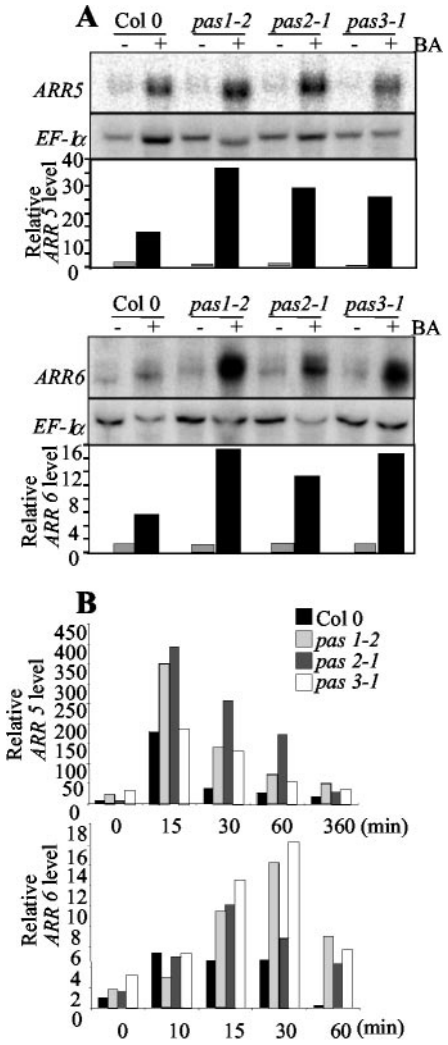

Figure 3. Cytokinin regulation of *ARR5* and *ARR6* in *pas* mutants. (A) *ARR5* and ARR6 mRNA levels in wild-type and *pas* mutant seedlings after 30 and 60 min cytokinin treatment, respectively. (B) Time-course expression analysis of *ARR5* (top) and *ARR6* (bottom) in wild-type and *pas* mutants. Seedlings were transferred 12 d after germination to liquid media for 2 d and treated with 10 μM BA for the time indicated. *ARR* hybridization signals were quantified and normalized with *EF-1α* as described in "Materials and Methods."

type (5-, 24-, 16-, and 23-fold for *ARR5* at 30 min and 4-, 13-, 8-, and 10-fold for *ARR6* at 60 min, for wild type, *pas1*, *pas2*, and *pas3*, respectively; Fig. 3A).

To investigate whether *pas* mutations modified not only the amplitude but also the timing of cytokinin response, time-course analysis of *ARR5* and *ARR6* expression was undertaken. *ARR5* was induced by cytokinins in wild-type treated seedlings in 15 min with a 19-fold induction and then mRNA levels declined rapidly, reaching noninduced levels after 60 min (Fig. 3B). *ARR6* mRNA showed a 4-fold induction by cytokinins after 10 min and then reached basal levels after 60 min. (Fig. 3B). Both genes were also induced by cytokinins in *pas* mutants but with a higher amplitude than in wild type. After a 15-min

induction, *ARR5* mRNA levels were 2-fold higher in *pas1* and *pas2* mutants compared with wild type, and after a 30-min induction, reached three to six times wild-type levels for the three *pas* mutants. Induction of *ARR5* in *pas3* mutants was similar to wild type after 15 min of treatment, but mRNA levels remained high after 60 min of induction. *ARR6* induction was also enhanced in the three *pas* mutants. *Pas3* mutants showed the highest response with a 4-fold wild-type level at 30 min. In all three mutants, *ARR6* expression was maintained at high levels even after 60 min of treatment, whereas wild-type mRNA levels returned to almost basal level.

ARR5 expression was also analyzed in planta by following the expression of an *ARR5::GUS* construct (Fig. 4A–E). *ARR5* expression in young seedlings was mainly expressed in apical and root meristems, but was also found in vascular tissues (root, hypocotyl, and cotyledons). Weak *ARR5* expression was also observed in cotyledons and mature leaves after prolonged staining (Fig. 4A; D'Agostino et al., 2000). Cytokinin treatment enhanced GUS staining in the meristem, confirming the northern results (Fig. 4, A and B, inserts). In the *pas* mutants, *ARR5::GUS* expression was also mainly localized in meristems, but the staining intensity was significantly increased compared with wild type in agreement with the northern results (Fig. 1, C–E). In young *pas* seedlings showing a strong phenotype, enhanced *ARR5::GUS* expression can be found in cotyledon and hypocotyl cells (data not shown). *ARR5::GUS* staining was also observed in the leaf vascular tissues and in the roots of *pas2* mutants and in leaves of *pas3* mutants (Fig. 4, D and E).

Cyclin D3.1 (*CYCD3*) Expression Is Not Enhanced in *pas* Mutants

Our results indicated that in the *pas* mutants, differentiated cells of the cotyledons or the hypocotyl were more competent for cell division. Moreover, we found that cytokinin primary responses were enhanced, suggesting that the *PAS* genes might control cell cycle regulation by cytokinins. *CYCD3* is one of the most well-characterized cell cycle genes regulated by cytokinins. Constitutive expression of *CYCD3* in transgenic plants led to cell proliferation in the absence of exogenous cytokinins (Riou-Khamlichi et al., 1999). In wild-type seedlings, *CYCD3* expression levels showed an almost 2-fold induction after cytokinin treatment (Fig. 5). *CYCD3* expression after cytokinin induction was slightly higher in the *pas2* mutant and lower in *pas1* and *pas3* mutants compared with wild type. To confirm this result, *CYCD3* expression was analyzed in planta using a *CYCD3::GUS* marker in wild-type and *pas* mutants.

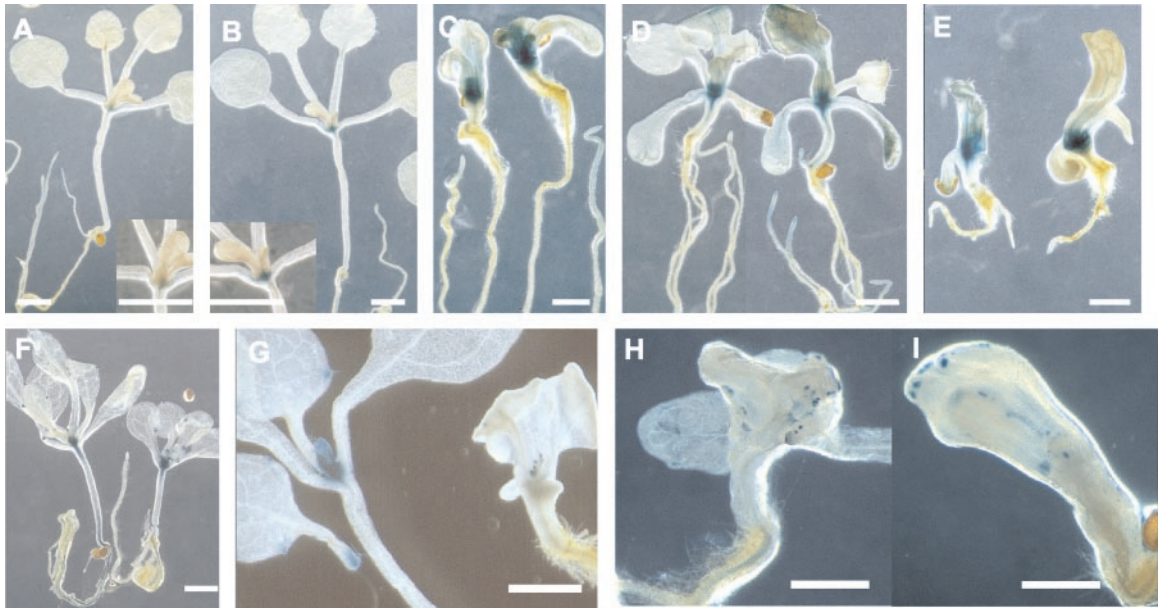

Figure 4. Expression of cytokinin-associated markers in *pas* mutants. (A) *ARR5::GUS* expression in a 15 d after germination wild-type seedling with an enlarged view of the corresponding meristematic zone in the insert. (B) *ARR5::GUS* expression in a 15 d after germination wild-type seedling treated for 2 h with 10 μM BA. An enlarged view of the corresponding meristematic zone is shown in the insert. (C) *ARR5::GUS* expression 15 d after germination in *pas1* seedlings treated with 10 μM BA (right) compared with control (left). (D) *ARR5::GUS* expression 15 d after germination in *pas2* seedlings treated with 10 μM BA (right) compared with control (left). (E) *ARR5::GUS* expression 15 d after germination in *pas3* seedlings treated with 10 μM BA (right) compared with control (left). (F) *CYCD3::GUS* expression 15 d after germination in wild-type seedlings grown in the presence of 5 μM BA (right) compared with control (left). (G) *CYCD3::GUS* expression in wild-type (left) and *pas1* (right) seedlings 15 d after germination. (H) *CYCD3::GUS* expression in *pas2* mutant. (I) *CYCD3::GUS* expression in *pas3-1* mutant. Scale bar represents 1 mm.

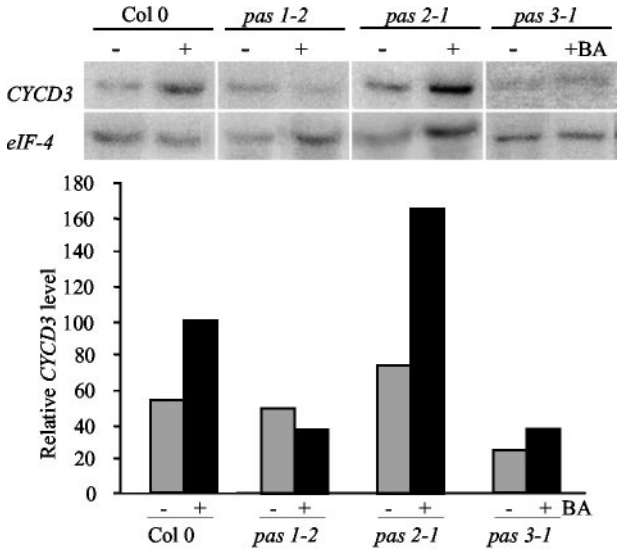

Figure 5. Cytokinin regulation of *CYCD3* expression in *pas* mutants. Steady-state mRNA levels of *CYCD3* in wild-type and *pas* mutants treated with 10 μM BA in liquid for 24 h. *CYCD3* hybridization signals were quantified and normalized with *eIF4A* as described in "Materials and Methods."

In wild type, *CYCD3* was mainly expressed in the apical meristem, leaf primordia, young leaves, and hydrathodes of older leaves (Fig. 4, F and G). Heterogeneous and weak staining was usually observed in *pas* mutants (Fig. 4, G–I). Some seedlings expressed *CYCD3* with a similar pattern to that of wild type, whereas most others showed no staining in apical meristems and leaf primordia but very localized foci of expression. Some stainings were localized in structures similar to the wild-type hydrathodes (Fig. 4G), whereas others were found in leaf cells clusters as for *CYCB1::GUS* staining (Fig. 4, H and I). *CYCD3::GUS* staining confirmed that *CYCD3* was not overexpressed in *pas* mutants. *CYCD3::GUS* staining was maintained in mutant root meristems (data not shown), probably explaining why *pas2* showed higher *CYCD3* expression than other *pas* mutants in northern experiments because *pas2* has an increased numbers of secondary root (Faure et al., 1998).

Auxin Induction of Primary Response Genes Is Reduced in *pas* Mutants

Cell dedifferentiation and proliferation are usually induced by a balanced auxin-to-cytokinin ratio. As *pas* mutants show an enhanced response to cytokinins, we investigated whether auxin responses were also modified. Auxin response was analyzed by the quantification of expression levels of primary response genes rapidly induced by auxin. We chose *indole-3-acetic acid4* (*IAA4*) and *IAA1* because they present a robust auxin regulation and they are among the few *IAA* genes expressed in shoot tissues (Abel et al., 1995).

The *IAA4* mRNA level was rapidly increased in wild-type seedlings, five and 10 times, respectively, after 15 min and 3 h of auxin treatment (Fig. 6A). *IAA4* mRNA basal levels were found to be lower in the three *pas* mutants compared with wild type. Auxin induction of *IAA4* was nonetheless observed in *pas* mutants, but with a lower magnitude (10, 7, 3, and 6 times for wild type, *pas1*, *pas2*, and *pas3*, respectively, relative to noninduced levels after 3 h of

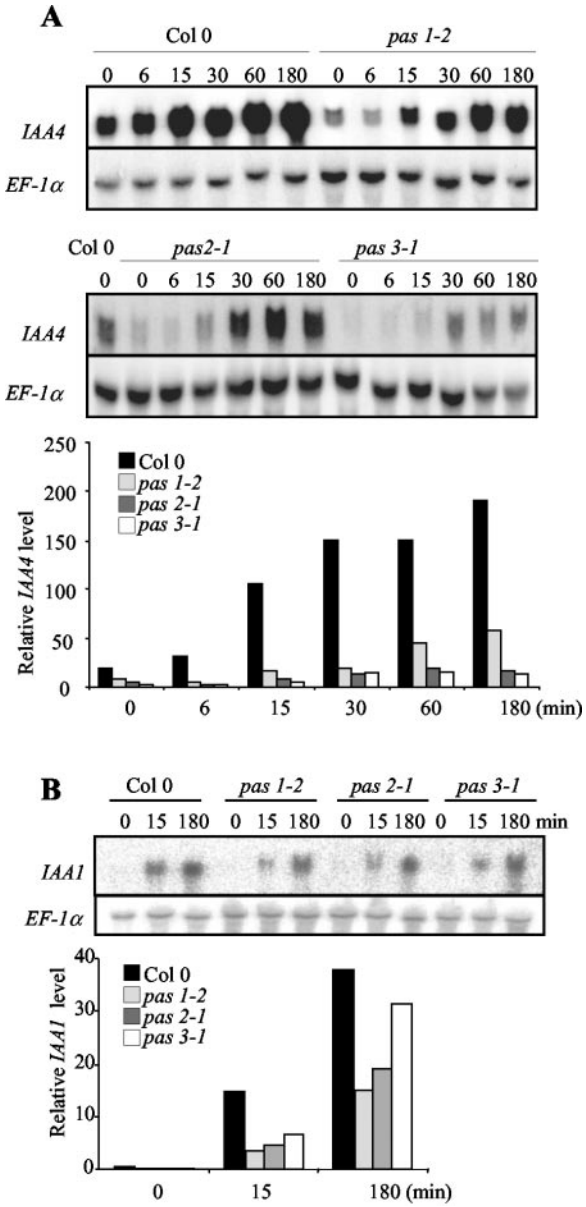

Figure 6. Auxin regulation of *IAA4* and *IAA1* gene expression in *pas* mutants. (A) Time-course analysis of steady-state *IAA4* mRNA levels in wild-type and *pas* seedlings. (B) Time-course analysis of steady-state *IAA1* mRNA levels in wild-type seedlings. Seedlings were transferred to liquid media 12 d after germination for 2 d and were treated with 20 μM IAA for the time indicated. *IAA* hybridization signals were quantified and normalized with *EF-1α* as described in "Materials and Methods."

treatment). A similar pattern of expression was also observed for *IAA1* with lower mRNA levels in the *pas* mutants (Fig. 6B). After a 15-min induction, *IAA1* levels were induced 82-, 62-, 49-, and 68-fold in wild type, *pas1*, *pas2*, and *pas3*, respectively. These results suggest that the expression of primary auxin response genes is also modified in the *pas* mutants.

DISCUSSION

The *pas* mutants are characterized by ectopic cell divisions, which are specifically enhanced by cytokinins. They provide an interesting genetic model to study hormonal regulation of cell differentiation and proliferation in planta. Mutations in *PAS* genes resulted into developmental defects that can be tracked back to the heart stage of embryogenesis during the transition from radial to bilateral symmetry (Faure et al., 1998).

In this study, we found that the disorganized cell divisions are associated with the deregulation of cell cycle marker genes like *CDKA* and *CYCB1*, suggesting that cells would be maintained in a state competent for cell proliferation. Ectopic figures of division are probably responsible for the increased layers of cells observed, for instance, in the cortex and the epidermis of the hypocotyl (Faure et al., 1998). Increased cell division competency in *pas* mutants was particularly pronounced in already undifferentiated cells such as meristematic cells as illustrated by the expression of the cell division markers *CDKA* and *CYCB1*. Previous observations found that *pas* apical meristems were often enlarged and presented a loose structure where the different layers and zones were difficult to distinguish (Faure et al., 1998). Alteration of *pas* meristem structure is correlated with the up-regulation of several *KNAT* genes. The loss of *PAS* function leads to an enlarged *KNAT2* expression zone comprising almost the entire apical meristem, confirming that most of the *pas* meristem are constituted by L3-like type cells. This enlarged *KNAT2* expression zone could be already seen in mature embryos, suggesting that an altered expression of *KNAT2* is an early effect of *pas* mutations. Increased propensity for cell division caused by the loss of *PAS* function is also illustrated by the suppression of strong *stm* phenotype by *pas2*. Loss of *STM* function leads to improper corpus/tunica organization in the embryo meristem and a functional meristem is never organized after germination (Barton and Poethig, 1993). *STM* loss of function could also be suppressed by a mutation in *ASYMMETRYC LEAVES 1* (*AS1*), which is a negative regulator of *KNAT* genes. Secondary suppressor screen of *as1 stm* double mutants revealed that *KNAT1* was involved in the restoration of meristem function in *stm* background (Byrne et al., 2002). Similarly, a mutation in *YABBY* that is associated with *STM* and *KNAT2* up-regulation led to partial suppression of *stm* phenotype (Kumaran et al., 2002). The suppression of *STM* dysfunction by *pas* mutations could be explained by the up-regulation of several *KNAT* genes and by the fact that there is some functional redundancy among the *KNAT* family members.

Several reports linked cytokinins to the expression of meristematic homeobox transcription factors (Rupp et al., 1999; Hamant et al., 2002). The *KNAT2* expression zone in cytokinin-treated wild type was enlarged as in *pas* mutants, suggesting that the deregulated *KNOX* expression in *pas* mutants could be related to an altered cytokinin response. Several other characteristics of *pas* phenotype suggest a defect in cytokinin responses. Earlier work has shown that, associated with their cytokinin-enhanced cell proliferation, *pas* mutants have two-dimensional protein profiles reminiscent to cytokinin-treated wild-type (Faure et al., 1998). The three *pas* mutants also show significant delay of senescence (Y. Harrar, unpublished data). Finally, *pas2* seedlings exhibited ectopic shoot formation, which is a phenotype observed in cytokinin-overproducing plants or cytokinin-treated callus (Bellec et al., 2002).

Cytokinin primary response of the *pas* mutants was enhanced as illustrated by the expression pattern of two A-type *ARRs*. The response of the *pas* mutants to cytokinins may be caused by the increased size or number of meristems in the mutants because *ARR5* was mainly expressed in the meristem. However, this seems unlikely because differences in tissue expression have not been correlated with the level of cytokinin response. Although *ARR5* is mainly expressed in the meristems and the vascular tissue, the RNA levels after cytokinin induction are higher in leaves and stems than in buds and young flowers (Brandstatter and Kieber, 1998). Furthermore, *ARR6* that is expressed in most tissues shows the strongest induction in leaves and not in buds (D'Agostino et al., 2000). Moreover, a higher number of responsive cells, as in *pas* meristems of the *pas* mutants, would lead to increased expression levels but with kinetics similar to that of the wild type. On the contrary, *pas* mutants show not only an increased amplitude of ARR gene expression, but also a delay in returning to basal levels. The modification of the amplitude of *ARR5* and *ARR6* expression but not the timing of the maximum cytokinin response suggests that the *pas* response to cytokinins could not only be explained by an higher number of responsive cells. The transient cytokinin induction of A-type ARRs is explained by the existence of a negative feedback regulatory loop in which the A-type *ARR* genes repress their own expression (Hwang and Sheen, 2001). The enhanced cytokinin response in *pas* mutants was also associated with a prolonged *ARR* expression. Such an expression pattern could not simply be the consequence of an increased amplitude of *ARRs* expression because *pas3* showed wild-type levels of *ARR5* induction, but its expression was nonetheless maintained

after a 1-h induction. The proposed model for the maintenance of A-type *ARR* expression after cytokinin induction in the *pas* mutants would be that *PAS* genes are required for the A-type *ARR* negative regulatory feedback loop.

As in A-type *ARRs*, *CYCD3* is also inducible by cytokinins and thus provides a valuable marker for cytokinin involvement in cell cycle regulation (Soni et al., 1995; Fuerst et al., 1996). Surprisingly, its expression was not enhanced in the *pas* or *tsd* mutants (Frank et al., 2002). The absence of *CYCD3* overexpression in the six known classes of mutants with tumorous development suggests that in contrast to mammals in which *CYCD* altered expression is very often associated with cancer (Prober and Edgar, 2001), plant *CYCD3* deregulation is not the main cause of cytokinin-driven tumor development.

Cell dedifferentiation and proliferation is usually caused by a balanced ratio of cytokinins and auxin. Callus-like development of *pas* seedlings in the presence of cytokinins was not found to be caused by a parallel increased of auxin sensitivity as judged by the phenotypic analysis of auxin-treated seedlings (Faure et al., 1998). Typical auxin responses such as secondary root formation in light-grown seedlings or hypocotyl peeling in dark-grown seedlings can be observed in *pas* mutants when exposed to auxin (Faure et al., 1998; C. Bellini, unpublished data). Auxin treatment could not compensate for *pas* apical phenotypes nor could it induce callus-like development in seedlings. The reduced induction of the early auxin-induced genes *IAA4* and *IAA1* suggests that the *pas* mutants have a reduced primary response to auxin. As several *IAA* genes have been involved as negative regulators of auxin response, a decreased induction of *IAA* genes in *pas* mutants could also be interpreted as an adaptive response to an enhanced cytokinin response by increasing auxin sensitivity (Tiwari et al., 2001; Park et al., 2002). The opposite case was illustrated for *axr3* mutant, which showed an increased auxin sensitivity and where an exogenous supply of cytokinins was able to complement many aspects of the mutant phenotype, demonstrating that increased cytokinin levels can compensate, to a certain degree, an increase in auxin sensitivity (Leyser et al., 1996).

Altogether, *PAS* genes appear as negative regulators of cell proliferation by repressing cell division or by inducing cell differentiation. The negative regulation of *KNAT* expression by *PAS* genes is probably involved in maintaining cells in a differentiated state, avoiding uncontrolled cell proliferation and tumor development. Competency for cell division is dependent on the cell response to cytokinins and auxin. *PAS* genes controlled the amplitude of cytokinin and auxin responses and thus represent new regulators linking hormone response to the control of cell division and differentiation. The elucidation of the biochemical function of PAS proteins and their corresponding protein networks should shed light on the mechanisms of hormonal control of cell proliferation and differentiation.

MATERIALS AND METHODS

Plant Material and Hormone Treatment

The *pas* mutants were isolated from an ethyl methane sulfonate-mutagenized population of the Columbia ecotype (Col-0; Faure et al., 1998). Seeds were sterilized and grown in vitro as described previously (Santoni et al., 1994) in a controlled environment chamber (irradiance 200 μE m^{2-} s^{-1}, 16 h of light, 60% humidity, 20°C day temperature, and 15°C night temperature). Mutant lines expressing *GUS* were produced by crossing wild-type lines expressing the *GUS* marker with the mutant lines. Three different progeny lines were tested in *GUS* staining to avoid background effect on *GUS* expression. *pas2/pas2; stm/stm* double mutants were obtained by selfing of the progeny of the *pas2/pas2* × *stm dgh6/+* (Aida et al., 2002) cross and were genotyped with *stm* primers (GAGACAGCAATTGATAGGAACAAT/ATGGTG-GAGGAGATGTGATCC).

For RNA analysis, mutant and wild-type plants were grown in vitro for 12 d and were then transferred to liquid Arabidopsis medium. After 2 d in liquid culture to avoid stress effects, culture medium was supplemented with 10 μM BA or 20 μM IAA for treated plants and dimethyl sulfoxide or ethanol, respectively, for control plants. Plants were harvested and stored in liquid nitrogen after different times of hormone induction before RNA extraction.

For GUS analysis, plants were grown for 10 to 15 d with or without 0.1 μM BA. A short 2-h induction was performed in liquid medium for *ARR5::GUS*-containing plants.

RNA Methods

Total RNA was extracted from seedlings as described previously (Verwoerd et al., 1989). Approximately 20 μg of RNA was separated in a denaturing 1.5% (w/v) agarose-formaldehyde gel and then transferred to GeneScreen (NEN Life Science Products, Boston) nylon membranes. Northern hybridization was performed in church buffer at 65°C with probes obtained by PCR: *EF-1*α (CATTTGGCACCCTTCTTCAC, CCACGAGTCTGTTCTTGAGGC), or by enzymatic digestion of plasmids: *ARR5*, *ARR6*, *CYCD3*, *IAA1*, and *IAA4*. Blots were washed at 55°C in 2× SSC, 0.2% (w/v) tetra-sodium diphosphate, and 0.5% (w/v) SDS, and were exposed to an Imaging Plate (FUJI, Tokyo) or to film (Eastman-Kodak, Rochester, NY). Quantification was performed with a phosphorimager (BAS 1500; FUJI), which has a higher sensitivity and a broader linear range of measurement. As the quality of images was not as good as x-ray films, each blot was also exposed to film (Eastman-Kodak) and the corresponding image was used for the illustration. Hybridization signals from different samples in a blot were normalized with *EF-1* α*A4* (Liboz et al., 1989) or *eIF4A* (Metz et al., 1992). To compare samples from different blots, a wild-type Col-0 control sample was loaded in each blot. Northern experiments were performed at least twice with independent plant samples.

RT-PCR Methods

RTs were performed from total mRNA or poly(A) mRNA after DNase treatment, using superscript II enzyme (Qiagen, Valencia, CA) according to standard protocol. Poly(A) mRNA was obtained from total mRNA using the oligotex mRNA Mini kit (Qiagen). RTs were tested and normalized with *EF-1*α*A4* primers. Each PCR was done on the same quantity of cDNA according to *EF-1* α*A4* amplification product intensity. For each gene tested by RT-PCR, *EF-1* α*A4* amplification was done from the same mix as an internal control. PCRs were performed using *STM* (CTTATGTCAATTGTCAGAAGG, ATGGTGGAGGAGATGTGATCC), *KNAT1* (TTCTTCTCTTCCATGTCACTTC, CTGTTGTCATGCTGGTATTCTT), *KNAT2* (CTTTTGTGTTTCTTCATATTCT, CGATTTTGGATTTGATGACACT), and *KNAT6* (GATAAGTCGGTTCTGATGATG, TATCTTATCTCCTTCAGTAGGGT) primers. PCR programs were chosen for each gene to recover PCR products during the exponential phase. RT-PCRs were done several times on two or three independent experiments.

Real-time PCR was done using *STM* (AGAGAATAGGCAGGAGCACAA, TGATGGTCCGATGTGTCCTATG), *EF-1αA4* (CGAAGGGTGGTGAAAGCAAGA, CTGGAGGTTTTGAGGCTGGTAT) primers. The PCR efficiency calculated for each couple of primers was similar, allowing us to express *STM* mRNA quantity as a percentage of *EF-1αA4* mRNA quantity.

GUS Staining

Histochemical analysis of the GUS reporter enzyme was performed as described previously (Molier et al., 1995). Sample tissues were fixed in 80% (w/v) ice-cold acetone for 10 min, washed three times with water, and placed under vacuum to increase the penetration of reaction buffer into the tissue. Samples were incubated at 37°C for 1 to 16 h in reaction buffer. Staining time was 1, 16, 16, 3, 1, and 16 h for *CDKA::GUS*, *CYCB1::GUS*, *CYCB1::db::GUS KNAT2::GUS*, *ARR5::GUS*, and *CYCD3::GUS* transgenic lines, respectively. Plant samples were destained in 70% (w/v) ethanol before observation. GUS fluorometric assay was performed as described by Elmayan et al. (1996) on 9 μg of total protein.

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