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The C Terminus of the Immunophilin PASTICCINO1 Is Required for Plant Development and for Interaction with a NAC-like Transcription Factor^{*[5]}

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PASTICCINO1 (PAS1) is a high molecular weight FK506-binding protein (FKBP) involved in the control of cell proliferation and differentiation during plant development. Mutations in the C-terminal region of *PAS1* result in severe developmental defects. We show here that the C-terminal domain of PAS1 controls the subcellular distribution of this protein. We also demonstrated *in vitro* and *in vivo*, by Forster resonance energy transfer, that this C-terminal region is required for interaction with FAN (FKBP-associated NAC), a new member of the plant-specific family of NAC transcription factors. PAS1 and FAN are translocated into the nucleus upon auxin treatment in plant seedlings. The nuclear translocation of PAS1 is dependent on the presence of the C terminus of the protein. Finally, we showed that FAN is involved in PAS1-regulated processes because FAN overproduction partly complemented the *pas1* phenotype. We suggest that PAS1 regulates the function of this NAC-like transcription factor by controlling its targeting to the nucleus upon plant cell division.

Immunophilins are a large family of proteins with peptidyl-prolyl *cis-trans*-isomerase (PPIase)⁵ activity (1) that bind to immunosuppressive drugs, such as FK506, rapamycin, and

cyclosporin A, mediating their effects. Immunophilins include cyclophilins and FK506-binding proteins (FKBPs). FKBPs, like other immunophilins, are found in all organisms and have many cellular activities (2–4). For example, the mammalian FKBP12, the smallest protein known to display PPIase activity and drug binding, mediates immunosuppression and modulates signal transduction pathways by interacting with ryanodine receptors, the inositol 1,4,5-triphosphate receptor, transforming growth factor receptor type I, and epidermal growth factor receptor (5–8). FKBP12 inactivation leads to severe developmental alterations and pronounced cardiac defects associated with changes in calcium flux regulation (8). Cells from FKBP12 knock-out mice display high levels of proliferation because of hyperactivity of the transforming growth factor receptor (9). The large mammalian FKBPs, FKBP52 and FKBP51, are associated with HSP90 in the native steroid receptor complex and modulate the activity of this complex (10, 11). The interaction between FKBP52 and dynein has been shown to facilitate import of the steroid receptor complex into the nucleus (10). Large FKBPs have also been found in complexes with transcription factors such as interferon-regulating factor 4 (IRF-4) (12) and Ying Yang 1 (YY1) (13). Very few functional studies are available concerning the cellular role of FKBPs. FKBP12 inactivation was investigated only in mammals (8, 9), and the function of FKBPs in yeast remains unclear, because a strain in which all the immunophilins were inactivated remained viable (14).

The first comprehensive analysis of all the immunophilins in a multicellular eukaryote was recently performed in the model plant *Arabidopsis thaliana* (15, 16). This plant was found to contain a family of 52 immunophilins, differing in length, domain organization, and predicted subcellular distribution. Characterization of mutants in high molecular weight immunophilins of *A. thaliana* showed that these molecules play an important role in plant development. Mutations in the FKBP *TWISTED DWARF* (*TWD*) gene lead to defects in cell elongation and disorientation of the growth of all plant organs (17). *TWD* is present in the plasma membrane and was found to be associated with HSP90 and the multidrug resistant-like ABC transporter AtPGP1 (17, 18). Studies of loss-of-function mutations in the gene encoding the cyclophilin CYP40 have shown that this molecule is required for the juvenile vegetative phase

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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⁵ The abbreviations used are: PPIase, peptidylprolyl *cis-trans*-isomerase; FKBP, FK506-binding protein; FRET, fluorescence resonance energy transfer; FAN, FKBP-associated NAC; TPR, tetratricopeptide repeat; GFP, green fluorescent protein; MBP, maltose-binding protein; RT, reverse transcription; NAM, no apical meristem; IRF4, interferon-regulating factor 4; GR, glucocorticoid receptor; mRFP, monomeric red fluorescent protein; RTS, rapid translation system.

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of shoot development (19). Finally, the large FKBP PASTIC-CINO1 (PAS1) has also been shown to be essential for plant development (20). *PAS1* is required for correct cell division and cell differentiation, as mutations in the gene encoding this FKBP (also known as AtFKBP70) caused ectopic cell division, leading to tumor growth in the presence of the plant hormone cytokinins (21). *PAS1* mutant plants grown in the light have short, thick hypocotyls with misshaped cotyledons that never expand (21). Mutant meristems are often disorganized but continue to generate leaves, although these leaves are abnormal and fused. Abnormal cell division and differentiation are associated with changes in responses to the plant hormones cytokinins and auxin (22). *PAS1* is mainly expressed in zones with high levels of mitotic activity, such as the shoot and root apical meristems (20). PAS1 has three FKBP12-like domains at the N terminus of the protein, three tetratricopeptide repeat (TPR) units, and a C-terminal domain. The TPR motif, a degenerate 34-amino acid repeat, is known to be involved in protein-protein interactions in many processes, including cell cycle regulation, RNA synthesis, protein transport, Ser-Thr dephosphorylation, and heat shock response (23). The C-terminal domain of PAS1, like that of its mammalian homolog, binds calmodulin *in vitro* in a calcium-dependent manner and may be considered to be a calmodulin-binding domain (24).

We carried out a functional analysis of PAS1 protein, evaluating the role of one of its domains. We demonstrated that the C-terminal region controlled the subcellular distribution of the protein and interactions with other proteins. This domain was able to recruit a member of the NAC family of transcription factors and to target this transcription factor to the nucleus. Our data provide new insight into the biochemical role of high molecular weight immunophilins.

EXPERIMENTAL PROCEDURES

GFP/DsRED/mRFP Fusion Proteins—The full-length (1901 bp) and C-terminally deleted (1555 bp) PAS1 cDNAs were inserted between a 2× 35S promoter and a poly(A) 35S terminator in pOL GFPS65C (25). The resulting 35S promoter-PAS1::GFP-35S terminator cassettes were introduced into the binary vector pCAMBIA 1300 to obtain the PAS1::GFP and PAS^{ΔC}::GFP constructs. Strep::PAS1 and Strep::PAS1^{ΔC} protein fusions were produced from the pIVEX4 vector (Roche Applied Science). The full-length FAN cDNA was amplified by RT-PCR and inserted into the GATEWAYTM-modified vector HispKM596 (Invitrogen) to produce the fusion protein of FAN with the maltose-binding protein (MBP::FAN). FAN cDNA was inserted behind the 35S promoter into the pGWB2 vector from Tsuyoshi Nakagawa (Research Institute of Molecular Genetics, Shimane University, Matsue, Japan). FAN cDNA was also cloned into pGDR to produce the fusion protein DsRED2:FAN (26). A DsRED-based vector was required for the acceptor bleaching FRET experiments because DsRED, unlike mRFP, has an excitation spectrum compatible with the emission spectrum of GFP and is sensitive to bleaching. The Gateway vectors pH7WGR2 and pH7RWG2 were used to generate mRFP:FAN fusion proteins (27).

BY2 Cell Culture, Synchronization, and Transformation—Suspension-cultured tobacco (*Nicotiana tabacum* L., cv. BY2) cells (28) were grown in modified MS basal medium (29) in the dark at 24 °C, on a rotary shaker (130 rpm). Cells were subcultured by transfer to fresh medium once every 7 days (1:30 dilution).

For transformation, 3 ml of BY2 cell suspension was incubated with 2 ml of MS medium containing 50 μl of a stationary phase culture of *Agrobacterium tumefaciens* strain C58C1, in the dark, for 36 h, without shaking. BY2 cells were thoroughly washed and cultured for 2 days in the dark (24 °C, 130 rpm). Cells were then incubated for 2 days with the antibiotic for selection (20 μg/ml hygromycin B, Duchefa). We obtained stable, independent transformants by plating cells at low density on agar-MS medium containing 200 mg/ml cefotaxime and the antibiotic for selection (20 or 40 μg/ml hygromycin) and incubating them in the dark (at 24 °C) for 4–6 weeks.

In Situ Hybridization—The DNA template for the antisense RNA probe was produced by PCR with the following oligonucleotides: for PAS1 (5'-GGTGAGGGCTGGGAATCTCC-3' and 5'-TGTAATACGACTCACTATAGGGCCAAGCCAGTCCAGTCTCTTGG-3') and FAN (5'-GGTACAAGTCAGAA-GGCACC-3' and 5'-TGTAATACGACTCACTATAGGGCCAACATTGCTATCAGAGCCAC-3'). The reverse primer includes the synthetic minimal T7 promoter in a 5'-position. Digoxigenin-labeled RNA probes were synthesized by incubating T7 RNA polymerase and 1 μg of DNA template in a final volume of 20 μl for 40 min at 37 °C, according to the instructions supplied by the manufacturer of the enzyme. Siliques were fixed, mounted in Histo-resin, and sectioned as described previously (30). The sections were dewaxed and mounted on slides. They were treated for 10 min with 10 μg/ml proteinase K and then for 10 min with anhydrous acetic acid/triethanolamide. Finally, the sections were dehydrated. Sections were hybridized overnight at 45 °C in mRNA *in situ* hybridization solution (Dakocytomation, Dako). Slides were washed by incubation for 30 min at 45 °C in 0.1× SSC, 0.5% SDS and then for 60 min at 45 °C in 2× SSC, 50% formamide. They were then incubated with RNase for 30 min (10 mg/ml in NTE buffer) and washed by incubation for 60 min at 45 °C in 2× SSC, 50% formamide. Digoxigenin-labeled probes were detected with alkaline phosphatase-coupled antibodies, as described previously (30).

Protein-Protein Interactions—The fusion proteins Strep::PAS1 and Strep::PAS1^{ΔC} were produced with the rapid translation system, RTS 100 *Escherichia coli* kit, according to the manufacturer's instructions (Roche Applied Science). Proteins were labeled with [³⁵S]methionine (Amersham Biosciences) and analyzed with a BAS 1500 (Fuji).

For MBP-tag/amylose affinity purification, the amylose matrix (100 μl/reaction) was incubated for 45 min with W buffer (100 mM Tris, pH 8, 150 mM NaCl, 1 mM EDTA, and protease inhibitors) supplemented with 3% bovine serum albumin and 0.1% Triton. BL21(DE3)pLysE cells expressing the MBP:FAN construct were induced by incubation with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 2 h at 24 °C. Induced cells were centrifuged and resuspended in W buffer. The cells were incubated for 30 min on ice in the presence of 0.1 mg/ml lysozyme, frozen at -70 °C, and thawed in the presence

of 40 $\mu\text{g}/\text{ml}$ of DNase I for 10 min on ice. The resulting suspension was centrifuged ($14,000 \times g$, 30 min, 4°C). The supernatant was incubated with amylose. The matrix was then washed and incubated with an RTS 100 reaction mixture containing the Strep: PAS1 protein. Proteins were eluted with 10 mM maltose and analyzed by Western blotting with an anti-PAS1 antibody. The CTTVSTERKRRWSEK-amide peptide from the C-terminal part of PAS1 was synthesized and used to elicit an antiserum (anti-PAS1) in rabbit (Biogenes).

For streptavidin tag/Strep-Tactin (IBA) affinity purification, the matrix was prepared as described above. An RTS 100 reaction mixture containing Strep: PAS1 protein or Strep: PAS1^{ΔC} was incubated with the Strep-Tactin matrix, with washing and elution according to the manufacturer's instructions (IBA). Eluted proteins were analyzed by Western blotting with an anti-MBP antibody (kindly provided by Jean-Michel Betton, Institut Pasteur France).

For pulldown assays, 10-day-old PAS1:GFP seedlings (1 g) were ground in liquid nitrogen, and 1–4 mg/ml protein was extracted in extraction buffer (60 mM Tris, pH 6.8, 20% glycerol, 10 mM dithiothreitol, 0.1% Triton, and protease inhibitors). The crude extract was incubated with a MBP:FAN amylose matrix. The proteins were washed, eluted, and analyzed by Western blotting with an anti-GFP antibody (Roche Applied Science).

Plant Materials and Transformation—*A. tumefaciens* strain C58C1 was electroporated with the pCAMBIA PAS1::GFP, PAS1^{ΔC}:GFP and pGWB2 35S::FAN binary vectors and used to transform plants as described by Clough and Bent (31). Heterozygous *pas1-3* plants were transformed, and primary transformants were selected *in vitro* in the presence of 15 mg/liter hygromycin B (Duchefa) and 50 mg/liter kanamycin (Duchefa) for the pCAMBIA and pCWB2 vectors, respectively.

Nicotiana benthamiana plants were grown in the greenhouse under the following conditions: 13 h of light, diurnal temperature of 25°C , and nocturnal temperature of 17°C . These plants were used for all agroinfiltration experiments. Leaves were infiltrated (32) with an exponential phase culture of *A. tumefaciens* strain C58C1 resuspended at an A_{600} of 0.5 in 0.04 g/ml saccharose. For coinfiltration experiments, equal volumes of the two cultures were mixed before agroinfiltration. Observations were performed 48 h after transformation.

Confocal Microscopy and FRET Measurements—Confocal microscopy was carried out with an inverted Leica TCS-SP2-AOBS spectral confocal laser scanning microscope (Leica). Samples were excited with a 488 nm argon laser with an emission band of 500–530 nm for GFP detection and with the 594 nm HeNe laser with an emission band of 600–635 nm for DsRED/mRFP detection. Nuclei were stained with 4 μM SYTO16 (Molecular Probes) and visualized with an emission band of 500 to 330 nm after excitation at 488 nm in sequential mode.

For FRET measurements, Leica FRET acceptor photobleaching was used according to the manufacturer's instructions. DsRED was photobleached in a region 4–8 μm in diameter, by four scans with the 543 nm laser at 100%, with a small illumination beam to increase bleaching efficiency (Leica Beam Expander). GFP fluorescence intensity was quantified before (donor 1 (d1)) and after (donor 1 (d2)) acceptor photobleaching,

and FRET efficiency was automatically determined by Leica software, using the $(d2 - d1)/d2$ equation.

Quantitative RT-PCR—Total RNAs were extracted and treated with DNase according to the RNeasy plant kit (Qiagen, Hilden, Germany). Reverse transcriptions were performed from total DNase-treated RNA with Superscript II enzyme (Qiagen) according to standard protocols. PCRs were performed on Roche Applied Science LightCycler with primers for *Arabidopsis* FAN (CC TCG TCA TTA TCC ACC TGT, GGG GAG AAA CTA CTC GCT GA) and *EF1 α* as control (CCA AGG GTG AAA GCA AGA AGA, CTG GAG GTT TTG AGG CTG GTA T).

RESULTS

Mutations in the C-terminal Domain of PAS1 Lead to Severe Developmental Defects—The *pas1* mutants were originally isolated on the basis of their early developmental phenotype. Two alleles were analyzed in more detail, as both the mutations concerned the C-terminal domain of the protein. The *pas1-1* allele was obtained from the Versailles T-DNA collection and resulted from an insertion in the 19th exon of the gene (20) (Fig. 1A). This insertion led to the deletion of 127 amino acids, corresponding to the last 12 amino acids of the third TPR motif and the entire C-terminal region. A second allele with a mutation in the C-terminal part of *PAS1* gene, *pas1-3*, was identified from a population subjected to ethyl methanesulfonate mutagenesis. The *pas1-3* mutation involves the replacement of G³⁴¹⁷ (from the ATG) by A at the intron 18 donor splicing site (Fig. 1A). This mutation results in partial splicing of the 19th exon, generating a smaller transcript, a translational frameshift, and a premature stop codon (Fig. 1, B and C). The predicted sequence of the PAS1 protein in the *pas1-3* mutant displayed a deletion of 134 amino acids, corresponding to the last 19 amino acids of the third TPR motif and the entire C-terminal domain (Fig. 1C).

The two alleles had similar developmental phenotypes, with a small, thick hypocotyl, finger-shaped cotyledons and small fused leaves (Fig. 1D). Both mutants displayed ectopic cell proliferation in the presence of cytokinins (data not shown). The identification of two mutations with similar phenotypes and modified PAS1 protein C termini suggests that this domain plays an essential role in plant development.

The C-terminal Domain Is Required for PAS1 Exclusion from the Nucleus—We investigated the intracellular distribution of PAS1, using a PAS1:GFP fusion protein produced under control of the cauliflower mosaic virus 35S promoter. The PAS1:GFP fusion was functional, and it complemented the *pas1-3* mutant (Fig. 2A). As the C-terminal region of PAS1 was required for the function of the protein, we investigated whether this region played an important role in controlling the subcellular distribution of the protein. A fusion protein consisting of the PAS1 protein deleted of the last 113 C-terminal amino acids fused to GFP was generated (PAS1^{ΔC}:GFP). This truncated fusion protein was produced under control of the 35S promoter in *Arabidopsis*. This protein was correctly produced (as indicated by GFP fluorescence) but did not complement the *pas1-3* mutant, demonstrating the functional importance of this C-terminal domain.

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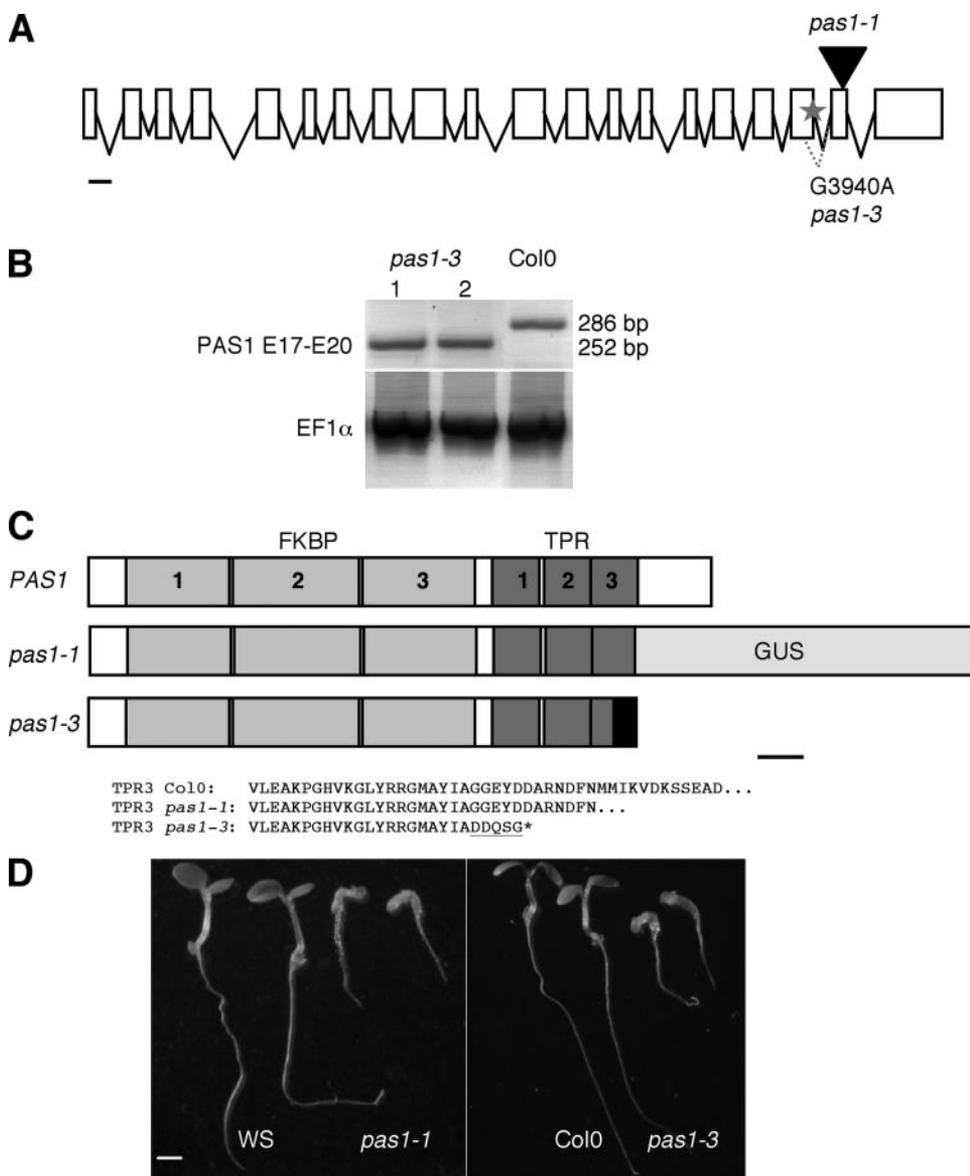


FIGURE 1. Mutations in the C-terminal domain of PAS1 alter seedling development. *A*, positions of *pas1-1* and *pas1-3* mutations in the *PAS1* gene. Exons are indicated as *open boxes*. The splicing defect induced by *pas1-3* is indicated with a *dashed line*, and the T-DNA insertion is shown as a *closed triangle*. The bar represents 100 bp. *B*, amplification by RT-PCR of exons 17–19 of the *PAS1* transcript from two independent RNA preparations (*lanes 1 and 2*) from the *pas1-3* mutant and the wild type (*lane Col0*). EF1 α amplification is shown as a control. *C*, the predicted proteins of the wild type, *pas1-1*, and *pas1-3* mutants are shown with the three FKBP domains (*light gray*), the three TPR domains (*dark gray*), and premature termination of the C-terminal domain (*black*). The bar represents 30 residues. The sequence details of the TPR 3 domain of the wild type (*Col0*), *pas1-1*, and *pas1-3* mutants is shown below. *D*, phenotype of 10-day-old seedlings of *pas1-1* (*left*) and *pas1-3* (*right*) mutants as compared with the wild type. The scale bar represent 1 mm.

The subcellular distribution of PAS1:GFP and PAS1^{ΔC}:GFP fusion proteins was analyzed in *Arabidopsis* seedlings root cells. The location of PAS1:GFP was mainly in the cytosol and excluded from the nucleus, whereas PAS1^{ΔC}:GFP was observed in both the compartments (Fig. 2*B*). The subcellular distribution of PAS1 was also confirmed in a different system, the tobacco BY2 cells. The PAS1:GFP fusion protein was also found in the cytosol of interphasic tobacco BY2 cells (Fig. 2*C, top*). However, during the exponential growth phase, during which cells were actively dividing, PAS1:GFP was occasionally detected in the nucleus (Fig. 2*C, middle, arrowheads*). Unlike PAS1:GFP, the PAS1^{ΔC}:GFP fusion protein was not excluded

from the nucleus and was present in the cytosol as well as the nucleus of BY2 cells, as in *Arabidopsis* plants (Fig. 2*C, bottom*). The C terminus of PAS1 therefore seems to be necessary for the nuclear exclusion of PAS1.

A Member of the NAC Transcription Factor Family Interacts with the C-terminal Domain of PAS1—We used a yeast two-hybrid approach to identify proteins interacting with PAS1. We used the full-length PAS1 protein as bait and screened 16 million clones from two *A. thaliana* cDNA libraries. We identified seven independent clones, one of which had a sequence similar to those of the NAC family of transcription factors and was subsequently named FAN (FKBP-associated NAC). We carried out *in vitro* pulldown assays to confirm the results obtained with the yeast two-hybrid system. We first assessed the ability of an MBP-FAN affinity matrix to capture the streptavidin-tagged PAS1 recombinant protein (Strep-PAS1). Strep-PAS1 was captured by MBP:FAN (Fig. 3*A, 1st lane*) but not by the amylose beads alone (*2nd lane*). This interaction was further confirmed in a pull-down assay with an MBP:FAN column and crude protein extract from BY2 cells expressing the 35S-PAS1:GFP construct. PAS1:GFP bound to the MBP:FAN column but not to the maltose matrix alone (Fig. 3*A, right*).

Yeast two-hybrid assays showed that the C terminus of PAS1 interacted most strongly with the FAN clone (data not shown). We therefore used *in vitro* assays to assess the involvement of the C-terminal domain of PAS1 in FAN binding. Strep-PAS1 and Strep-PAS1 deleted of the C-terminal domain (Strep-PAS1^{ΔC}) were produced *in vitro* (Fig. 3*B, left*), and the proteins were bound to Strep-Tactin beads. MBP:FAN was retained on the Strep-PAS1 column (Fig. 3*B, right, 2nd lane*) but was only weakly retained on Strep-PAS1^{ΔC} columns (Fig. 3*B, right, 3rd lane*) and Strep-Tactin beads alone (Fig. 3*B, 1st lane*). These data suggest that the C terminus of PAS1 is required for interaction with FAN.

NAC transcription factors were named after the no apical meristem (NAM) protein of petunia (33), and the *Arabidopsis* ATAF1 and *Cup Shape Cotyledons2* (*CUC2*) genes (34). They have a characteristic conserved N-terminal domain of about

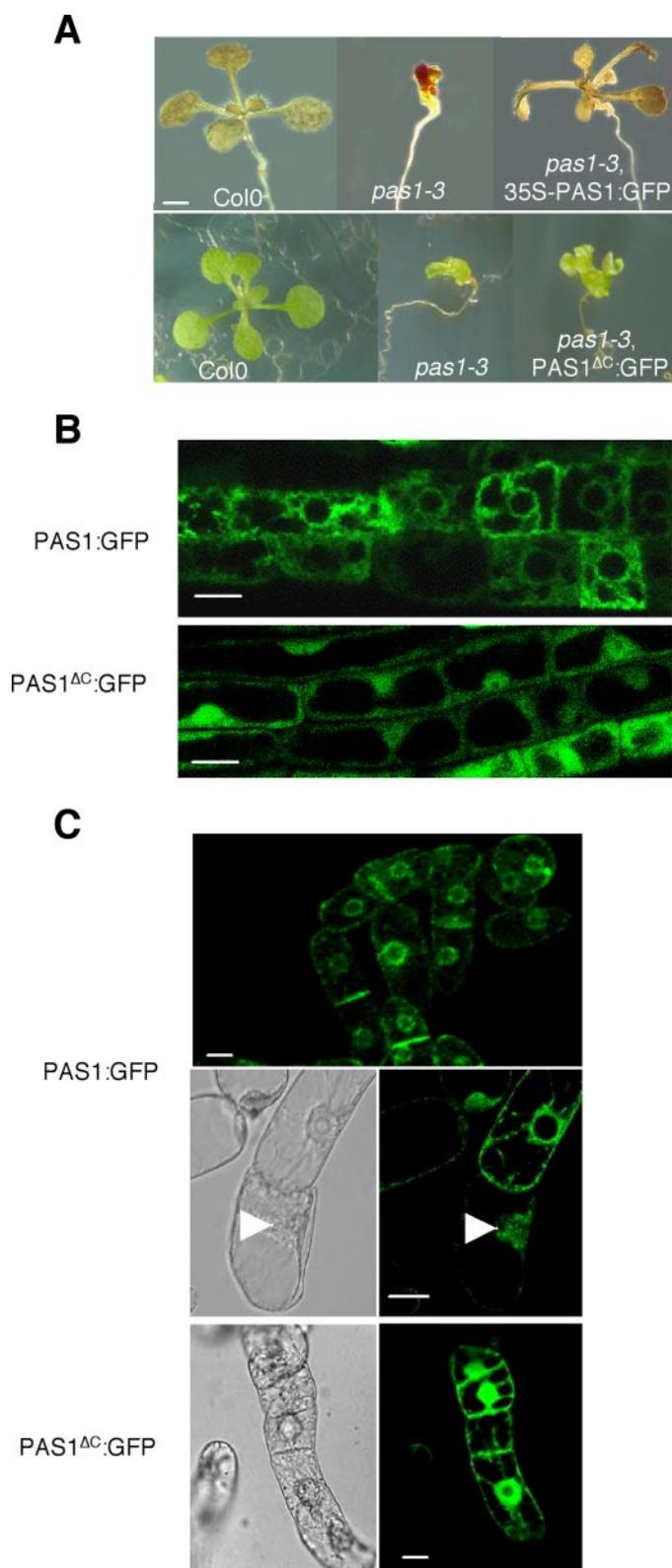


FIGURE 2. The C-terminal domain of PAS1 is required for nuclear exclusion. *A*, expression of the 35S-PAS1:GFP (top) construct but not of the 35S-PAS1 Δ C::GFP construct (bottom) complements the *pas1-3* mutant. The images on the left and in the middle correspond to the untransformed wild type and the *pas1-3* mutant. The bar represents 1 mm. *B*, subcellular distribution of PAS1:GFP and PAS1 Δ C::GFP fusion proteins in *Arabidopsis*. Fluorescence confocal micrograph of root tip cells from PAS1:GFP (top) and PAS1 Δ C::GFP (bottom) stable transgenic lines. *C*, subcellular distribution of PAS1:GFP and PAS1 Δ C::GFP fusion proteins in stable transgenic BY2 cell lines. The bars represent 20 μ m in *B* and *C*.

150 amino acids, defined as the NAC domain (35). The clone isolated by two-hybrid screening corresponded to residues 18–239 of the At5g22290 gene. The NAC domain of the FAN protein was 85, 49, 49, 48, 46, and 41% identical to the NAC domains of NAC2, AtNAM, CUC2, NAM, NAC1, and CUC1, respectively (Fig. 3C). Computer analyses of the expression profile of FAN revealed that this gene was expressed at low but constant levels during most stages of plant development, with the exception of the final stages of seed development where FAN was highly expressed. Interestingly, *pas1* mutants display major defects in embryo development (20, 21). We therefore hypothesized that the *PAS1* and *FAN* genes would be coexpressed in the developing embryo. *In situ* hybridization confirmed that both genes were indeed expressed in the same cells of mature embryos (Fig. 3D).

In Vivo Interaction between PAS1 and FAN Requires the C-terminal Domain—We then investigated whether FAN could interact with PAS1 *in vivo*. We first coexpressed constructs encoding PAS1:GFP and a DsRED:FAN fusion protein transiently in *N. benthamiana* leaves. As shown in *Arabidopsis* and BY2 cells, PAS1:GFP was localized in the cytoplasm of *N. benthamiana* epidermal cells (Fig. 3E, top). DsRED:FAN was also localized in the cytoplasm and colocalized with PAS1:GFP (Fig. 3E). Following the coexpression of PAS1 Δ C::GFP and DsRED::FAN, PAS1 Δ C::GFP was found in both the cytoplasm and nucleus, as expected, and DsRED:FAN was detected in the cytoplasm. In contrast to what was observed with the PAS1:GFP fusion, PAS1 Δ C::GFP and DsRED:FAN were seldom, if ever, colocalized.

We then used fluorescence resonance energy transfer (FRET) technology to demonstrate the direct interaction between PAS1 and FAN *in vivo*. The GFP-DsRED combination was shown previously to be a suitable donor-acceptor pair for FRET in living plant cells (36). We evaluated FRET efficiency by a method based on an increase in donor fluorescence after photochemical destruction of the acceptor in *N. benthamiana* leaf cells coexpressing the PAS1:GFP and DsRED:FAN constructs (Fig. 3E). We determined the fluorescence intensity of PAS1:GFP in a region 4–8 μ m in diameter localized in the cytoplasm (Fig. 3F, inset, green circle). DsRED:FAN was then photobleached to achieve at least 40–60% acceptor bleaching, which is sufficient for FRET measurement and protection of the GFP signal against quenching. We then measured the fluorescence intensity of PAS1:GFP before and after photobleaching (Fig. 3F, inset). In more than 30 independent photobleaching experiments, a significant increase in the fluorescence of PAS1:GFP, represented by FRET efficiency, of up to 22% was observed (Fig. 3F). This increase in GFP fluorescence after photobleaching was not seen in cells coexpressing the PAS1 Δ C::GFP and DsRED:FAN constructs. Mean FRET efficiency for the PAS1:GFP/DsRED:FAN constructs was 7.7 versus 1.4% for the PAS1 Δ C::GFP and DsRED:FAN constructs. Colocalization and FRET analysis demonstrated that PAS1 interacted *in vivo* with FAN and that its C terminus was required for this interaction.

In Dividing Cells, PAS1 and FAN Are Colocalized in the Nucleus—Some FKBP in animals have been shown to shuttle between different subcellular compartments. The regulatory

Function of the C Terminus of the Immunophilin PAS1

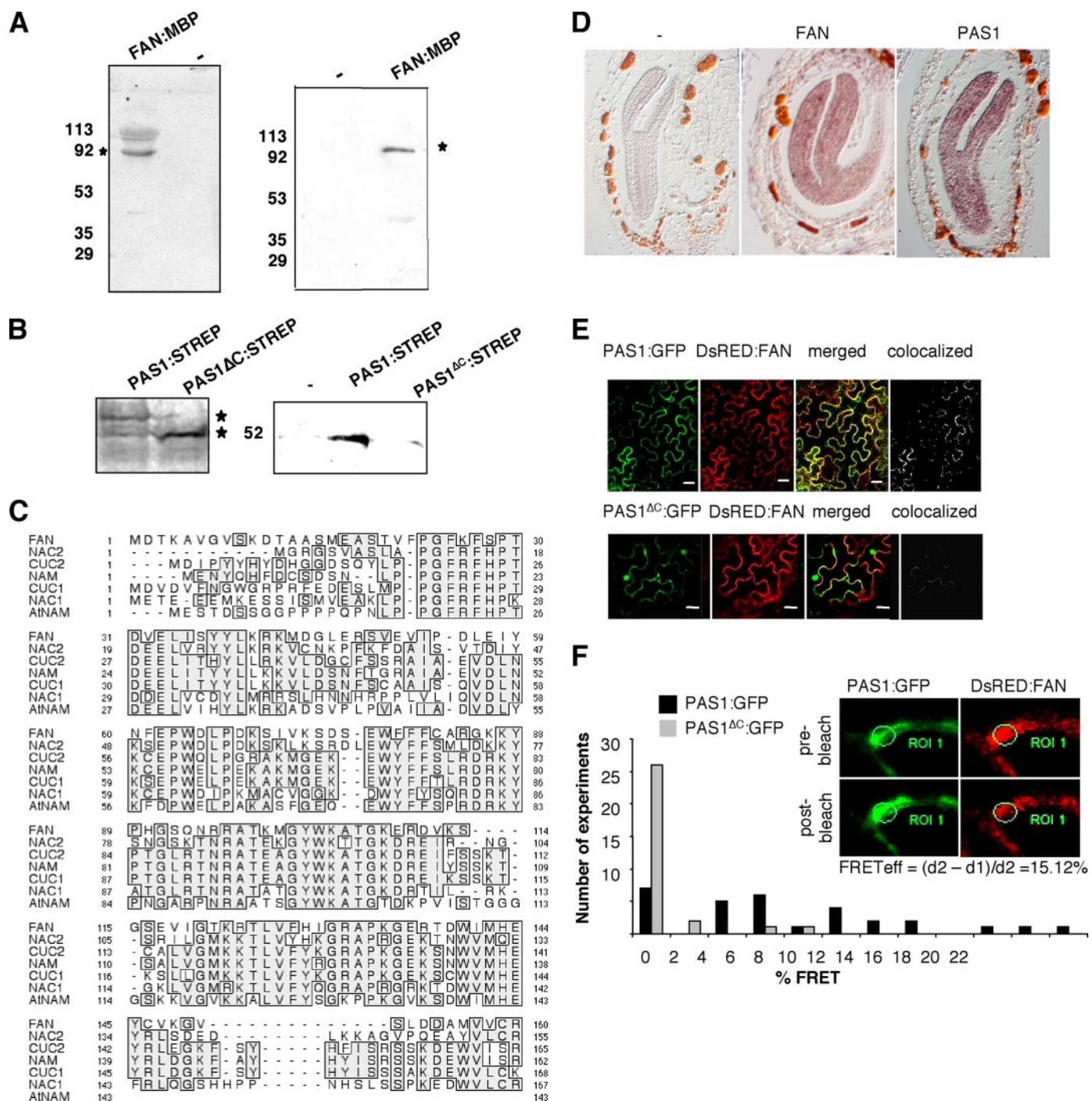


FIGURE 3. PAS1 interacts *in vitro* and *in vivo* with a member of the NAC family of transcription factors. *A*, PAS1 interacts with FAN *in vitro*. The RTS Strep-PAS1 protein was retained by the recombinant MBP:FAN protein fixed on amylose beads (MBP:FAN) and not by the beads alone (–) (top left). Western blotting was carried out with an anti-PAS1 antibody, as described under “Experimental Procedures.” The PAS1:GFP fusion protein from 35S-PAS1:GFP *Arabidopsis* plants bound to the MBP-FAN column and not to the matrix alone (–) (top right). The PAS1:GFP fusion protein was detected by immunoblotting with anti-GFP antibodies. *B*, *in vitro* interaction between FAN and PAS1 requires the C-terminal domain of PAS1. [³⁵S]Methionine-labeled Strep: PAS1 and Strep: PAS1^{ΔC} proteins were produced with the RTS kit and analyzed with a PhosphorImager (left panel). The recombinant MBP:FAN protein was retained by the full-length Strep: PAS1 but not by Strep: PAS1^{ΔC} or the beads alone (right panel). The MBP:FAN fusion protein was detected by immunoblotting with anti-MBP antibodies. *C*, alignment by ClustalW of the NAC domains of FAN (At5g22290) with those of NAC2 (At5g04410), AtNAM (At1g52880), CUC2 (At5g53950), NAC1 (At1g56010), CUC1 (At3g15170), and NAM (CAA63102). *D*, PAS1 and FAN are present in mature embryos. *In situ* hybridization was performed in the absence of or with a FAN or PAS1 probe. *E*, PAS1:GFP colocalized with DsRED:FAN in *N. benthamiana* epidermal cells whereas PAS1^{ΔC}:GFP did not (top). Fluorescence confocal micrographs showing the cytoplasmic location of PAS1:GFP and DsRED:FAN. The colocalization of GFP and DsRED signals is shown in yellow on the merged images and was confirmed by a two-dimensional cytofluorogram, as shown by the white dots representing pixels with equal value in the two detection channels (green and red) (far right images). The scale bar represents 20 μm. *F*, *in vivo* protein-protein interaction was monitored by acceptor bleaching FRET (inset). Fluorescence confocal micrographs of PAS1:GFP (left) and DsRED:FAN (right) were taken before (top) and after (bottom) photobleaching of the acceptor (DsRED) in the 4–8-μm diameter region of interest (ROI; shown as a green circle). Regions of interest were chosen in cytoplasmic region with stable fluorescent signals. The fluorescence intensity of PAS1:GFP was measured in the bleached region of interest before (donor 1 (d1)) and after (donor 2 (d2)) photobleaching. FRET efficiency was estimated with the ratio (d2 – d1)/d2. The histogram shows the distribution of FRET efficiency in 30 independent cells from several independent cotransformations with either the PAS1:GFP/DsRED:FAN or PAS1^{ΔC}:GFP/DsRED:FAN constructs (bottom).

role of PAS1 may involve the nuclear targeting of transcription factors such as FAN. Indeed, the C-terminal domain controls the subcellular distribution of PAS1 and is involved in its interaction with FAN. FAN, like PAS1, is excluded from the nucleus in differentiated cells. Finally, mutations in the C-terminal domain were associated with cell proliferation and cell de-differentiation during early plant development. It therefore appears likely that the subcellular distributions of FAN and PAS1 are modified following specific physiological changes, such as cell de-differentiation and division.

Stable *Arabidopsis* transgenic lines expressing mRFP fusion protein-encoding constructs were produced to allow the colocalization of PAS1 or FAN with the DNA vital green stain SYTO16. Analysis of transgenic *Arabidopsis* plants expressing mRFP: PAS1 or mRFP: FAN constructs showed that these two fusion proteins did not seem to colocalize with nuclear DNA (Fig. 4, A and B, top) with a distribution very similar to that observed for GFP fusion proteins (Fig. 3E). The subcellular distribution of the fusion proteins was further analyzed by quantifying RFP and SYTO16 fluorescence across the cell (Fig. 4C). Although in *N. benthamiana* epidermal cells both proteins seemed to be excluded from the nucleus, in transgenic *Arabidopsis* root cells, some RFP fluorescence, mainly for mRFP: FAN, could still be detected in the nucleus (Fig. 4, B and C). We then applied exogenous auxin to the seedlings to promote cell de-differentiation and division. The treatment of *Arabidopsis* seedlings with 2 μ M 1-naphthaleneacetic acid for 48 h led to auxin accumulation in the root tip (supplemental Fig. 1A) and to ectopic cell division along the root axis (supplemental Fig. 1B). Auxin treatment led to an increase in mRFP: PAS1 and mRFP: FAN accumulation in the nucleus, as illustrated by the colocalization of the RFP signal with the DNA vital dye SYTO16 (Fig. 4, A and B, bottom panels). The accumulation of fluorescence in the nucleus was confirmed by quantification of RFP and SYTO16 fluorescence across the cell (Fig. 4D). The nuclear accumulation of these two fusion proteins was observed in cells located above the primary root tip. Thus, PAS1 and FAN are able to accumulate into the nucleus in physiological conditions associated with cell division and cell de-differentiation.

FAN Overproduction Partially Complements the *pas1* Phenotype—The major defects in embryonic and vegetative development caused by deletion of the C terminus of PAS1 may result from defective interaction between PAS1 and FAN. We investigated whether FAN expression was functionally related to *pas1* phenotype, by overexpressing FAN in the wild type and *pas1-3* genetic backgrounds. Wild type T2 transformants showed no obvious developmental phenotype (data not shown). Kanamycin-resistant mutants were selected from T2 families of heterozygous *pas1* mutants transformed with the 35S-FAN construct. Among the 13 independent transgenic lines, 3 lines segregating *pas1-3* mutations were analyzed further. First, FAN mRNA levels was quantified in the transgenic *pas1-3* mutants by quantitative RT-PCR (Fig. 5B). FAN expression was increased 3- and 8-fold compared with *pas1-3* mutant in the 2 lines A15 and A20. Transgenics mutants from A15 and A20 lines developed almost normal leaves, very different from

those of the untransformed *pas1-3* seedlings (Fig. 5B). Conversely, the mutants from A18 line did not show clear overexpression of FAN and as expected showed a much weaker restoration of leaf development. Although leaf development of *pas1-3* mutants seems to be correlated with the expression level of FAN, lateral root development absent in *pas1-3* mutants was not restored by FAN overexpression (data not shown). The partial compensation of the *Pas1* post-embryonic phenotype resulting from FAN overexpression suggests that FAN is probably involved in some PAS1-regulated processes.

DISCUSSION

The first evidence for a functional relationship between PAS1 and cell division was provided by the observation that mutations in the C-terminal domain lead to strong developmental phenotypes primarily involving uncoordinated and ectopic cell division (20–22). The *pas1-1* and *pas1-3* alleles encode predicted proteins with a modified C terminus. We were unable to assess the stability of the mutant proteins directly because the available antibodies against PAS1 were directed against a peptide absent from both alleles. However, we have two pieces of evidence supporting the fact that the deletion of the C-terminal domain is directly involved in the phenotype and does not simply lead to an unstable protein. First, the *pas1-1* mutation created an in-frame fusion of the PAS1 N terminus with GUS protein. Detection of the hybrid PAS1:GUS transcript and GUS activity demonstrated the production of a stable truncated PAS1 protein (20). Second, the PAS1 protein deleted of its C-terminal domain, expressed as a PAS1^{ΔC}:GFP fusion protein, was stable, as it could be monitored directly by GFP fluorescence. These reconstruction experiments comparing the complementation effects of PAS1:GFP and PAS1^{ΔC}:GFP demonstrated that the C-terminal domain of PAS1 was required for PAS1 function. C-terminal deletion also prevented the nuclear exclusion of PAS1, as it led to detection of the protein in the nucleus in *Arabidopsis* BY2 cells and *N. benthamiana* epidermal cells. The subcellular distribution of PAS1 is probably important for the function of this protein as a nuclear location seems to be associated with cell division. In *Arabidopsis* roots, PAS1 is mainly cytosolic but accumulates in the nucleus if cells are treated with auxin, which promotes cell proliferation in the root. In tobacco, PAS1 could not be detected in the nuclei of highly differentiated and non-dividing epidermal cells, but this protein was detected in the nuclei of actively dividing BY2 cells. The presence of PAS1^{ΔC}::GFP in the nucleus was not simply caused by the smaller size of the fusion protein (85.3 kDa rather than 98.4 kDa for PAS1:GFP), because DsRED:FAN, which is even smaller (62.4 kDa) was also excluded from the nucleus in the three different expression systems tested. The accumulation of PAS1:GFP in the nucleus in some cells also suggests that an active regulatory mechanism is involved in controlling subcellular distribution.

The C-terminal domain was also found to be involved in protein-protein interaction. It allows calmodulin binding *in vitro* (24), and we show here that it is also involved in interactions with a

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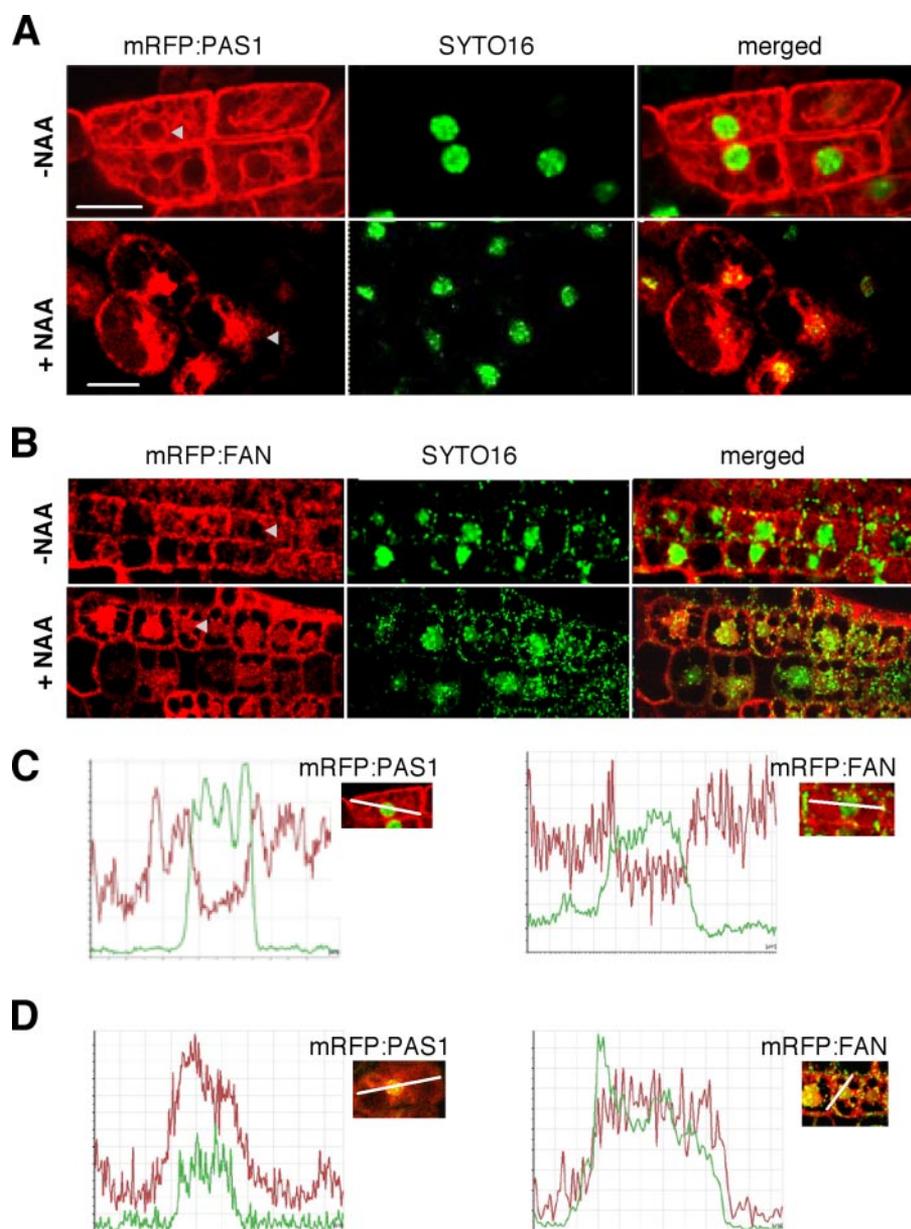


FIGURE 4. Regulation of the subcellular distributions of PAS1 and FAN. *A*, fluorescence confocal micrographs of transgenic mRFP: PAS1 *Arabidopsis* root with (bottom) and without (top) auxin treatment. *B*, fluorescence confocal micrographs of transgenic mRFP: FAN *Arabidopsis* root with (bottom) and without (top) auxin treatment. Nuclear DNA was stained with the vital dye SYTO16. *C* and *D*, quantification of mRFP (red) and SYTO16 (green) fluorescence across a cell (white line, right inset) indicated by an arrowhead in *A* and *B*. The fluorescence of mRFP: PAS1 (left) and mRFP: FAN (right) was measured from control (*C*) and auxin-treated plant (*D*). For all the panels, the scale bar is 20 μm .

protein resembling the plant-specific transcription factors of the NAC family. Interactions between PAS1 and FAN were detected *in vitro* and by pull-down assays. FAN like PAS1 was present in the cytosol in differentiated cells and in the nucleus in dividing cells. *In vivo* confirmation of protein interaction was obtained by FRET experiments. These experiments also confirmed that the GFP/ DsRED fusion protein was a potentially efficient tool for investigating protein-protein interaction.

Differential intracellular distributions associated with specific protein interactions have been described for other immunophilins. The best described example is FKBP52, which, like CYP40, is part of the steroid receptor complex in the

cytosol. FKBP52 and PAS1 have similar protein structures, including three FKBP domains, three TPR motifs, a calmodulin-binding domain, and a nuclear localization signal (4, 20). FKBP52 interacts with HSP90 in the glucocorticoid receptor (GR) complex via its TPR motif and with dynein via its PPIase domain (37). The PPIase domain of FKBP52 also inhibits the dexamethasone-dependent translocation of GFP-GR from the cytoplasm to the nucleus (38). Immunofluorescence and fractionation experiments showed hormone-induced translocation of the hormone-generated GR-HSP90/FKBP52-

dynein complex from the cytoplasm to the nucleus, a step preceding dissociation of the complex within the nucleus and the conversion of GR to the DNA-binding form (38). Interestingly, the binding of high molecular weight immunophilins to hsp90 via TPR domains and to dynein via PPIase domains was demonstrated with the wheat FKBP52 (39, 40). Moreover the 77-kDa wheat FKBP was found to accumulate specifically into the nucleus upon heat shock (41). Even if PAS1 and FKBP51/52 have similar protein structures, the nuclear targeting activity involved different sub-domains. Although the FKBP52/51 N-terminal PPIase domains are mainly involved in nuclear targeting of proteins, PAS1 seems to use its C-terminal domain. The PAS1 C-terminal domain is unusually long compared with the structurally similar high molecular weight FKBP52, ROF1, ROF2, and TWD. The C-terminal domains were either predicted, for ROF1 and ROF2, or demonstrated, for TWD and PAS1, to bind calmodulin (24, 42). TWD interacts with P-glycoprotein ABC transporter AtPGP1 and AtPGP19 via its PPIase domain and with the multidrug resistance associated ABC transporters AtMRP1 and AtMRP2 via its TPR domain (42). Interestingly, the TPR domain of PAS1 but not of ROF1 interacts also with AtMRP1 (42).

Several transcription factors have been identified as targets of mammalian FKBP52. Ying Yang1 (YY1) is a transcriptional activator or repressor involved in the regulation of gene activity in many embryonic, differentiating, and nondividing cell types. YY1 forms a complex with a nuclear FKBP, FKBP25, and with the histone deacetylases HDAC1 and

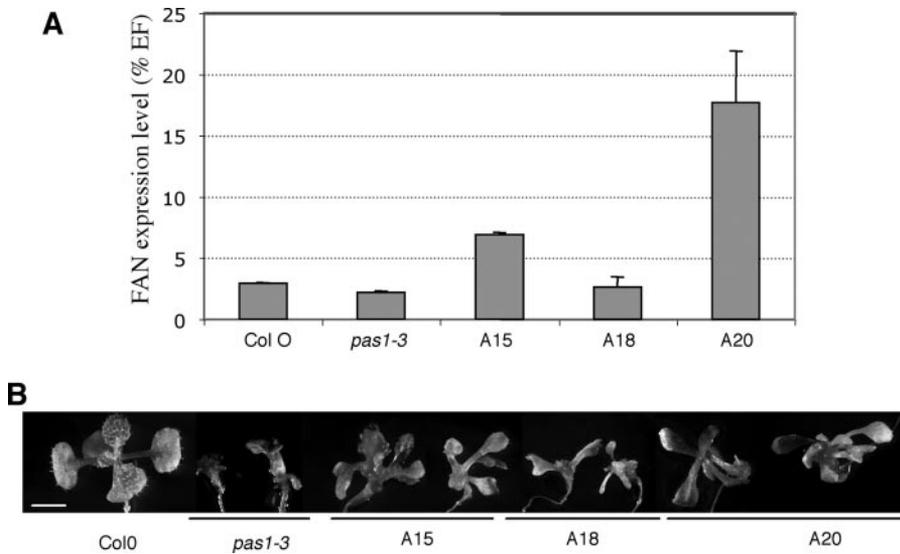


FIGURE 5. **Partial complementation of the *pas1* phenotype by the overexpression of FAN.** *A*, quantitative analysis by RT-PCR of FAN expression level in wild type (*Col0*), *pas1-3* mutant, and three independent transgenic 35S-FAN, *pas1-3* mutant seedlings (*A15*, *A18*, and *A20*). FAN expression was normalized against EF1 α expression. *B*, phenotype of *pas1-3* transgenic seedlings expressing a 35S-FAN construct described in *A* (right), compared with the mutant *pas1-3* (middle) and the wild type (left). The bar represents 1 mm.

HDAC2 to form a histone deacetylase-binding transcriptional complex regulating YY1 DNA binding activity (13). IRF4 is a transcription factor involved in B-cell proliferation and differentiation. This factor is deregulated in multiple myeloma. IRF4 is associated with FKBP52, and its DNA binding and transcriptional activities are negatively regulated by PPIase activity (12). In all cases, interactions between FKBP52 and transcription factors occur solely in the context of the regulation of cell division and differentiation. FKBP52 has been shown to act as negative regulators of cell proliferation in FKBP12-deficient and tumor-bearing mice (9, 43). Conversely, FKBP52 has been identified as a direct target of MYC, a transcription factor inducing normal and neoplastic cell proliferation (44). A direct link between the role of FKBP52 in cell proliferation and the regulation of protein distribution was revealed by the demonstration that FKBP38 inhibits apoptosis by targeting and anchoring Bcl-2 and Bcl-x(L) to mitochondria (45).

Similarly, PAS1 controls the targeting of a potential transcription factor involved in cell proliferation. FAN overexpression partially compensates for the defects in leaf development caused by uncoordinated and ectopic cell division in the apical meristem and leaf primordia (21, 22). Several members of the NAC family are involved in regulating cell proliferation in the meristem. One of the first members of this family to be identified, the product of the petunia no apical meristem (*NAM*) gene, positively regulates floral organ development and embryonic shoot apical meristem formation (33). Conversely, the *CUC* genes define the limits of the meristem by down-regulating cell proliferation (33, 34, 46–48). Mutations in the NAC family genes *NAM*, *cupuliformis* (*CUP*), or in both *CUC1* and *CUC2* are associated with organ fusion during embryo development (33, 34). The phenotype of *cup* mutants differs from those of *nam* and *cuc1 cuc2* mutants in that marked organ fusion is observed

throughout development (46). The post-embryonic organ fusion described in *pas* mutants may therefore be caused by changes in the expression of one or several NAC genes or in the activity of their products (21, 49). Finally, several studies have suggested that the products of NAC genes exert their effects at the transcriptional level (50, 51).

According to our data, we can propose a simple model in which PAS1 regulates FAN activity by targeting the protein to the nucleus, where it inhibits cell division. The overexpression of FAN in *pas1-3* mutants partly compensates for the absence of interaction with PAS1, by allowing some diffusion of the FAN protein into the nucleus, decreasing the frequency of ectopic cell division. The characterization of the PAS1-FAN complex

during cell division or de-differentiation associated with the confirmation of their putative transcriptional activity and target genes will help with the understanding of the PAS1 mode of action.

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The C Terminus of the Immunophilin PASTICCINO1 Is Required for Plant Development and for Interaction with a NAC-like Transcription Factor

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