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Components and targets of the PINOID signaling complex in Arabidopsis thaliana

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Chapter 2

The multi-functional scaffold PINOID Binding Protein 2 interacts with both cytoskeletal proteins and transcriptional regulators

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SUMMARY

The Arabidopsis PINOID (PID) protein serine/threonine kinase is a key regulator of plant growth that modulates the polar transport of the phytohormone auxin by controlling the polar subcellular localization of PIN auxin efflux facilitators. This chapter focuses on PID Binding Protein 2 (PBP2), a likely scaffold protein that - based on its possible localization at the cytoskeleton in onion cells - was considered as a promising link between PID and PIN vesicle trafficking. The presumed scaffold function of PBP2 was apparent, since the protein consists of two domains that are known to mediate protein-protein interactions: an amino-terminal Bric-a-brac, Tramtrack and Broad Complex/Pox virus and Zinc finger (BTB/POZ) domain, and a carboxy-terminal Transcriptional Adaptor putative Zinc Finger (TAZ) domain. In contrast to previous results, *in vitro* phosphorylation assays showed that PBP2 is not a phosphorylation target of PID, but that instead PID activity is repressed by PBP2. *In vitro* pull down assays suggested that PID interacts with the BTB/POZ domain, and transient expression of both proteins fused to GFP in Arabidopsis protoplasts suggested that this interaction occurs in the cytoplasm. The likely scaffold function of PBP2 indicated that other PBP2 interacting proteins (PBP2IPs) play a role in the PID signaling pathway. In an yeast two-hybrid screen sixteen putative PBP2IPs were identified that classified as cytoskeletal proteins, transcription factor-like proteins or proteins with an enzymatic function in primary metabolism. Three PBP2IPs, the putative microtubule-associated PBP2 BINDING MYOSIN-LIKE PROTEIN (PBMP), the transcription factor-like PBP2 BINDING MYB PROTEIN (PBMYB), and an uncharacterized AUXIN-INDUCIBLE PBP2 BINDING PROTEIN (APBP), were analyzed in more detail. *In vitro* pull down assays showed that PBMP and PBMYB interact with the C-terminal TAZ domain containing portion of PBP2, whereas APBP interacts with the N-terminal part of PBP2. *In vitro* phosphorylation assays did not show any evidence that these PBP2 partners are phosphorylated by PID, implying that PBP2 is not a scaffold for PID substrates. Further analysis did not provide evidence that PBMYB and PBMP are part of the PID signaling complex. In contrast, for APBP we concluded that it may be involved in modulating flowering time, and that it possibly competes with PID for the interaction with PBP2, and as a consequence activates the kinase by relieving it from PBP2-mediated repression. Overall, our data indicate that PBP2 is a scaffold protein with multiple functions, one of which is to be recruited to the PID signaling complex to regulate PIN polar targeting.

Abbreviations: APBP, auxin-inducible PBP2 binding protein; BTB/POZ, bric-a-brac, tramtrack and broad complex/Pox virus and zinc finger domain; CC, coiled-coil domain; F-actin, actin filament; GFP, green-fluorescent protein; GST, glutathione-S-transferase; IAA, indole-3-acetic acid; MBP, myelin basic protein; MPSS, massively parallel signature sequencing; NPA, 1-N-naphthylphthalamic acid; PAT, polar auxin transport; PBK, PBP2 binding kinesin; PBMP, PBP2 binding myosin-like protein; PBMYB, PBP2 binding MYB domain protein; PBP, pinoid binding protein; PBP2IP, PBP2 interacting protein; PID, pinoid; PM, plasma membrane; RRM, RNA recognition motif; SCF, SKP1/Cullin/F-box; TAZ, transcriptional adaptor putative zinc finger domain

INTRODUCTION

The plant hormone auxin plays a central role in plant growth and development. A distinctive feature of this compound concerns its transport in a polar fashion from sites of biosynthesis to sites of action. The unraveling of the molecular mechanism behind this polar transport started with the molecular characterization of the Arabidopsis *pin-formed 1* (*pin1*) mutant, that is defective in polar auxin transport (PAT), and was named after its pin-shaped inflorescences that lack flowers and bracts (1, 2). The *PIN1* gene appeared to encode a transmembrane protein that – due to its polar subcellular localization and its apparent role in PAT – was considered to be a likely candidate for the auxin efflux carrier in the chemiosmotic model for PAT proposed in the 1970s. The Arabidopsis genome encodes eight PIN proteins, for several of which the polar subcellular localization was correlated with the direction of auxin efflux (3-5). The polar localization of PIN proteins was shown to be maintained by recycling of PIN-containing vesicles from endosomal compartments to the plasma membrane (PM) along the actin cytoskeleton (6).

Another Arabidopsis mutant that develops pin-shaped inflorescences is *pinoid* (*pid*) (7). Cloning of *PINOID* identified a gene encoding a plant-specific protein kinase (8), whose ectopic expression causes phenotypic changes that can be partly rescued by application of PAT inhibitors. This and other observations led to the hypothesis that PID is a positive regulator of PAT (9).

Despite their shared involvement in PAT and the phenotypic similarities between the corresponding loss-of-function mutants, the true relationship between PID and PIN1 remained elusive until recently, when it was shown that the polar subcellular targeting of PIN proteins is determined by threshold levels of PID (10). The strong phenotypes observed in either loss- or gain-of-function PID lines imply that PID-mediated phosphorylation is an important step in the control of PIN polar targeting and, as a consequence, in the directionality of the auxin flow in patterning processes.

Although there is clear regulation of PIN protein localization by PID activity, the molecular mechanism behind it is far from being understood. In order to uncover the link between these two proteins, a yeast two-hybrid screen was performed using

PID as bait, and one of the interactors identified was PINOID BINDING PROTEIN 2 (PBP2) (11). The function of this protein is still unknown, but its primary amino acid sequence shows the presence of two conserved protein-protein interaction domains. One is a Transcriptional Adaptor putative Zinc Finger (TAZ) domain (12), and the other is a 'Bric-a-brac, Tramtrack and Broad Complex/Pox virus and Zinc finger (BTB/POZ) domain that is known to mediate both homo- and heterodimerization (13, 14). The Arabidopsis genome encodes at least seventy-six BTB domain proteins that can be classified in eleven major families according to their domain architecture (15). BTB proteins seem to be involved in a broad range of processes, such as phototropic growth (16, 17), systemic acquired resistance (18) and targeted proteolysis (19, 20).

Proteins containing both a BTB/POZ domain and a TAZ domain are only found in plants and the Arabidopsis genome encodes four homologs of PBP2 corresponding to gene models At3g48360, At1g05690, At4g37610 and At5g67480. PBP2 and its homologous proteins share 60% or more of similarity at the amino acid level (Robert, unpublished data) (11).

Preliminary experimental data suggested that PBP2 had a role as regulator of PID activity when complexed with this protein kinase. Weak phosphorylation of PBP2 was observed in *in vitro* phosphorylation assays with PID, and the presence of PBP2 strongly inhibited PID auto-phosphorylation (11). Moreover, bombardment of onion cells with a *35S::GFP-PBP2* construct suggested that the corresponding fusion protein is associated with the cortical cytoskeleton (11). Similar experiments with tobacco cell suspension cultures showed, however, that PBP2-GFP is localized in the nucleus (21). Assuming that both observations are correct, PBP2 could have a dual role acting both in the nucleus and at the cortical cytoskeleton in the cytoplasm. Histochemical staining of Arabidopsis seedlings transgenic for a *PID-GUS* fusion construct indicate that PINOID localizes in the cytoplasm of vascular cells (9), suggesting that this is the sub-cellular region where the interaction between PBP2 and PID takes place.

In order to further elucidate the role of PBP2 in the PID signaling, we first repeated the *in vitro* phosphorylation assays. These experiments confirmed that PBP2 downregulates PID kinase activity. However, in contrast to previous assays, the results indicated that PBP2 is not a phosphorylation target of PID. Since PBP2 has two protein interaction domains, its function could be to bring PID in proximity of its phosphorylation targets. To test this hypothesis, we performed an yeast two-hybrid screen using PBP2 as bait. This identified several interacting proteins that classified as cytoskeletal proteins, transcription factor-like proteins or proteins with an enzymatic function in primary metabolism. *In vitro* protein pull-down experiments showed that the N-terminal BTB domain containing portion of PBP2 interacts with PID and one of its interactors, APBP, whereas the C-terminal TAZ domain

comprising part interacts with transcription factors or cytoskeletal proteins. None of the newly identified PBP2 binding proteins were phosphorylation targets of PID, implying that PBP2 does not function as scaffold for PID-mediated protein modification. The interaction of PBP2 with both cytoskeleton-associated and nuclear proteins suggests a functional multiplicity, which will be discussed in light of the known role of PID in directing PIN polar targeting.

MATERIALS AND METHODS

Molecular cloning and constructs

Molecular cloning was performed following standard procedures (22). The yeast two-hybrid bait plasmid pAS2-PBP2 was obtained by cloning a *PBP2 PstI/SalI*-blunted fragment derived from pSDM6014 into pAS2 digested with *PstI/XmaI*-blunted. The histidine tagged PID construct was created by excising the *PID* cDNA with *XmnI-SalI* from pSDM6005 (11) and cloning it into pET16H (pET16B derivative, J. Memelink, unpublished results) digested with *BamHI*, blunted and subsequently digested with *XhoI*. The *35S::PID-GFP* construct was generated by amplifying the *PID* cDNA using the primers 5'-TTAATATGACTCACTATAGG-3' and 5'-GCTCACCATAAAGTAATCGAACGC-3' and the *eGFP* coding region using the primers 5'-GATTACTTTATGGTGAGCAAGGGC-3' and 5'-TCAATCTGAGTACTTGTA CAG-3'. Both PCR products were used together with outer primers in a new PCR reaction to generate the *PID-GFP* fragment, which was cloned into pUC28 digested with *NcoI/HincII*. The resulting pUC28-PID-GFP was digested with *EcoRI/StuI*-blunted and the *PID-GFP* fragment was ligated into *EcoRI/SmaI* digested pART7. Construction of histidine- and GFP-tagged PBP2 vectors are described by Benjamins (11). The GST-tagged PBP2 fusion (plasmid pGEX-*PBP2*) was generated by digesting pSDM6014 (11) with *XhoI/SmaI* and cloning the *PBP2* cDNA into pGEX-KG (23). The plasmid for production of a GST-tagged PBP2 BTB/POZ domain was created by digesting pGEX-*PBP2* with *NdeI,* filling in with Klenow and re-ligating. This created a stop codon at position 220 aa of the protein. The plasmid encoding the GST-tagged PBP2 TAZ domain was created by deleting the *NcoI* fragment encoding the BTB/POZ domain from pGEX-*PBP2.* The *PBMP* cDNA was amplified by PCR using the primers 5'-ACGCTTGTCGACTATATGTATGAGCAGCAGCAACAT-3' and 5'-CGGGATCCAAACAACCCAAGGA GAGAAATATC-3', and the resulting PCR fragment was digested with *BamHI/SalI* and cloned into the corresponding sites in pBluescriptSK+. His-PBMP and GFP-PBMP were obtained by cloning *PBMP BamHI/SalI* and *PBMP SalI/NotI* fragments into the plasmids pET16B (Novagen) and $pTH2^{BN}$ (derived from pTH2 plasmid described by Chiu and co-workers (24)) digested with *XhoI/BamHI* and *XhoI/NotI*, respectively. The *PBMYB* cDNA was amplified by PCR using the primers 5'-CCGCTCGAGTTGTGTCCGCCGGTATATGA-3' and 5'- CGGGATCCTTGGTTCCAAACTTAATCTTCA GG-3', and subsequently ligated into the pGEM-T cloning vector (Promega). The *PBMYB* fragment was excised from the resulting plasmid with *XhoI* and *NotI* and cloned into $pTH2^{BN}$ using the corresponding enzymes, giving rise to *GFP-PBMYB*. His-PBMYB-CT was generated by ligating the *PBMYB-CT NdeI/XhoI* fragment derived from the original pACT2-*PBMYBCT* yeast two-hybrid clone into pET16B digested with the corresponding restriction enzymes. Finally, the His-APBP and GFP-APBP fusion proteins were obtained by cloning the *APBP NdeI/XhoI* and *APBP BglII* fragments (derived from the pACT2-APBP yeast two-hybrid clone) into the corresponding restriction sites of the vectors pET16B and $pTH2^{BN}$, respectively.

Yeast two hybrid screen

Using the Matchmaker II yeast two-hybrid system and *Saccharomyces cerevisiae* strain PJ69-4A (Clontech), an *Arabidopsis thaliana* cDNA library fused to the GAL4 activation domain (pACT2) was screened at 20°C with PBP2 fused to the GAL4 DNA binding domain (pAS2) as bait. The cDNA library

was constructed from RNA samples isolated from Arabidopsis root cultures in a one to one mix of untreated roots and roots treated for 24 hours with the auxin analog 1-naphthaleneacetic acid (1-NAA) (25). The positive clones were analyzed by colony hybridization as described in the Hybond-N+ Membrane Manual (Amersham Biosciences) and in the work of Memelink and co-workers (26).

In vitro pull down experiments

GST tagged full-length PBP2, its deletion versions (GST-BTB/POZ and GST-TAZ) or GST protein alone were used in pull down assays with histidine (his)-tagged PBP2 interactors (H-interactors). Cultures of *E. coli* strain BL21 containing one of the constructs were grown at 37ºC to OD_{600} 0,8 in 50 ml LC supplemented with antibiotics. The cultures were then induced for 4 hours with 1 mM IPTG at 30ºC, after which cells were harvested by centrifugation (10 min. at 4.000 RPM in tabletop centrifuge) and frozen overnight at -20ºC. Precipitated cells were re-suspended in 2 ml Extraction Buffer (EB: 1x PBS, 2 mM EDTA, 2 mM DTT, supplemented with 0,1 mM of the protease inhibitors PMSF - Phenylmethanesulfonyl Fluoride, Leupeptin and Aprotinin, all obtained from Sigma) for the GST-tagged proteins or in 2 ml Binding Buffer (BB: 50 mM Tris-HCl pH 6,8, 100 mM NaCl, 10 mM $CaCl_2$, supplemented with PMSF 0,1 mM, Leupeptin 0,1 mM and Aprotinin 0,1 mM) for the his-tagged PBP2 interactors and sonicated for 2 min. on ice. From this point on, all steps were performed at 4ºC. Eppendorf tubes containing the sonicated cells were centrifugated at full speed (14.000 RPM) for 20 min., and the supernatants were transferred to fresh 2 ml tubes. H-interactors supernatants were left on ice, while 100 µl pre-equilibrated Glutathione Sepharose resin (pre-equilibration performed with three washes of 10 resin volumes of 1x PBS followed by three washes of 10 resin volumes of 1x BB at 500 RCF for 5 min.) was added to the GST- fusion protein containing supernatants. Resin-containing mixtures were incubated with gentle agitation for 1 hour, subsequently centrifugated at 500 RCF for 3 min. and the precipitated resin was washed 3 times with 20 resin volumes of EB. Next, all H-interactors supernatants (approximately 2 ml per interactor) were added to GST-fusions-containing resins, and the mixtures were incubated with gentle agitation for 1 hour. After incubation, supernatants containing GST resins were centrifugated at 500 RCF for 3 min., the new supernatants were discarded and the resins subsequently washed 3 times with 20 resin volumes of EB. Protein loading buffer was added to the resin samples, followed by denaturation by 5 min. incubation at 95^0C. Proteins were subsequently separated on a 12% polyacrylamide gel prior to transfer to an Immobilon^{TM}-P PVDF (Sigma) membrane. Western blots were hybridized using a horse radish peroxidase (HRP)-conjugated anti-pentahistidine antibody (Quiagen) and detection followed the protocol described for the Phototope-HRP Western Blot Detection Kit (New England Biolabs).

In vitro phosphorylation assays

All proteins used in *in vitro* phosphorylation assays were his-tagged for purification from several (usually five) aliquots of 50 ml cultures of *E. coli*. strain BL21 which were grown, induced, pelleted and frozen as described above for the *in vitro* pull down experiments. Each aliquot of frozen cells pellet was resuspended in 2 ml Lysis Buffer (25 mM Tris-HCl pH 8,0; 500 mM NaCl; 20 mM Imidazol; 0,1% Tween-20; supplemented with 0,1 mM of the protease inhibitors PMSF, Leupeptin and Aprotinin) and subsequently sonicated for 2 min. on ice. From this point on, all steps were performed at 4ºC. Sonicated cells were centrifugated at full speed (14.000 RPM) for 20 min, the new pellets were discarded, and supernatants from all aliquots of the same construct were transferred to a 15 ml tube containing 100 µl of pre-equilibrated Ni-NTA resin (pre-equilibration performed with three washes of 10 resin volumes of Lysis Buffer at 500 RCF for 5 min.). Supernatant and resin were incubated with gentle agitation for 1 hour. After incubation, supernatant containing Ni-NTA resin was centrifuged at 500 RCF for 3 min., the new supernatant was discarded and the resin subsequently washed: 3 times with 20 resin volumes of Lysis Buffer, once with 20 resin volumes of Wash Buffer 1 (25 mM Tris.Cl pH 8,0; 500 mM NaCl; 40 mM Imidazol; 0,05% Tween-20) and once with 20 resin volumes of Wash Buffer 2 (25 mM Tris-HCl pH 8,0; 600 mM NaCl; 80 mM Imidazol). In between the washes, the resin was centrifugated for 5 min. at 500 RCF. After the washing steps, 20 resin volumes of Elution Buffer (25 mM Tris.HCl pH 8,0; 500 mM NaCl; 500 mM Imidazol) was added to the resin and incubated for 15 min. with gentle agitation. The resin was

centrifugated for 3 min. at 500 RCF, and the supernatant containing the desired protein was diluted a 1000-fold in Tris Buffer (25 mM Tris.HCl pH7,5; 1 mM DTT) and concentrated to a workable volume (usually 50 µl) using Vivaspin microconcentrators (10 kDa cut off, maximum capacity 600 µl, manufacturer: Vivascience). Glycerol was added as preservative to a final concentration of 10% and samples were stored at -80ºC.

Approximately 1 µg of each purified his-tag protein (PID and substrates) in maximal volumes of 10 µl were added to 20 µl kinase reaction mix, containing 1x kinase buffer (25 mM Tris-HCl pH 7,5; 1 mM DTT; 5 mM $MgCl_2$) and 1 x ATP solution (100 µM $MgCl_2$/ATP; 1 µCi ^{32}P-γ-ATP). Reactions were incubated at 30ºC for 30 min. and stopped by the addition of 5 µl of 5 x protein loading buffer (310 mM Tris-HCl pH 6.8; 10 % SDS; 50% Glycerol; 750 mM β-Mercaptoethanol; 0,125% Bromophenol Blue) and 5 min. boiling. Reactions were subsequently separated over 12,5% acrylamide gels, which were washed 3 times for 30 min. with kinase gel wash buffer (5% TCA – Trichoroacetic Acid; 1% $Na_2H_2P_2O_7$), coomassie stained, destained, dried and exposed to X-ray films for 24 to 48 hours at -80ºC using intensifier screens.

Protoplast transformations

Protoplasts were obtained from *Arabidopsis thaliana* Col-0 cell suspension cultures that were propagated as described by Schirawski and co-workers (27). Protoplast isolation and PEG-mediated transformation followed the protocol described originally by Axelos and co-workers (28) and adapted by Schirawski and co-workers (27). The transformations were performed with 20 µg of plasmid DNA, after which the protoplasts were incubated for at least 16h. Images were obtained by laser scanning confocal microscopy.

Plant growth and lines

Seeds were germinated and seedlings grown *in vitro* on MA medium (29) supplemented with antibiotics or other compounds when required, at 21°C, 50% relative humidity and a 16 hours photoperiod of 2500 lux. Flowering Arabidopsis plants were grown on substrate soil, in growth rooms at 20°C, 40% relative humidity and a 16 hours photoperiod of 2500 lux.

The Arabidopsis mutant lines N620810 and FLAG_371C08 with T-DNA insertions in the *PBMP* and *APBP* genes, respectively, were obtained from the Salk Institute (N620810) and INRA (FLAG_371C08). For the PCR identification of the mutant alleles, we used the primers 5'-GAAATGATGCA AACATTTGGCG-3', 5'-TCTGGGTTTGGGGACGATAGC-3' and 5'-TGGTTCACGTAGTGGGCCATCG-3' for the *pbmp* allele N620810, and 5'-CATGCCCTTACACATTTCCACA-3', 5'-TGATGAGGCTCG TAGCTTCCG-3' and 5'-CGTGTGCCAGGTGCCCACGGAATAGT-3' or 5'-CTACAAATTGCCTTTTCTT ATCGAC-3' for the *apbp* FLAG_371C08 allele.

RESULTS

PBP2 could be a regulator of PINOID activity

Previous *in vitro* phosphorylation experiments suggested that PBP2 is a phosphorylation target of PID and that PBP2 significantly inhibits PID auto-phosphorylation (11). New experiments using Myelin Basic Protein (MBP) as phospho-substrate confirmed the negative effect of PBP2 on both auto- and trans-phosphorylation activity of PID (Figures 1A and 1B). The previously observed weak labeling of PBP2 in the presence of PID (11) was only found occasionally (Figure 1B). Control reactions, in which PBP2 was incubated with radio-labeled ATP alone showed the same weak labeling of PBP2 (Figure 1B), indicating that PBP2 binds ATP at low-affinity. The results imply that PBP2 is not a target of PID phosphorylation, but instead functions as a regulator of PID activity.

PINOID binds the BTB/POZ domain containing part of PBP2 *in vitro*

BTB domain proteins are known as scaffold- or linker-proteins that organize protein complexes (30). PBP2 has two typical protein-protein interaction domains, and to test which of them binds to PID, GST-tagged full length PBP2, or the GST-tagged BTB/POZ or TAZ domain alone (Figure 1C) were incubated *in vitro* with histidine-tagged PID, and protein complexes were pulled down with glutathione beads. Western blot analysis using anti-His antibodies showed that PID efficiently binds the BTB/POZ domain containing part, whereas the TAZ domain containing part only pulls down background levels of the kinase (Figure 1D). In view of the established role of the BTB and TAZ domains in protein-protein interaction, this result suggests that PID interacts with the BTB/POZ domain, and that PBP2 indeed acts as a scaffold that – through its TAZ domain - recruits proteins that are phosphorylation targets of PID or that regulate PID activity.

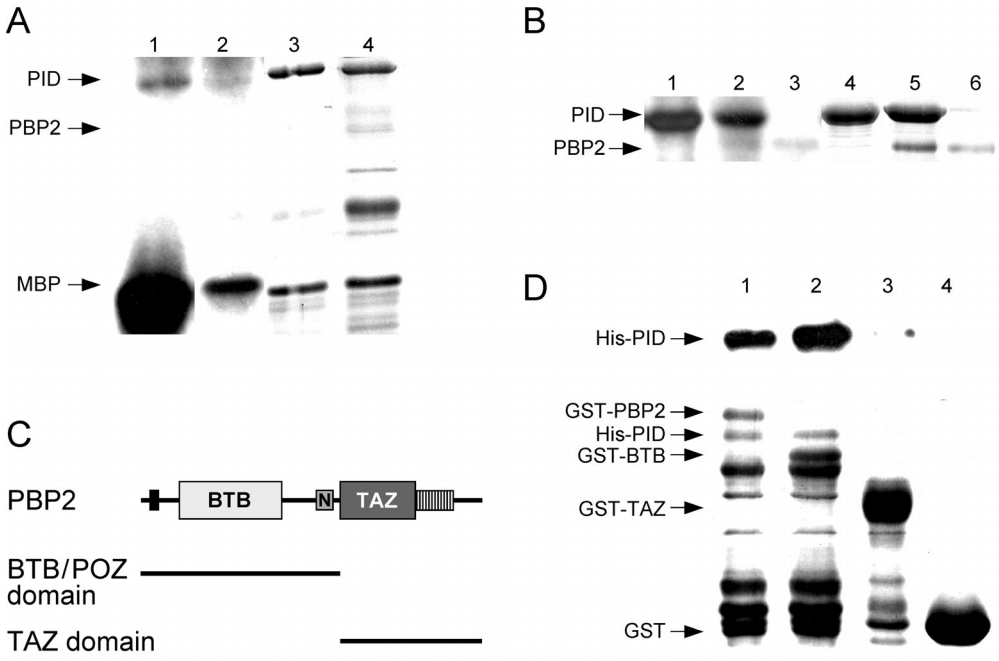
PINOID and PBP2 co-localize in the cytoplasm of Arabidopsis protoplasts

In order to identify the subcellular compartments in which PID and PBP2 are localized, we transformed the plasmids *35S::PID-GFP* and *35S::GFP-PBP2* into Arabidopsis protoplasts. PID-GFP primarily localized at the plasma membrane, but in 50% of the protoplasts also cytoplasmic localization was observed (Figure 1E). GFP-PBP2 was nuclear localized in 80% of the protoplasts, whereas 20% of the protoplasts showed cytoplasmic localization (Figure 1E). The nuclear localization of PBP2 was reported previously (21), and corroborates the presence of a functional nuclear localization signal in the protein (Figure 1C). Based on these and previous results (10) it can be hypothesized that PID-mediated regulation of PIN polar targeting occurs through direct interaction between PINs and the PID protein kinase at the plasma membrane. PID, however, is also found in the cytoplasm, where PBP2 possibly down-regulates its activity through its interaction with the protein kinase. The predominant nuclear localization of PBP2 may relate to another function of PBP2 that is unrelated to PID. An interesting possibility is that PBP2 and PID alter each others subcellular localization when co-expressed in plant cells.

Identification of PBP2 interacting proteins suggests multiplicity in PBP2 function

In order to obtain more insight into the protein complexes that involve PBP2, an yeast two-hybrid screen was performed using PBP2 as bait. Yeast cells were co-transformed with the full-length cDNA of PBP2 fused to the GAL4 DNA binding domain (bait) and a cDNA library prepared from a 1:1 mixture of uninduced and NAA-induced root cultures fused to the GAL4 activation domain (prey library). Selection for three different markers resulted in 78 positive clones from an

estimated total of 1,4x10^6 transformants (Table 1), which corresponds to a near-complete screening of the original mRNA population (31).
Following the subsequent selection steps, twenty-eight apparently distinct positive clones were obtained, which after sequence analysis appeared to represent sixteen



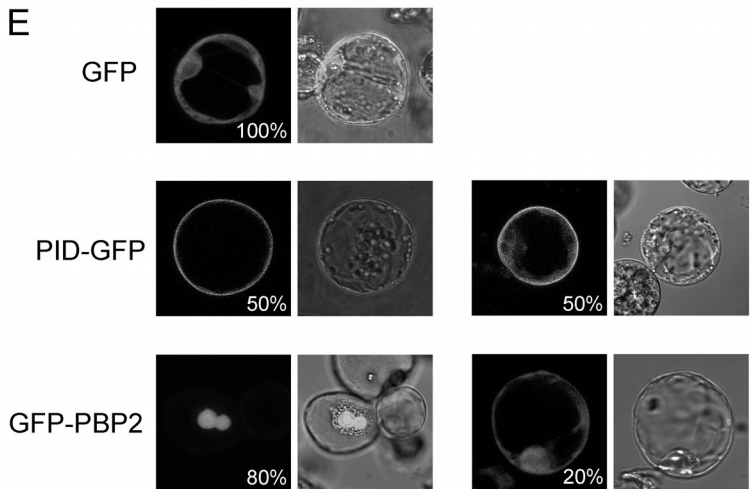


Figure 1. PID binding to the BTB domain portion of PBP2 negatively regulates PID kinase activity. (A) Autoradiograph (1 and 2) and coomassie stained gel (3 and 4) of a phosphorylation assay containing PID and MBP (lanes 1 and 3); or PID, PBP2 and MBP (lanes 2 and 4). (B) Autoradiograph (1 to 3) and coomassie stained gel (4 to 6) of a phosphorylation assay containing PID (lanes 1 and 4), PID and PBP2 (lanes 2 and 5) or PBP2 alone (lanes 3 and 6). (C) Schematic representation of PBP2 (365 aa) and the two deletion derivatives containing either the BTB/POZ- or the TAZ domain. The N-box and the striped box indicate the positions of respectively an NLS and a putative calmodulin binding site (Du and Poovaiah, 2004). (D) *In vitro* pull-down of his-tagged PID with GST-tagged PBP2 (lane 1), GST-tagged BTB domain (lane 2) or -TAZ domain (lane 3) containing portions of PBP2 or GST alone (lane 4). Top: immunodetection of his-tagged PID. Bottom: coomassie stained gel with the positions of the different input proteins indicated. (E) Arabidopsis protoplasts transformed with *35S::GFP* (top), *35S::PID-GFP* (middle) or *35S::GFP-PBP2* (bottom). Per construct one or two couples of a fluorescence image (left) and a merged transmission light and fluorescence images (right) of a representative protoplast are shown.

unique cDNAs (Table 1). A BLAST sequence comparison with the NCBI database showed that, although the function of several of the PBP2 interactors is still unknown, most of the encoded proteins contain reasonably well-characterized domains, thereby allowing the assignment of hypothetical functions. Based on this analysis, PBP2 interactors can be roughly classified in three groups: i) proteins involved in gene expression regulation, ii) cytoskeletal proteins and iii) proteins with a specific enzymatic function in primary metabolism (Table 2). Curiously, the most frequent interactor of PBP2 that was represented by almost 50% of the His, Ade and α-Gal positive yeast colonies, as determined by the subsequent analysis steps, does not fall in any of these groups (Table 2) due to insufficient functional information.

Table 1. Yeast two-hybrid screen for PBP2 interactors.

Transformants	+His	+Ade	+His +Ade +α-GAL	Molecular analysis **	Colony hybridization	Sequencing - Final unique clones
$1{,}4x10^6$	510*	196*	78*	48	28	16

* Colonies with positive phenotype concerning the respective selection marker

** Selection steps consisting of PCR followed by restriction analysis to eliminate redundant clones

Despite the finding of a considerable number of proteins that interact with PBP2, only few of them were chosen for further research. The choice was mainly based on the reliability of interaction with PBP2 and the likelihood that the protein participates in the PID signaling pathway. In particular, the PBP2 interactors of the class of enzymatic proteins were excluded for further analysis, since a direct link with the

elusive PID signaling pathway was unclear or unlikely. Concerning the remaining proteins, a functional relationship between PID, PBP2 and the PBP2-interactors can be explained by three hypotheses: i) PBP2 acts as a scaffold to recruit phosphorylation targets of PID; ii) the three proteins are part of a functional complex in which PID does not phosphorylate the PBP2-interactor; iii) PBP2 interacts with PID and the PBP2-interactor independently but as part of the same regulatory pathway. Below, these possibilities will be discussed for the selected PBP2-interactors in context of their possible functions.

PBP2 Interacts with Putative Cytoskeletal Proteins

Of the sixteen PBP2 interactors identified, five are likely components of the cellular cytoskeleton (Table 2). Since it has recently been demonstrated that PID activity directs the subcellular localization of PIN proteins (10) and the localization of PIN proteins is regulated and maintained by vesicle trafficking along the cytoskeleton, the cytoskeletal PBP2 interactors may be part of the PID signaling complex.

Two of the putative cytoskeletal proteins are homologous proteins that have a typical N-terminal microtubule motor domain and thus belong to the super-family of kinesins. The proteins were named PBP2 BINDING KINESIN 1 and 2 (PBK1 and 2, respectively) and their detailed functional analysis will be presented in Chapter 3.

The third putative cytoskeletal PBP2 interactor contains three Armadillo repeats (At3g22990). Comparison of these repeats with the Pfam database showed that they are found in proteins involved in vacuolar targeting of macromolecules via microtubuli.

The possible cytoskeletal function of the fourth PBP2 interactor is indicated by its internal CXC box (At5g25790). In Drosophila, the CXC box is present in kinesins associated with the spindle apparatus during meiosis and fertilization (32, 33). In Arabidopsis, CXC boxes are found in proteins such as TSO1 and CURLY LEAF, whose functions are related to cytokinesis and cell elongation, respectively (34-36).

Although At3g22990 and At5g25790 seem to be clearly linked with the cytoskeleton, there are virtually no data concerning their true function, making an association with the actual molecular function of the PID kinase a difficult task. As a consequence, they were not studied in further detail.

PBP2 Binding Myosin-like Protein suggests association of PBP2 to the microtubule cytoskeleton

The fifth cytoskeleton-related PBP2 interactor, PBMP (for PBP2 Binding Myosin-like Protein), is a protein of unknown function that contains a long coiled-coil domain (Figure 2A). Long coiled-coil (CC) domains are present in proteins involved in a variety of functions, for example attachment of protein complexes to cellular structures such as the Golgi, the nuclear envelope or centrosomes. This domain is

Table 2. Proteins interacting with PBP2 in the Yeast Two-Hybrid System.

CLASS	GENE MODEL	ASSIGNED NAME	FEATURES	# CLONES*	PROTEIN (aa)	MIN. SIZE (aa)**	PBP2 PORTION ****
Cytoskeleton Related	At2g21300	PBP2 Binding Kinesin 2 (PBK2)	Kinesin motor domain	4	862	257	TAZ
	At4g38950	PBP2 Binding Kinesin 1 (PBK1)	Kinesin motor domain	2	836	286	n.d.
	At3g22990	-	Armadillo-repeat	1	460	379	n.d.
	At5g25790	-	CXC box	1	408	161	n.d.
	At5g08120	PBP2 Binding Myosin-like Protein (PBMP)	Long coiled-coil domain	2	326	247	TAZ
Regulator of Gene Expression	At5g41020	PBP2 Binding MYB domains protein (PBMYB)	MYB DNA binding domains	2	588	164	TAZ
	At3g56510	-	Transcription co-factor like; RRM domain	2	266	266	n.d.
	At4g08150	KNAT1	KNAT1 transcription factor	1	398	386	n.d.
	At3g53920	-	SIGC transcription factor	1	571	179	n.d.
	At3g09850	-	G-patch, RRM and ATP-binding site	1	781	771	n.d.
	At2g43190	-	ribonuclease P family protein	1	296	226	n.d.
Enzymatic Proteins	At3g53120	-	glutamyl-tRNA reductase	3	217	217	n.d.
	At2g46200	Putative Chromatin-Remodeling PBP2 Binding Protein (CROP)	Zinc Metalloenzyme	2	382	338	BTB/POZ
	At3g03420	-	Zinc Metalloprotease domain	1	194	98	n.d.
	At3g23940	-	Dehydratase protein	1	608	254	n.d.
Auxin-Inducible	At2g39870	Auxin-Inducible PBP2 Binding Protein (APBP)	Auxin inducible gene	30	330	330	BTB/POZ
Not further characterized***	-	-	-	23	-	-	-

*Number of times a gene was found among the 78 His, Ade and α-GAL positive clones (Table 1).
**Minimal number of carboxi-terminal amino acids encoded by the shortest positive cDNA clones.
***These 23 clones were not further characterized because they either autoactivated in yeast, encoded truncated or out-of-frame proteins or failed to promote yeast growth when back-transformed.
**** Portion of PBP2 with which the interactor binds, as determined by *in vitro* pull-down assays.

also found in proteins of the intermediate filaments, which together with actin and microtubules are involved in enhancing structural integrity, cell shape, and cell and organelle motility. Finally, CC domains are present in proteins related to nuclear and chromosomal organization, microtubule structure and organization and in proteins related to targeting to membrane systems (37). PBMP has previously been identified in a screen for Arabidopsis proteins related to cytoskeleton in *Schizosaccharomyces pombe* (38). In this screen, an Arabidopsis cDNA library was expressed in *S. pombe* cells, and transformed cells displaying cytoskeletal defects, such as the ones expressing PBMP, were isolated and characterized. This observation, combined with the fact that PBMP contains a long coiled-coil domain, led to its initial naming as myosin-related protein. That this initial naming may not be entirely correct was suggested by the fact that the tobacco ortholog MPB2C is associated with microtubules, and seems to function in the inter- or intra-cellular transport of macromolecules (39). The experimental data suggesting that PBMP has a function in cytoskeleton-related processes and the finding that it interacts with PBP2, a putative cytoskeletal protein that binds PID, leads us to speculate that PBMP could play a role in the PID signaling pathway that determines the polar targeting of PIN proteins.

To confirm the data from the yeast two-hybrid screen, *in vitro* protein pull-down experiments were performed using GST-tagged full length PBP2, or the GST-tagged BTB/POZ or TAZ domain containing portion alone, together with his-tagged PBMP. The results showed that PBMP preferentially binds the C-terminal TAZ domain containing part of PBP2 (Figure 2B). Interestingly, this observation and the fact that PID likely interacts with the BTB domain (Figure 1D) fit to the model that PBP2 acts as a scaffold protein.

Subsequently we tested the possibility that PBMP is a phosphorylation target of PID*. In vitro* phosphorylation experiments showed that PBMP is not phosphorylated by PID either in the presence or absence of PBP2 (Figure 2C). As observed before, PID kinase activity is inhibited in the presence of PBP2.

Even though PBMP is not a phosphorylation target of PID, it may still have a function in the PID signaling pathway. PBMP could affect the subcellular localization of PID, or be involved in altering cytoskeletal properties as part of the PID-PBP2-PBMP complex. Since a requirement for such function involves that PBMP is localized in the same subcellular compartments as PID and PBP2, we transformed a *35S::GFP-PBMP* construct to Arabidopsis protoplasts. GFP-PBMP was found to localize in the cytoplasm (Figure 2D). Considering that PID and PBP2 were also present in this compartment (Figure 1E), it is possible that the three proteins form a complex. Although PBMP is predicted to be microtubule-associated, its overexpression in Arabidopsis protoplasts may have prevented us to observe the

microtubule-specific pattern that has previously been reported for its tobacco ortholog (39).

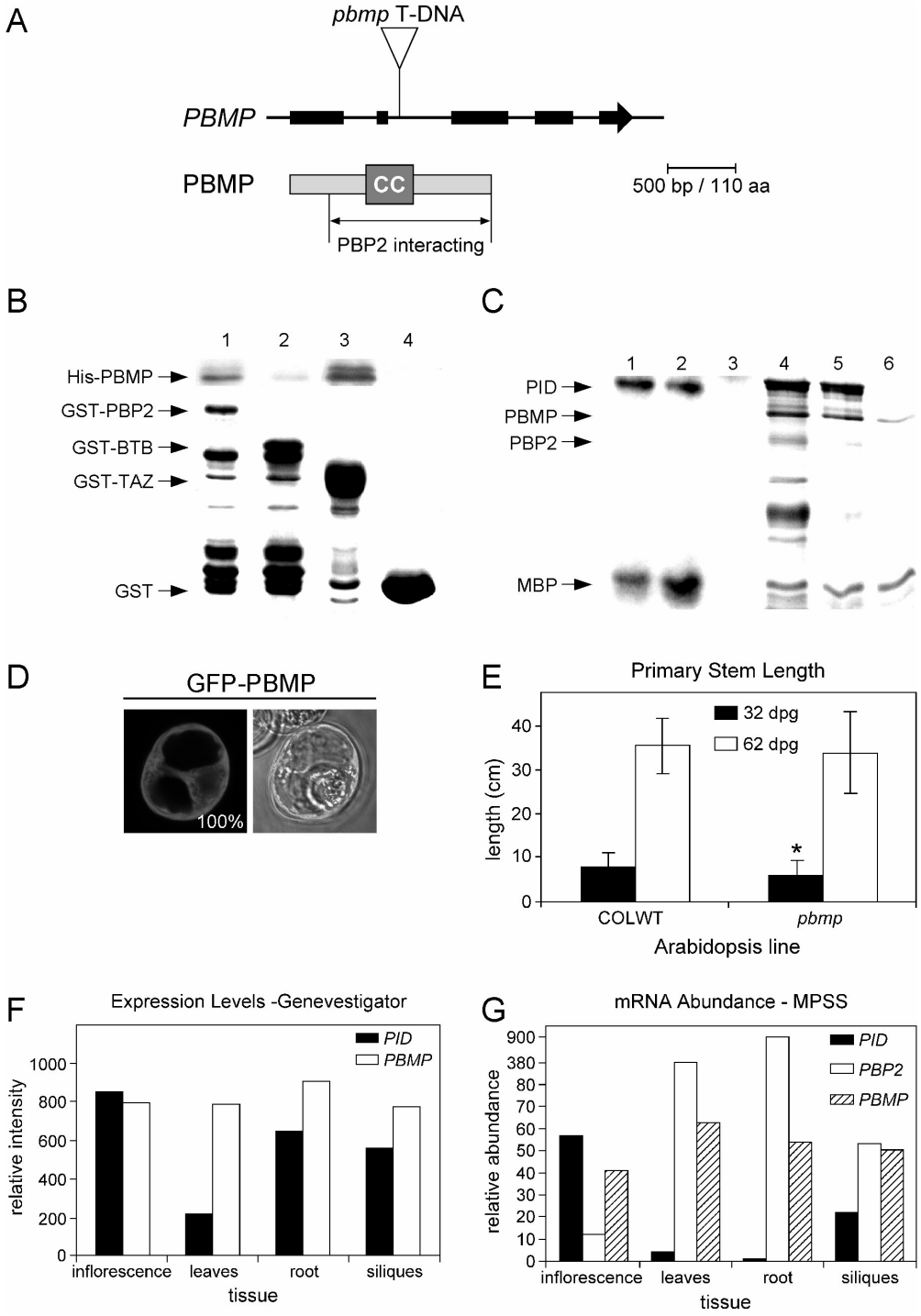
If PBMP is crucial for proper functioning of PID, *pbmp* loss-of-function may lead to phenotypes related to those of the *pid* mutant. A mutant Arabidopsis line was obtained from the Salk Institute with a T-DNA insertion in the second intron of the gene (Figure 2A). Unfortunately, no striking mutant phenotypes were observed in *pbmp* seedlings, even when they were grown on 0,1 µM IAA or 0,3 µM NPA. After bolting, the young primary inflorescence of mutant plants was significantly shorter compared to wild type (Figure 2E). In fully matured plants, however, the inflorescence length did not significantly differ from wild type (figure 2E), suggesting that the shorter primary inflorescence is caused by a delay in bolting rather than by a defect in elongation growth. Experimental data from publicly available microarray and MPSS (Massively Parallel Signature Sequencing) datasets (40, 41) (Figures 2F and 2G, respectively) show that PBMP is constitutively expressed at moderate levels in most Arabidopsis tissues, including the inflorescence, therefore partly corroborating phenotypes observed in the *pbmp* insertion mutant plants. These same data indicate that PBP2 is also expressed in inflorescences, although at reduced levels, suggesting that both PBMP and PBP2 proteins are present in the same cells as PID.

The data presently shown suggest that PID, PBMP and PBP2 could form a complex, since the first two interact with different domains of PBP2, and the three proteins are expressed in the same tissues and co-occur in the same subcellular compartment. However, the lack of clear mutant phenotypes of *pbmp* mutant line prevents us to speculate on a function for such a complex. The fact that there is no significant PBMP homolog and thus no clear redundancy in gene function in Arabidopsis indicates that PBMP can not play an important role in PID action. The *in vivo* occurrence and the exact function of a complex involving PID, PBP2 and PBMP therefore remain to be investigated.

PBP2 Interacts with Regulators of Gene Expression

Six of the PBP2 interactors are putative or known transcriptional regulators or have domains related to RNA recognition and binding (Table 2). This finding, together with the observed nuclear localization of PBP2 (Figure 1E), implies a role for PBP2 in transcription regulation.

Two of the PBP2 interactors contain an RRM motif (At3g56510 and At3g09850; Table 2), which has been found in different types of RNA-related proteins, such as heterogeneous nuclear ribonucleoproteins, small nuclear ribonucleoproteins and pre-RNA and mRNA associated proteins (42). The protein corresponding to gene model At3g56510 is relatively similar to the mouse TATA-BINDING-BINDING PROTEIN (ABT1), which was found to be associated with mouse TATA-BINDING


A
pbmp T-DNA
PBMP
PBMP
CC
500 bp / 110 aa
PBP2 interacting
B
1 2 3 4
His-PBMP
GST-PBP2
GST-BTB
GST-TAZ
GST
C
1 2 3 4 5 6
PID
PBMP
PBP2
MBP
D
GFP-PBMP
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E
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leaves
root
siliques
tissue
G
mRNA Abundance - MPSS
PID
PBP2
PBMP
relative abundance
0 10 20 30 40 50 60 70 80 380 900
inflorescence
leaves
root
siliques
tissue

Figure 2. PBMP is a constitutively expressed protein that interacts with the TAZ domain portion of PBP2. (A) Schematic representation of the Arabidopsis *PBMP* gene (top) and the PBMP protein (bottom). Exons of *PBMP* are represented by thick black lines and the position of the T-DNA insertion in the Arabidopsis mutant line N620810 from the Salk Institute is indicated. For PBMP the coiled-coil domain (CC) as well as the PBP2 interacting portion are indicated. (B) *In vitro* pull-down of his-tagged PBMP with GST-tagged PBP2 (lane 1), GST-tagged BTB domain (lane 2) or -TAZ domain (lane 3) containing portions of PBP2 or GST alone (lane 4). Top: immunodetection of his-tagged PBMP; Bottom: coomassie stained gel with the positions of the different input proteins indicated. (C) Autoradiograph (1 to 3) and coomassie stained gel (4 to 6) of phosphorylation assays showing that PBMP is not a phosphor-substrate of PID. The relative positions of PID, PBP2, PBMP and MBP are indicated. Lanes 1 and 4: PID, PBP2, PBMP and MBP; Lanes 2 and 5: PID, PBMP and MBP; Lanes 3 and 6: PBMP and MBP. (D) Arabidopsis protoplasts transformed with *35S::GFP-PBMP.* A fluorescence image (left) and a merged fluorescence and transmission light images (right) of a representative protoplast are shown. (E) The primary inflorescence stem of young *pbmp* loss-of-function mutants is significantly shorter (star) than wild type plants, but in adult plants there is no significant difference. (F and G) Gene expression data available through Genevestigator (F) and Arabidopsis MPSS (G) databases show that PBMP is constitutively expressed in Arabidopsis.

PROTEIN *in vitro* and shown to act as transcriptional activator (43). The PBP2 interactor corresponding to gene model At3g09850 contains an RRM motif coupled to a G-Patch domain. A G-Patch domain is characterized by the presence of seven highly conserved glycines, and is found in a number of RNA binding proteins, and in proteins that contain RNA binding domains (44). The combination of G-Patch and RRM domains has been described for DNA repair and RNA recognition proteins (45).

A PBP2 interactor that represents a well-characterized transcription factor is KNAT1. This protein belongs to the KNOTTED-class of homeodomain proteins, and phenotypes of the *knat1/brevipedicellus* loss-of-function mutants (46) imply that it is an important regulator of the growth and cell differentiation of the inflorescence stem, pedicel, and style in *Arabidopsis*. KNAT1 was shown to traffic between cells and it was suggested to play a role in the intercellular trafficking of macromolecules (47). Interestingly, plants overexpressing KNAT1 were shown to ectopically produce meristems, implying a role for this protein in meristem maintenance and organogenesis (48). Since both PID and KNAT1 are regulators of organogenesis at the inflorescence meristem, it is possible that PBP2 plays a role in this process as well. The interaction of KNAT1 with PBP2 in the yeast two-hybrid system, however, was very weak, and expression of this transcription factor fused to the GAL4 activation domain in the yeast strain PJ69-4A promoted background growth on selective medium. Based on these results and the fact that KNAT1 was identified only once, we decided to exclude this protein from further studies.

The observation in protoplasts that PID-GFP seems to be cytoplasmic or plasma membrane-localized implies that this kinase and transcription factors cannot be part of the same protein complex. At this stage we cannot exclude, however, that the

subcellular localization of PID is tissue dependent. Therefore, it could be possible that PID is nuclear localized in cells of different organs, in which case the interaction with KNAT1 or other PBP2 partners related to transcriptional regulation becomes more likely.

PBP2 Binding MYB-domain protein possibly represents a cell-cycle-related transcription factor

One of the transcription factor-like PBP2 interactors that was identified twice contains four MYB DNA binding domains at the carboxy-terminal portion and a lysine-rich region at the amino-terminal portion, which also comprises a putative NLS (Figure 3A). The protein was named PBP2 Binding MYB-domain protein (PBMYB). PBMYB shows reasonable similarity (43%) with a mouse homolog of CYCLIN-D BINDING MYB LIKE TRANSCRIPTION FACTOR 1 (DMP1). DMP1 is a three MYB domain protein that seems to be involved in the regulation of cell cycle arrest, probably through inhibition of S-phase entry (49).

The interaction between PBP2 and PBMYB was confirmed by *in vitro* protein pull-down assays. The his-tagged C-terminal portion of PBMYB (PBMYB-CT) was efficiently pulled down with GST-tagged PBP2 and -TAZ domain, and less efficiently with the GST-tagged BTB domain, suggesting that PBMYB-CT preferably binds the TAZ domain containing portion of PBP2 (Figure 3B).

Transformation of Arabidopsis protoplast with the *35S::GFP-PBMYB* construct showed that PBMYB is a nuclear protein, as expected for a transcription factor (Figure 3C).

Additional phosphorylation assays using PID, PBP2 and PBMYB did not provide any evidence that the kinase is able to phosphorylate the C-terminal portion of PBMYB (Figure 3D). As we have not yet been able to test the full length cDNA in this assay, we can not exclude that the N-terminus may interfere with the interaction or that this part of the protein is phosphorylated by PID.

Massive Parallel Signature Sequencing (MPSS) data (40) reveal that *PBMYB* is expressed at moderate to high levels in different Arabidopsis tissues (Figure 3E) but, like *PID*, its expression is highest in the inflorescence. These data indicate that PBMYB, PBP2 and PID may be part of the same signaling pathway involved in cell division in the inflorescence. On the other hand, PBMYB localization is restricted to the nucleus, and the fact that PID-GFP is cytoplasmic or plasma membrane localized implies that the proteins can hardly be part of the same protein complex. Considering that, as mentioned above, the subcellular localization of PID could be tissue dependent, it could be possible that PID is nuclear localized in cells of different organs, in which case the interaction with PBMYB would be allowed. The *in vivo* existence and functionality of the PID-PBP2-PBMYB complex remains to be investigated.

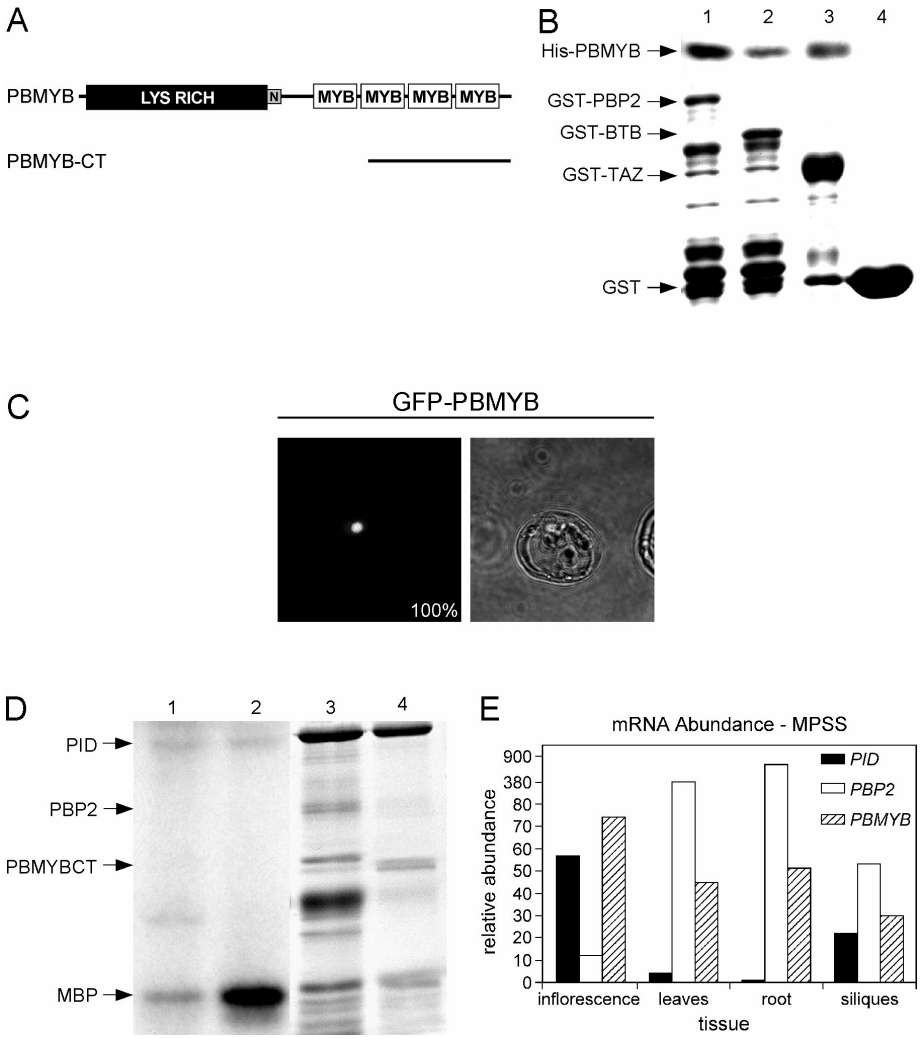

Figure 3. PBMYB interacts with the TAZ domain portion of PBP2 and shows highest expression in inflorescences. (A) Schematic representation of PBMYB (588 amino acids) and the 217 amino acids C-terminal part (PBMYB-CT) that interacts with PBP2 in the yeast two-hybrid system and was used in *in vitro* pull down assays. The lysine rich region, the NLS (N) and the MYB domains are represented by boxes. (B) *In vitro* protein pull-down of his-tagged PBMYBCT with GST-tagged PBP2 (lane 1), GST-tagged BTB domain (lane 2) or -TAZ domain (lane 3) containing part of PBP2 or GST alone (lane 4). Top: immunodetection of his-tagged PBMYB-CT. Bottom: coomassie stained gel with the positions of the different input proteins indicated. (C) Arabidopsis protoplasts transformed with *35S::GFP-PBMYB.* A fluorescence image (left) and a merged fluorescence and transmission light images (right) of a representative protoplast are shown. (D) Autoradiograph (1 and 2) and coomassie gel (3 and 4) showing the relative positions of PID, PBP2, PBMYBCT and MBP, and autophosphorylation and transphosphorylation activities of PID. Lanes 1 and 3: PID, PBP2, PBMYBCT and MBP; Lanes 2 and 4: PID, PBMYBCT and MBP. (E) In the expression data retrieved from the Arabidopsis MPSS Database, higher expression of PBMYB and PID, but not of PBP2, occurs in Arabidopsis inflorescences.

The Auxin-inducible PBP2 Binding Protein may compete with PID for PBP2 interaction

The most abundant interactor of PBP2, representing almost 50% of the positive clones identified in the screen was named Auxin-inducible PBP2 Binding Protein (APBP; Figure 4A). This protein seems to be unique to plants, as the only clear ortholog of APBP has been identified in rice (*Oryza sativa*). In Arabidopsis, the protein shows significant homology with the protein predicted by gene model At3g55690 (APBPH; Figure 4B), but this homology is confined to a stretch of 138 amino acid residues (aa 28 to 165 in APBP; Figure 4B), suggesting that this conserved region represents a functional domain. Apparently, the conserved domain is somehow important for the interaction with PBP2, since all the six sequenced yeast two-hybrid clones comprise the coding region for this part (Figure 4A). APBP and APBPH have no other conserved domain that could provide insight into their function.

Analysis of publicly available micro array data (TAIR Database) indicates that *APBP* gene expression is induced upon auxin stimulation (data not shown), and that it is strongly expressed in the shoot apex of Arabidopsis (Figure 4C) (41). Interestingly, a very similar expression pattern is observed for PID (Figure 4C) (9, 41), suggesting that the proteins are present in the same cells and that they possibly participate in the same pathway.

In vitro phosphorylation assays did not provide evidence that PID phosphorylates APBP, either in presence or absence of PBP2 (Figure 4D). *In vitro* protein pull-down assays showed that APBP interacts strongly with both full length PBP2 and its BTB domain containing part (Figure 4E). The fact that PID also interacts with the BTB domain (Figure 1D) makes it less likely that APBP and PID co-exist in the same protein complex with PBP2.

A function of APBP could be to repress the PID-PBP2 interaction, but for that, all three proteins must be present in the same subcellular compartments. Transformation of the *35S::GFP-APBP* construct into Arabidopsis protoplasts showed that GFP-APBP, like PID-GFP and GFP-PBP2, localizes to the cytoplasm (Figure 4F).

To further look into the function of APBP, we identified an Arabidopsis mutant line with a T-DNA insertion in the first exon of the corresponding gene (Figure 4A). Plants of the *apbp* mutant line develop significantly bigger leaves (both petiole and leaf blade; Figure 4G). In addition, the *apbp* mutant plants show a delay in bolting, which explains the shorter primary inflorescences at 28 days post germination (Figure 4G). The fact that the *apbp* inflorescences are longer at 46 days post germination excludes that mutant plants are defective in elongation growth. The combination of such phenotypes suggests that APBP could negatively regulate vegetative development thereby enhancing bolting, allowing the plant to enter the

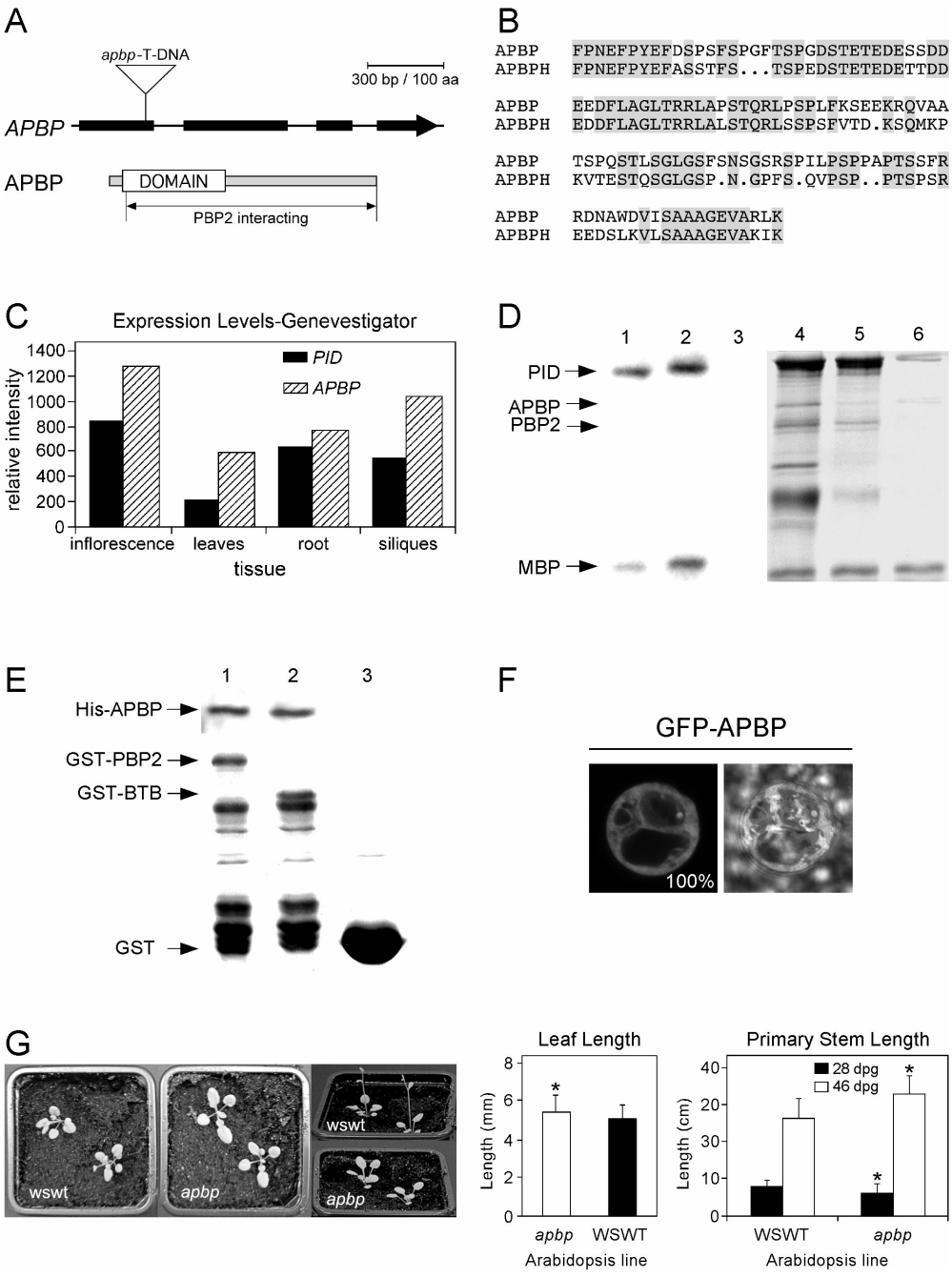
A
apbp-T-DNA
300 bp / 100 aa
APBP
APBP
DOMAIN
PBP2 interacting
B
APBP FPNEFPYEFDSPSFSPGFTSPGDSTETEDESSDD
APBPH FPNEFPYEFASSTFS...TSPEDSTETEDETTDD
APBP EEDFLAGLTRRLAPSTQRLPSPLFKSEEKRQVAA
APBPH EDDFLAGLTRRLALSTQRLSSPSFVTD.KSQMKP
APBP TSPQSTLSGLGSFSNSGSRSPILPSPPAPTSSFR
APBPH KVTESTQSGLGSP.N.GPFS.QVPSP..PTSPSR
APBP RDNAWDVISAAAGEVARLK
APBPH EEDSLKVLSAAAGEVAKIK
C
Expression Levels-Genevestigator
PID
APBP
relative intensity
1400
1200
1000
800
600
400
200
0
inflorescence
leaves
root
siliques
tissue
D
1
2
3
4
5
6
PID
APBP
PBP2
MBP
E
1
2
3
His-APBP
GST-PBP2
GST-BTB
GST
F
GFP-APBP
100%
G
wswt
apbp
apbp
wswt
Leaf Length
Length (mm)
8
6
4
2
0
apbp
WSWT
Arabidopsis line
Primary Stem Length
28 dpg
46 dpg
Length (cm)
40
30
20
10
0
WSWT
apbp
Arabidopsis line

Figure 4. APBP interacts with the BTB/POZ domain of PBP2 and it is highest expressed in inflorescences. (A) Top: *APBP* gene. Exons are shown as thick lines and the T-DNA insertion is indicated by the arrowhead. Bottom: APBP protein. APBP conserved domain as well as PBP2 interacting portion are indicated. (B) Alignment of the conserved domain shared by APBP and APBP homolog (APBPH encoded by *At3g55690*). Identical residues are shaded. (C) According to Genevestigator Database, APBP generally follows the same expression pattern as PID and is highest expressed in inflorescences. (D) Autoradiograph (1 to 3) and coomassie gel (4 to 6) showing the relative positions of PID, PBP2, APBP and MBP, and autophosphorylation and transphosphorylation activities of PID. Lanes 1 and 4: PID, PBP2, APBP and MBP; Lanes 2 and 5: PID, APBP and MBP; Lanes 3 and 6: APBP and MBP. (E) *In vitro* protein pull-down of his-tagged APBP with GST-tagged PBP2 (lane 1), GST-tagged BTB domain (lane 2) containing part of PBP2 or GST- protein (lane 3); Top: immunodetection of his-tagged APBP; Bottom: coomassie stained gel showing the different input of proteins. (F) Arabidopsis protoplasts transformed with *35S::GFP-APBP*. A fluorescence image (left) and a merged fluorescence and transmission light images (right) of a representative protoplast are shown. (G) *apbp* grow more their rosette leaves (figures left and center and graph) and initially grows shorter primary inflorescence (28 dpg; figure right and graph) which at latter stages become more elongated than WT (46 dpg; graph). Stars indicate statistically significant variations.

reproductive phase by shortening the generation time. Such a regulatory process could be triggered, for example, under conditions where nutrients or light are rate limiting.

In summary, a few interesting facts were observed for the APBP protein: APBP and PID show very similar expression patterns; APBP and PID bind the PBP2 BTB/POZ domain; APBP, PBP2 and PID localize in the cytoplasm of Arabidopsis protoplasts; APBP knock-out mutants show longer vegetative stage, indicating that APBP could be involved in promoting shorter generation time. These observations, combined with the fact that mild PID overexpressing plants show shorter generation time, lead to the speculation that APBP may possibly compete with PID for the interaction with PBP2, and as a consequence activates the kinase by relieving it from PBP2-mediated repression. This hypothesis, however, remains to be further assessed.

DISCUSSION

The function of PID has been tightly correlated with the regulation of PAT, since activity of the kinase determines the subcellular polar localization of proteins belonging to the PIN family of auxin efflux facilitators. To further elucidate the molecular mechanism behind PID-dependent basal-to-apical switch in PIN polar targeting, we studied the functional relationship between PID and PID Binding Protein 2. Our research specifically focused on this PID interactor since preliminary experiments suggested that it may be a cytoskeleton-associated protein, and cytoskeletal elements such as F-actin have been shown to be essential for the proper localization of PINs (6). Here we further characterized the interaction

between PID and PBP2, and performed a yeast two-hybrid screen using PBP2 as bait in an attempt to identify proteins that are partners of PBP2 and putative PID regulators or phosphorylation targets.

PID binding to the BTB/POZ domain portion of PBP2 represses its kinase activity

In our efforts to unravel the function of PBP2 in the PID signaling pathway, several *in vitro* assays were performed employing both proteins. *In vitro* phosphorylation experiments showed that PBP2 inhibits both the auto- and transphosphorylation activity of PID. Moreover, with *in vitr*o protein pull-down experiments we could demonstrate that PID likely binds to the BTB/POZ domain portion of PBP2. To date, an inhibitory role of BTB/POZ domain proteins has been shown only for transcriptional regulators (50-52), and our observation that PBP2 represses PID kinase activity therefore probably reveals a new functional aspect of BTB/POZ domain proteins.

Besides acting as repressors, BTB domain proteins have been shown to act as scaffolds that organize protein complexes (20, 30, 53). In this chapter we describe the identification of sixteen putative PBP2 interacting proteins, and the more detailed analysis of the nature of the interaction between PBP2 and a selection of these proteins is in line with a role of PBP2 as scaffold protein. For example, we showed that several PBP2 binding proteins interact with the C-terminal TAZ domain portion, while PID interacts with the N-terminal BTB/POZ domain portion of PBP2 (Figure 5). These results raise the possibility that the PBP2 scaffold recruits PID phosphorylation targets or connects PID with other functional structures. Such scaffold- and phosphorylation-enabling function has been described for 14-3-3 proteins, that through dimerization create two protein-protein interaction domains which facilitate phosphorylation activity of certain kinases on their specific substrates (54). However, the fact that none of the tested PBP2 interactors is a phosphorylation substrate of PID, and the observation that PBP2 represses PID kinase activity, rather suggest that the scaffold function of PBP2 is employed to regulate the activity or the subcellular localization of PID (Figure 5).

Our protoplast transformation experiments suggest that PID is predominantly localized at the PM, which is in line with its function as regulator of the polar subcellular targeting of the transmembrane PIN auxin efflux facilitators (10). In fact, this co-localization opens the possibility that PID mediates its effect through direct phosphorylation of PIN proteins. In 50% of the protoplasts, PID-GFP also shows a cytoplasmic localization, and since GFP-PBP2 is also present in the cytoplasm, it is likely that both proteins interact in this subcellular compartment. In this view, it will be interesting to test in co-transformation experiments if PBP2 - through its interaction with PID - alters the subcellular localization of this kinase.

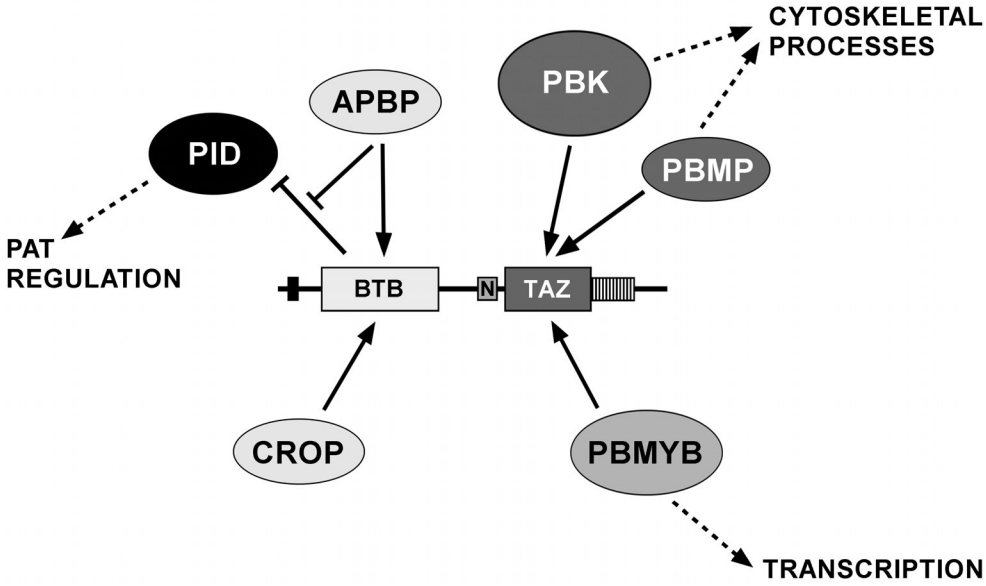

Figure 5. PBP2 is a multifunctional scaffold protein that connects PID-related and -unrelated pathways. PBP2 interacting proteins are shown for which the interaction domain in PBP2 has been mapped by *in vitro* pull down experiments (see Figures 1 to 4 and Table 2 for further details). PID kinase activity is repressed by PBP2. APBP possibly competes with PID for its interaction with the BTB domain of PBP2, thereby activating the PID kinase.

In this chapter, the inhibitory effect of PBP2 on PID activity was only demonstrated *in vitro*. Presently, crosses between mutant Arabidopsis lines with altered expression levels of *PID* or *PBP2* are being analyzed and preliminary observations confirm the inhibitory role of PBP2 on PID activity *in vivo* (Robert, unpublished data). These data combined with *in vivo* protein pull downs will more conclusively address the functional relationship between PID, PBP2 and the PBP2 interacting proteins.

PBP2 as a multi-functional scaffold protein

BTB/POZ domain proteins are known to interact with a wide diversity of proteins (55). In line with these earlier observations, our yeast two-hybrid screen has identified a wide range of PBP2 interacting proteins that can be roughly classified into three classes: cytoskeletal proteins, transcriptional regulators and proteins with enzymatic activity.

Several reports describe the interaction of BTB proteins with the cytoskeleton in various organisms and correlate this interaction with a change in stability and dynamics of F-actins (55). Accordingly, it has been speculated that the PBP2-

related *Caenorhabditis elegans* MEL26 protein promotes cytokinesis by reducing the activity of the cytoskeletal protein POD-1, possibly by blocking the F-actin cross-linking capability of POD-1 (56). In spite of that, from the apparent functions of most of the cytoskeletal partners of PBP2, it is only possible to hypothesize that in Arabidopsis cells PBP2 is involved in microtubular trafficking. Whether PBP2 reduces the activity of its cytoskeletal interactors in a similar way as MEL26 does towards POD-1 remains to be investigated.

The interaction between BTB proteins and transcription factors has also been well documented. It has been consistently shown, for example, that BTB/POZ and C2H2 zinc finger domains containing proteins mediate transcriptional repression (50-52). Therefore, it is inevitable to speculate that the function of PBP2 towards its transcription factor partners is to repress them. The role of PBP2 in transcription regulation is not contradictory with its previously observed cytoskeletal localization. In fact, such dual cytoplasmic-nuclear localization of PBP2 is supported by its potential partner KNAT1, whose rice ortholog KNOX1 has been found to be present in both cellular compartments (47, 57). Considering the well established fact that regulation of transcription factor activity can be performed by their nuclear uptake, it is plausible that PBP2 acts as transcription factor transporter. Alternatively, PBP2 could perform different functions in the cytoplasm and in the nucleus. The actual role of PBP2 in transcription regulation has to be studied in further detail.

Not much is known about the relationship between BTB proteins and catalytic enzymes. The most informative example consists in the demonstration that isoforms of the mouse BTB/POZ protein NAC1 recruit histone deacetylases for transcriptional repression (58). The role of PBP2 towards its enzymatic interactors is yet to be determined.

As discussed above, our recent findings on the function of PID (10) could explain a hypothetical relationship between PID and cytoskeletal activity. Our observations that PBP2 interacts with putative cytoskeletal proteins may indicate that PBP2 represents the actual link between the PID and the cytoskeleton. However, none of the PBP2 binding proteins described in this chapter could unequivocally be related to PID activity, making this connection unclear. The observation that several proteins that are likely to play a role in unrelated processes interact with PBP2, leads us to conclude that PBP2 is a multi-functional scaffold protein that connects different networks and participates in both PID-related and -unrelated signaling pathways. This view is again supported by the extremely well described case of the 14-3-3 scaffold proteins, which are known to play a role in a large variety of processes such as the regulation of ATP production, DNA conformation and cell cycle (59, 60). Which PBP2 interactors are truly part of the PID signaling complex remains to be addressed.

APBP could possibly be an activator of the PID protein kinase

The most frequent interactor of PBP2, the APBP protein, does not classify to any of the pre-defined groups of PBP2 partners. APBP does not contain any acknowledged domain or signal-peptide, and it is therefore impossible to assign a clear cellular function to this protein. Publicly available microarray data, combined with *in vitro* and *in vivo* data provided in this chapter tend to favor the hypothesis that APBP competes with PID for binding the BTB domain of PBP2. By competing with PID, APBP could release PBP2-induced PID inhibition, thereby activating the kinase. The fact that both APBP and PID have the same expression profile may indicate tight feed-back of PID activity control. Crosses between mutants and overexpression lines and *in vitro* phosphorylation assays using titrated quantities of PBP2, APBP and PID could help to clarify this model.

PBP2 does not seem to interact with calmodulins or CUL3

Previously, a calmodulin, two fsh/Ring class transcription factors and CULLIN3 have been reported as interactors of PBP2 (20, 21). Curiously, none of these proteins were identified in our screen, nor did we identify PID itself as an interactor of PBP2. The fact that these putative PBP2 interactors were not identified may be explained by the fact that the root-specific cDNA library that was used for our screen insufficiently represented the indicated proteins. For some of the putative PBP2 interactors there are however alternative explanations why they were not represented by the positive clones.

The absence of PID among the PBP2IPs may be explained by two previous observations: i) yeast two-hybrid interaction tests using the GAL4AD-PID fusion always resulted in poor growth on selective medium (data not shown), indicating that PID is relatively toxic to yeast when fused to the GAL4 activation domain; ii) when PID (bait) and PBP2 (target) were transformed to yeast, optimal growth was never reached upon selective pressure for the interaction, suggesting that PID and PBP2 bind weakly in this system, or that PID as bait is also mildly toxic. As shown in this chapter, the *in vitro* interaction between PID and PBP2 is strong and stable, corroborating our previous findings that these two proteins do interact (11).

The binding of PBP2 to a calmodulin was identified in a screen that used an Arabidopsis library as target and potato Calmodulin 6 (Cam6) as bait. The interaction between PBP2 and the Arabidopsis ortholog of Cam6 was never tested, indicating that the significance of this interaction in Arabidopsis remains to be addressed.

In several organisms BTB domain proteins have been demonstrated to interact with CULLIN3 (CUL3). The BTB/CUL3 complex forms a new class of E3 ligases, in which the BTB domain protein acts as the SCF E3 ligase SKP1/F-BOX protein sub-complex, by interacting with CUL3 through its BTB domain, while recruiting

ubiquitination and proteolysis targets through its second domain (19, 20, 53, 61, 62). Not many BTB protein ubiquitination targets have been identified, but in *Caenorhabditis elegans* it has been shown that during the meiosis-to-mitosis transition the microtubule-severing protein MEI-1/katanin is recruited for degradation by the BTB protein MEL26 (19, 62). Recently, a report suggested that also PBP2 interacts with CUL3 in *in vitro* pull down assays (20). However, neither CUL3 nor its homologs were found in the yeast two-hybrid screen described in this chapter. Our observations are corroborated by the work of Gingerich and co-workers (63) and Dieterle and co-workers (15), who showed that the PBP2 class of BTB domain proteins does not interact with CUL3 to participate in proteolysis. Such finding, combined with our own results, assign a debatable character to the conclusion that PBP2 is part of the CUL3-containing E3 ubiquitin ligase complex.

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