PINOID-Mediated Signaling Involves Calcium-Binding Proteins

René Benjamins1, Carlos S. Galván Ampudia, Paul J.J. Hooykaas, and Remko Offringa*


The plant hormone auxin is a central regulator of plant development. In Arabidopsis, the PINOID (PID) protein serine/threonine kinase is a key component in the signaling of this phytohormone. To further investigate the biological function of PID, we performed a screen for PID-interacting proteins using the yeast two-hybrid system. Here, we show that PID interacts with two calcium-binding proteins: TOUCH3 (TCH3), a calmodulin-related protein, and PID-BINDING PROTEIN 1 (PBP1), a previously uncharacterized protein containing putative EF-hand calcium-binding motifs. The interaction between PID and the calcium-binding proteins is significant because it is calcium dependent and requires an intact PID protein. Furthermore, the expression of all three genes (PID, TCH3, and PBP1) is up-regulated by auxin. TCH3 and PBP1 are not targets for phosphorylation by PID, suggesting that these proteins act upstream of PID. PBP1 was found to stimulate the autophosphorylation activity of PID, and calcium influx and calmodulin inhibitors were found to enhance the activity of PID in vivo. Our results indicate that TCH3 and PBP1 interact with the PID protein kinase and regulate the activity of this protein in response to changes in calcium levels. This work provides the first molecular evidence for the involvement of calcium in auxin-regulated plant development.

The plant hormone auxin plays a central role in plant growth and development and has therefore been the subject of study for more than seven decades. Auxin is unique among the plant hormones in that it is actively transported in a polar fashion from its sites of biosynthesis. Polar auxin transport has been generally recognized as a major determinant underlying the action of this hormone, as shown by its involvement in developmental processes such as vascular differentiation and tropic growth (Luschnig et al., 1998; Mattson et al., 1999; Rashotte et al., 2000). The pin formed 1 (pin1) mutant of Arabidopsis is defective in polar auxin transport and develops pin-like inflorescences (Okada et al., 1991; Gälweiler et al., 1998). The PIN1 gene is part of a small gene family that encodes transporter-like membrane proteins. In accordance with their proposed function as efflux carriers in polar auxin transport, the cellular localization of these proteins was shown to be polar (Gälweiler et al., 1998; Müller et al., 1998). Recently, cycling of PIN-containing vesicles from endosomal compartments to the plasma membrane along the actin cytoskeleton was found to underlie the polar localization of PIN proteins (Geldner et al., 2001).

Loss-of-function pinoid (pid) mutants phenocopy pin mutants (Bennett et al., 1995), and phenotypic changes caused by ectopic expression of the PID protein kinase (Christensen et al., 2000) can be partially rescued by application of polar auxin transport inhibitors (Benjamins et al., 2001). Based on these observations, we proposed that PID is a positive regulator of polar auxin transport, although some aspects of PID activity can also be explained by feedback regulation on auxin signaling (Christensen et al., 2000; Benjamins et al., 2001). As protein kinases are signal transduction components per se, we refer to PID as a component in auxin signaling.

In 1973, dela Fuente and Leopold (dela Fuente and Leopold, 1973) suggested a role for calcium in the regulation of polar auxin transport. More than a decade later, Hasenstein et al. (1986) showed that calcium induces a transient inhibition of root elongation in a manner similar to treatment with low concentrations of auxin. The authors suggested that auxin action on root growth is mediated by an auxin-induced increase in the level of cytosolic free calcium ([Ca2+]cyt), which in turn induces growth responses. This hypothesis was verified in a later study in which auxin was shown to induce an increase in [Ca2+]cyt within minutes after its application (Gehring et al., 1990). Evidence for the role of calcium in polar auxin transport came from gravistimulation studies. After gravistimulation, [Ca2+]cyt peaks were found to coincide with the basipetal movement of auxin at the lower side of the root from the root tip toward the elongation zone (Lee et al., 1984). Moreover, roots were found to curve toward a calcium-containing agar block and away from a block containing the calcium-chelating agent EGTA (Lee et al., 1983). These observations indicate that the variations in [Ca2+]cyt during root gravitropic response are cou...
pled to the direction of auxin transport, and they suggest that auxin transport is directed by local increases in [Ca\(^{2+}\)]\(_{\text{cyt}}\), or that [Ca\(^{2+}\)]\(_{\text{cyt}}\) peaks are induced by increased auxin levels in cells at the lower side of the root tip. Recently, Plieth and Trewavas (2002) used transgenic seedlings expressing aequorin to show that [Ca\(^{2+}\)]\(_{\text{cyt}}\) is transiently increased in roots upon gravistimulation. However, evidence against a role for calcium in gravitropism has also been reported (Legue et al., 1997). This underlines the complications that are encountered in determining the exact role of calcium in a auxin-regulated processes, because calcium is involved in a large number of other cellular processes, such as ethylene action (Lau et al., 1977), stomatal opening (Wood et al., 2000), vesicle aggregation (dela Fuente and Parra, 1995), and plant defense (Lecourieux et al., 2002).

Here, we describe the interaction of PID with two different calcium-binding proteins (CBPs), one of which is TOUCH3 (TCH3), a calmodulin-related protein involved in touch response (Braam and Davis, 1990) and the other being PID-BINDING PROTEIN 1 (PBP1), which contains putative EF-hand calcium-binding motifs. Our data show that these interactions are specific and calcium dependent, thereby indicating a role for calcium in the regulation of PID activity.

RESULTS

PID Interacts with CBPs

The yeast two-hybrid system was used to screen two Arabidopsis cDNA libraries for proteins that interact with the PID protein Ser/Thr kinase. Three independent transformation experiments, each yielding a saturating number of transformants, identified 25 positive clones that did not show autoactivation after retransformation with the empty pAS2-1 vector. These 25 positive clones represent three different genes. Here, we present the analysis of two of these genes, which encode CBPs (Table I). One of the CBP genes, TCH3 (At2g41100), was identified previously by Braam and Davis (1990) in a screen for genes that are up-regulated in response to mechanical stimuli such as wind and touch. TCH3 (324 amino acids) is designated a calmodulin-related protein because it has six EF-hand calcium-binding sites in contrast to the four EF-hand calcium-binding sites that are normally found in calmodulins (Braam, 1992). The second gene we identified, PINOID BINDING PROTEIN 1 (PBP1, At5g54490), encodes a previously uncharacterized protein of 127 amino acids with three possible EF-hand calcium-binding motifs. The sequence of two of these EF-hands does not completely match the canonical EF-hand consensus sequence, suggesting that these calcium-binding motifs may not be functional (Fig. 1). However, EF-hands are often found to work in pairs, and the function of the nonperfect EF-hands can be enhanced through the cooperative action of the perfect EF hand, resulting in high-affinity binding of calcium (Ikura, 1996).

In the plating assays (data not shown) and the \(\beta\)-galactosidase activity assay, the interaction between PBP1 and PID was stronger than the interaction between TCH3 and PID (Fig. 2B). To determine the specificity of the interaction, two mutations were introduced into the PID coding region in the pAS-PID vector. One mutation results in a small deletion of amino acids 69 to 82, whereas the other mutation deletes most of the catalytic domain of the PID protein kinase (Fig. 2A, amino acids 82–283). Both deletions significantly reduced the interaction between PID and PBP1 or TCH3 (Fig. 2B). The deletions in PID cannot be used to map the interaction sites because they may affect the general protein structure of PID and lead to conformational changes that make binding of the interactors less efficient. However, the results do imply specificity of interaction between the protein kinase and the CBPs.

## Table I. Interactors of PID identified by yeast two-hybrid screening

<table>
<thead>
<tr>
<th>Interactor</th>
<th>Two-Hybrid cDNA Librarya</th>
<th>Green parts</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCH3</td>
<td>2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PBP1</td>
<td>2</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* See “Materials and Methods.”

Figure 1. Protein sequence and calcium-binding motifs of PBP1. A, Protein sequence of PBP1. Putative EF-hands (underlined) were identified by scanning multiple databases (http://www.expasy.ch). B, Alignment of EF-hands present in PBP1 with the consensus sequence for this structural feature (Marsden et al., 1990).
Interaction of TCH3 and PBP1 with PID Is Calcium Dependent

To confirm the interactions detected in the yeast two-hybrid system, we performed in vitro pull-down assays with glutathione S-transferase (GST):PID. In these experiments, His-tagged versions of TCH3 and PBP1 interacted specifically with PID; even both proteins were present in the same extract (Fig. 3). The stronger interaction between PBP1 and PID observed in the yeast two-hybrid system was also observed in the in vitro pull-down assays with GST:PID. However, the stronger signal observed in the pull-down assays is partly due to the fact that the production of His-tagged PBP1 in E. coli is more efficient than that of the His-tagged TCH3 protein.

When the 10 mM calcium chloride in the binding buffer was replaced by 10 mM EGTA or 10 mM magnesium chloride, no HIS:TCH3 signal was detected with the anti-HIS antibody on the resulting western blot (Fig. 4). For PBP1, the binding to GST:PID was significantly reduced in the absence of calcium, but was not completely abolished (Fig. 4). These findings indicate that TCH3 binding to PID is calcium dependent, whereas the interaction between PID and PBP1 is enhanced by calcium.

**PID, TCH3, and PBP1 Are Auxin-Regulated Genes**

One criterion for a possible functional interaction between two proteins is that they colocalize in the same cells and/or tissues. TCH3 was previously...
shown to accumulate in cells or tissues that are exposed to mechanical strain, such as attachment points of secondary and cauline leaves to the stem, but the protein can also be found in xylem cells (Antosiewicz et al., 1995). The TCH3 transcript level was shown to be up-regulated by light, heat shock, and auxin treatment (Braam and Davis, 1990; Braam, 1992; Sistrunk et al., 1994; Antosiewicz et al., 1995). Expression analysis of the TCH3 gene confirmed that the gene is auxin inducible and is expressed throughout the plant (Fig. 5; Antosiewicz et al., 1995). PID transcription levels are also up-regulated by external auxin application, and the expression pattern of PID seems to overlap with that of TCH3 in the vasculature of the root and in flower buds (Antosiewicz et al., 1995; Benjamins et al., 2001). The highest levels of TCH3 expression were observed in flower buds where the PID mRNA is also most abundant. Interestingly, TCH3 expression was strongly up-regulated in seedlings of the pid::En310 loss-of-function mutant, and the auxin inducibility of TCH3 was slightly reduced in the 35S::PID overexpression background. These results suggest that in seedlings, there is feedback regulation on TCH3 expression by PID.

RNA-blot analysis showed that the PBP1 transcript was not detectable in wild-type tissues, and was not detectable in the 35S::PID gain-of-function and pid loss-of-function mutants. PBP1 mRNA was only weakly detectable in roots of Arabidopsis seedlings after IAA treatment (Fig. 5A). RT-PCR analysis again detected the highest PBP1 expression in auxin-treated seedling roots, but a slight increase in expression was also detectable in seedling shoots after auxin treatment (Fig. 5B). PBP1 expression was unchanged in 35S::PID and pid mutant backgrounds, implicating that PBP1 expression is not dependent on PID. These results indicate that PBP1 expression, as with PID and TCH3 expression, is responsive to auxin.

TCH3 and PBP1 Are Not Phosphorylation Targets of PID

In vitro kinase assays using GST:PID and His-tagged TCH3 and PBP1 were performed to determine whether TCH3 and PBP1 are targets for phosphorylation by PID. Christensen et al. (2000) previously demonstrated that a GST:PID fusion protein shows autophosphorylation activity in vitro. However, the trans-phosphorylation capacity of PID has not yet been demonstrated. Therefore, we began by testing the capacity of PID for trans-phosphorylation using myelin basic protein (MBP) as an artificial substrate. GST:PID phosphorylates MBP, showing that the GST:PID protein is also active as a protein kinase (Fig. 6). Next, we determined whether PID phosphorylates TCH3 and PBP1 by coincubating GST:PID with the His-tagged versions of TCH3 or PBP1 in the presence of calcium. GST:PID did not phosphorylate TCH3 or PBP1, whereas autophosphorylation of GST:PID was still detectable. Interestingly, autophosphorylation of GST:PID was reproducibly enhanced in the presence of PBP1 (Fig. 6). Therefore, our findings suggest that TCH3 and PBP1 are not direct downstream targets of PID phosphorylation. Moreover, the increase in autophosphorylation of GST:PID in the presence of PBP1 suggests that this CBP is an upstream regulator of PID activity.
Calcium Negatively Regulates PID Activity in Vivo

Seedlings of 35S::PID lines show agravitropic growth of the hypocotyl and the root, and a few days after germination, the primary root tip loses its meristematic identity and eventually collapses (Benjamin et al., 2001). If calcium acts as a second messenger in PID signaling in vivo, then it would be expected that the effects of PID overexpression are influenced by inhibitors of the calcium signaling pathway. Therefore, we used the 35S::PID root collapse phenotype as a bioassay to monitor the effect of such inhibitors on PID activity.

Wild-type seeds, and seeds of two overexpression lines, 35S::PID#1 and 35S::PID#21, were germinated on medium with the calmodulin-inhibitor tetracain (TC) and the calcium channel blockers, GdCl₃ and LaCl₃, and 3 d after germination, the number of seedlings with collapsed roots was counted. Germination of 35S::PID seeds on medium containing 0.1 mM TC or different concentrations of the calcium channel blockers LaCl₃ and GdCl₃ (0.10–0.25 mM) significantly increased the percentage of collapsed root tips (Fig. 7). The concentrations used did not inhibit growth of wild-type (Colombia [Col-0]) seedlings (data not shown), whereas higher concentrations of TC (>0.25 mM) or of the calcium channel blockers (0.50 mM or higher) did inhibit wild-type root growth. Germination on medium lacking calcium could not be tested because this severely affects the growth rate of roots of (wild-type) seedlings. These results indicate that the application of calcium signaling inhibitors can, in a narrow concentration window, significantly enhance the effect of PID activity in the 35S::PID background (t-test: P > 0.05). The data imply that PID activity is negatively regulated by calcium and/or calmodulins. This function may be performed by one of the PID-interacting CBPs. However, we cannot exclude that the compounds used here interfere with pathways that act in parallel with PID signaling, and, therefore, we consider these data as a first indication of the functionality of the PID-CBP interaction. Future research, including in vivo pull-down experiments and double mutant studies, will further confirm the role of the CBPs in PID action.

Figure 6. Phosphorylation activity of GST:PID. A, Autophosphorylation of GST:PID (first lane), and phosphorylation of MBP (second lane). No phosphorylation of PBP1 or TCH3 was observed (third and fourth lanes, respectively). B, Parallel nonlabeled protein samples were run on a gel and were checked for the presence of the two His-tagged-interacting proteins by western blotting using anti-His antibodies. C and D, A second protein gel stained with Coomassie Blue detects MBP (C) and shows equal loading of the GST:PID fusion protein (D).

Figure 7. Effect of calcium influx and calmodulin inhibitors on root tips of two 35S::PID lines (5). Seedlings of 35S::PID lines 1 and 21 were grown on normal MA medium and MA medium containing 0.1 mM TC (calmodulin inhibitor), 0.25 mM GdCl₃, or 0.25 mM LaCl₃ (calcium influx inhibitors). The timing of collapse of the primary root meristem was determined by counting the number of seedlings with collapsed root meristems 3 d after germination. The experiments were performed three times for line 1 and two times for line 21. The averages of the experiments are shown ± the se of the mean.
DISCUSSION

The protein Ser/Thr kinase PID was previously shown to be a component in auxin signaling (Christensen et al., 2000; Benjamins et al., 2001). To obtain more insight into the signaling pathways that involve PID, we characterized proteins that interact with PID in a yeast two-hybrid system.

Two of the proteins identified as PID interactors are CBPs, one of which is the calmodulin-related protein encoded by the touch-responsive gene TCH3 (Braam and Davis, 1990). The other protein, PBP1, contains three putative EF-hand calcium-binding sites. The significance of the interaction of these CBPs with PID was supported by the repeated identification of cDNAs corresponding to these CBPs in the two-hybrid screens, by the calcium dependency of the interactions in vitro pull-down assays, and by the fact that the interaction in the yeast two-hybrid system only occurred with the intact version of the PID protein. Moreover, PID, TCH3, and PBP1 are all up-regulated by treatment with auxin and are expressed in overlapping tissues, indicating that the spatial and temporal distribution of the proteins in the plant could permit the interaction to occur in vivo.

PID belongs to the plant-specific ACG group VIII family of protein Ser/Thr kinases (Christensen et al., 2000; Benjamins et al., 2001), of which only a few members have been studied in detail. Two of these ACG group VIII kinases, NPH1/PHOT1 and NPL1/PHOT2 act as UV-A/blue light receptors in directing phototropic growth and chloroplast movement, respectively (Christie et al., 1998; Jarillo et al., 2001). Baum et al. (1999) showed that photoactivation of NPH1/PHOT1 induces a rapid transient increase in [Ca$^{2+}]_{cyt}$. This observation and the interaction of PID with two CBPs make it tempting to speculate that PID activity may also induce a rapid transient increase in [Ca$^{2+}]_{cyt}$. Auxin treatment is known to induce an elevation of [Ca$^{2+}]_{cyt}$ (Gehring et al., 1990), and PID could be involved in inducing this initial calcium peak. However, the relatively slow increase in PID mRNA after auxin treatment (Benjamins et al., 2001) suggests that this calcium peak can only occur in cells where PID is already present, and that the PID proteins that are produced in response to auxin then cause a second wave of increased [Ca$^{2+}]_{cyt}$.

Based on the in vitro phosphorylation assays, we conclude that TCH3 and PBP1 are not downstream targets of phosphorylation by PID, but rather, they act as upstream regulators of PID activity. TCH3, PBP1, and PID are auxin-responsive genes, and although we do not know the exact timing of auxin-induced TCH3 and PBP1 expression, this observation suggests that the respective gene products are present in cells with relatively high auxin levels. PBP1 is expressed at a low level, but its interaction with PID is relatively strong, even in the absence of calcium. PBP1 appears to enhance the autophosphorylating activity of PID in vitro. These results suggest that PBP1 acts as a cofactor to positively regulate PID activity in specific tissues; however, it is not known if autophosphorylation activates the PID protein kinase. TCH3 is expressed at a much higher level than PBP1. The observation that the TCH3-PID interaction is completely dependent on the presence of calcium, and that a calmodulin inhibitor enhances PID activity, suggests that TCH3 negatively regulates PID activity. TCH3 expression is elevated in the pid loss-of-function background, implying that in seedlings, PID regulates its own activity through feedback control of TCH3 expression.

Our observations suggest a fine-tuned mechanism underlying the control of PID activity, including transcriptional control by auxin of the three genes (PID, PBP1, and TCH3), feedback regulation, autophosphorylation, and the interference of the two CBPs with the activity of the kinase. The identification of TCH3 as a PID-interacting protein also suggests a mechanism linking touch responses and calcium to auxin transport. TCH3 is proposed to be involved in tissue reinforcement and cell expansion through interference with vesicular transport (Braam et al., 1997). Considering the involvement of vesicular trafficking along actin filaments in the localization of auxin efflux carriers, this could indicate a role for TCH3 and PID in this process and thereby in auxin transport (Geldner et al., 2001).

Calcium and auxin have been proposed as regulators of the same cellular processes, including the establishment of cell polarity, growth, and vesicular transport along actin filaments and secretion (dela Fuente and Parra, 1995; Chen et al., 1999; Hepler et al., 1997). Considering the involvement of vesicular trafficking along actin filaments in the localization of auxin efflux carriers, this could indicate a role for TCH3 and PID in this process and thereby in auxin transport (Geldner et al., 2001).

Calcium peaks during root gravitropism were suggested. Later, Evans and coworkers (Evans et al., 1992; Young and Evans, 1996) found evidence for the involvement of calcium in gravistimulated movement of auxin in root tips. Although this stimulation can be indirect, it indicates that there is a link between changes in calcium peaks and auxin transport. The authors suggest that gravity-induced calcium asymmetry is important in establishing auxin asymmetry during the root gravitropic response. Other reports corroborate the long-thought crosstalk between auxin and calcium signaling. For example, Yang and Poovaiah (2000) identified a small auxin up-regulated RNA protein as a calmodulin-binding protein, whereas Okamoto et al. (1995) described the isolation of an auxin-regulated calmodulin gene from mung bean (Vigna radiata).
In conclusion, the identification of the PID-interacting proteins TCH3 and PBP1 has brought us closer to the dissection of the biological function of this protein kinase in auxin-mediated plant growth and development. The fact that PID, a component in auxin signaling, exhibits calcium-dependent interactions with CBPs reveals for the first time to our knowledge how the auxin response may be coupled to the second messenger calcium. Further functional and expression analysis of TCH3 and PBP1 is needed to determine the dynamics of the interaction with PID and the exact role of these interactors in the PID signaling pathway.

MATERIALS AND METHODS

Two-Hybrid Interaction

The Matchmaker yeast two-hybrid system (Clontech, Palo Alto, CA) was used to screen two Arabidopsis cDNA libraries fused to the GAL4-activation domain (pACT) with a PID::GAL4-DNA-binding domain (pAS2-1) fusion. One cDNA library was constructed using mRNA isolated from green parts of 6-week-old flowering Arabidopsis plants. The second cDNA library was constructed using a 1:1 ratio of mRNA from auxin-treated (1 μM 1-naphthaleneacetic acid for 24 h) and wild-type roots of 10-d-old Arabidopsis seedlings. The yeast strain Pj69-4a (James et al., 1996) containing pAS2-1-PID was transformed with each of the pACT cDNA libraries (Gietz et al., 1992), and was screened for His and adenine auxotrophy. Growth rates of transformants were compared with controls (the wild-type strain and the wild-type strain containing only pAS2-1-PID). When screened for adenine auxotrophy, reddening of the colonies was used as an indicator of the strength of interaction. Screens were performed at 20°C and 30°C. α-Galactosidase assays in liquid yeast cultures were performed as described (Meier et al., 2000).

RNA Purification, Northern-Blot, and RT-PCR Analysis

Total RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) or using the method described by Jakkola et al. (2001). Northern-blot analysis was performed using approximately 10 μg of RNA per sample as previously described (Memelink et al., 1994). For RT-PCR purposes poly-A RNA was isolated from tissues of 7-d-old Arabidopsis (Col-0) seedlings using the QuickPick mRNA isolation kit described (Memelink et al., 1994).

ACKNOWLEDGMENTS

We thank Johan Memelink and Bert van der Zaal for providing the green part and the auxin-induced root-specific two-hybrid cDNA libraries, respectively, Peter Hock and Tobias Sieberer for help with the figures and RT-PCR analysis, respectively, Dolf Weijers and Haico van Attikum for valuable discussions, and Christian Luschnig and Kim Boutilier for valuable comments on the manuscript.

Received January 6, 2003; returned for revision February 4, 2003; accepted April 4, 2003.

LITERATURE CITED


Plant Physiol. Vol. 132, 2003 Copyright © 2003 American Society of Plant Biologists. All rights reserved.