IBR5, a Dual-Specificity Phosphatase-Like Protein Modulating Auxin and Abscisic Acid Responsiveness in Arabidopsis

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Auxin is an important plant hormone that plays significant roles in plant growth and development. Although numerous auxin-response mutants have been identified, auxin signal transduction pathways remain to be fully elucidated. We isolated ibr5 as an Arabidopsis indole-3-butyric acid–response mutant, but it also is less responsive to indole-3-acetic acid, synthetic auxins, auxin transport inhibitors, and the phytohormone abscisic acid. Like certain other auxin-response mutants, ibr5 has a long root and a short hypocotyl when grown in the light. In addition, ibr5 displays aberrant vascular patterning, increased leaf serration, and reduced accumulation of an auxin-inducible reporter. We used positional information to determine that the gene defective in ibr5 encodes an apparent dual-specificity phosphatase. Using immunoblot and promoter-reporter gene analyses, we found that IBR5 is expressed throughout the plant. The identification of IBR5 relatives in other flowering plants suggests that IBR5 function is conserved throughout angiosperms. Our results suggest that IBR5 is a phosphatase that modulates phytohormone signal transduction and support a link between auxin and abscisic acid signaling pathways.

INTRODUCTION

Auxins are an essential class of phytohormones that influence many aspects of plant growth and development. At the molecular level, auxins influence cell division, cell elongation, and cell differentiation (Davies, 1995). At the macroscopic level, auxins direct vascular development, promote apical dominance and lateral root formation, and mediate gravitropism and phototropism (Davies, 1995). Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are two endogenous auxins that can be interconverted (reviewed by Ludwig-Müller, 2000; Bartel et al., 2001).

Many auxin responses are mediated by changes in gene expression, and some of these are controlled by transcription from Auxin-Response Elements (AuxREs). Auxin-Response Factors (ARFs) were isolated initially because they bind AuxREs (Ulmasov et al., 1997a); they can either activate or repress transcription (Ulmasov et al., 1999). Aux/IAA proteins can heterodimerize with ARFs and repress the ability of activating ARFs to promote transcription (Ulmasov et al., 1997b; Tiwari et al., 2001). Increased auxin levels enhance the degradation rate of Aux/IAA proteins via ubiquitin-mediated degradation, allowing activating ARFs to increase the transcription of auxin-responsive genes (reviewed by Rogg and Bartel, 2001; Kepinski and Leyser, 2002).

Genetic analysis of auxin-response mutants in Arabidopsis has led to the identification of proteins necessary for IAA influx and efflux (reviewed by Muday and DeLong, 2001), several ARFs and Aux/IAA proteins (reviewed by Liscum and Reed, 2002), and proteins that mediate and regulate ubiquitin-dependent proteolysis (reviewed by Rogg and Bartel, 2001; Kepinski and Leyser, 2002). Moreover, biochemical experiments have revealed that the association of Aux/IAA proteins with a ubiquitin-protein ligase can be stimulated by auxin (Gray et al., 2001). Although it is likely that this binding leads to Aux/IAA protein degradation in vivo, the molecular signaling cascade by which auxin promotes Aux/IAA protein binding to the ubiquitin protein ligase is not understood, and the regulation of the components downstream of these initial events is only beginning to be elucidated (Xie et al., 2000, 2002).

Abscisic acid (ABA) is another phytohormone that affects many aspects of growth and development, including shoot and root growth, stomatal closure, storage protein synthesis, and seed dormancy (Davies, 1995). Gaps remain in our understanding of ABA signaling, although the transcription factors necessary for ABA sensitivity and the phosphatases that modulate ABA responses have been identified (reviewed by Finkelstein et al., 2002). Like auxin signaling, ABA responses depend on regulated ubiquitin-dependent proteolysis of a key transcription factor (Lopez-Molina et al., 2001, 2003). Analysis of Arabidopsis mutants has revealed interactions between ABA and other signaling pathways, including those for sugar, ethylene, and auxin (reviewed by Fedoroff, 2002; Gazzarrini and McCourt, 2003). Indeed, an emerging theme of phytohormone responses is that many such responses cannot be reduced to simple linear pathways that connect inputs and outputs but may more closely resemble interaction webs (Moller and Chua, 1999; Gazzarrini and McCourt, 2003).

Mutants that respond aberrantly to multiple phytohormones may be used to elucidate the connections between interacting phytohormone-response pathways. Here, we report the characterization of ibr5, an Arabidopsis mutant defective in specific auxin and ABA responses. ibr5 is less sensitive to inhibitory
concentrations of exogenous auxins and ABA and appears to be less sensitive to endogenous auxin. \textit{IBR5} encodes a 257–amino acid protein with \textasciitilde{}35\% identity to known dual-specificity mitogen-activated protein kinase (MAPK) phosphatases. Dual-specificity phosphatases often dephosphorylate signaling components; therefore, \textit{IBR5} may modulate auxin and ABA signal transduction pathways.

\section*{RESULTS}

\textbf{ibr5 Displays Attenuated Responses to Auxin and ABA}

In an attempt to isolate auxin signaling components, we performed a screen for mutants resistant to the inhibitory effects of exogenous auxin on root elongation, focusing on mutants with slightly weaker auxin phenotypes than seen in previously isolated auxin-response mutants. \textit{ibr5-1} was isolated from a pool of ethyl methanesulfonate–mutagenized seeds as an \textit{IBA}-response mutant (Zolman et al., 2000). Analysis of \textit{ibr5} after backcrossing revealed general auxin resistance. For example, \textit{ibr5} was less sensitive than the wild type to primary root elongation inhibition caused by the natural auxins IAA and IBA (Figure 1A); the synthetic auxins 2,4-D (Figure 1A), 2,4-dichloro-phenoxybutyric acid, and 1-naphthaleneacetic acid; and auxin transport inhibitors, including 1-naphthylphthalamic acid and 2,3,5-triiodobenzoic acid (data not shown). A strong allele of the auxin-response mutant \textit{axr1} (Estelle and Somerville, 1987; Lincoln et al., 1990) generally was more resistant than \textit{ibr5} to the root elongation inhibition caused by these auxins (Figure 1A) and by auxin transport inhibitors (data not shown), consistent with the idea that \textit{ibr5} is a weak auxin-response mutant. The \textit{ibr5} defects were recessive (data not shown), suggesting that the mutant lesion results in a loss of function.

To examine the specificity of \textit{ibr5} auxin-response defects, we examined root elongation inhibition by other phytohormones. \textit{ibr5} showed decreased sensitivity to ABA (Figure 1B) but displayed wild-type sensitivity to the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid, the cytokinin 6-benzylaminopurine, the brassinosteroid epibrassinolide, the growth hormone nocyclopropane-1-carboxylic acid, the cytokinin 6-benzylamino-1-glucuronidase (Guilfoyle, 1999). Analysis of \textit{ibr5} DR5-GUS lines grown on unsupplemented medium revealed decreased GUS expression in the shoot apex, root tips, hydathodes, true leaves (Figure 3D), and sepals (data not shown), suggesting that \textit{IBR5} is necessary for full responsiveness to endogenous auxin.

In an attempt to identify specific genes misregulated in the \textit{ibr5} background, which might include targets of \textit{IBR5}-modulated signaling, we performed whole-genome microarray analysis using RNA prepared from 7-day-old \textit{ibr5-1} and wild-type seedlings. Comparison of the results from three independent preparations each of \textit{ibr5-1} and wild-type RNA revealed no significant (\textasciitilde{}2.5-fold) and reproducible alterations in mRNA accumulation of the \textasciitilde{}22,000 genes analyzed (data not shown). Therefore, at 7 days, any gene expression changes in \textit{ibr5-1} seedlings may be local, as seen with the \textit{DR5}-GUS reporter (Figure 3D), and not apparent in whole seedling RNA.

\textbf{IBR5 Encodes a Putative Dual-Specificity Phosphatase}

We identified the \textit{IBR5} gene using map-based positional cloning (see Methods). \textit{ibr5-1} was mapped to the top of chromosome 2, between the markers \textit{AIR3} and \textit{F5G3} (Zolman et al., 2000). We refined the \textit{ibr5-1} position to a 41–kb region containing most of BAC T103 and one end of BAC F7D11 (Figure 4A). We then used a candidate gene approach to identify the mutant gene. We sequenced a putative phosphatase (At2g04550) within this region and found a C-to-T mutation in the first exon, changing a Gin at position 42 to a premature stop codon in the \textit{ibr5-1} mutant and destroying an AcI restriction site (Figure 4C).

To confirm that this gene was responsible for the \textit{ibr5-1} phenotypes, we transformed the wild-type \textit{IBR5} gene, driven by its own promoter, into \textit{ibr5-1} plants and assayed for rescue of the mutant phenotypes. The resulting transgenic plants displayed wild-type root elongation on the auxins IAA, IBA, 2,4-D, and 1-naphthaleneacetic acid; on the auxin transport inhibitors than wild-type seedlings on un-supplemented medium (Figures 2B and 2C). Moreover, the few lateral roots in \textit{ibr5-1} elongated less than those in the wild type (Figure 2D). These phenotypes also were seen in \textit{axr1} (Figures 2A to 2C) (Estelle and Somerville, 1987; Lincoln et al., 1990) and other auxin-response mutants, such as \textit{axr4} (Hobbie and Estelle, 1995). In contrast to these defects in light-grown seedlings, \textit{ibr5} hypocotyls and roots elongated normally in the dark (data not shown).

\textit{ibr5} mutants also displayed vascularization defects. Wild-type seedlings typically formed continuous veins in cotyledons and leaves (Figure 3A), whereas \textit{ibr5}, like \textit{axr1}, often formed discontinued veins and spurs (Figure 3A). Adult \textit{ibr5} plants were slightly smaller than wild-type plants and had epinastic leaves (Figure 3C). In addition, \textit{ibr5} had serrated rosette (base) and cauleine (inflorescence stem) leaves, in contrast to the smoother margins of wild-type leaves (Figure 3B).
1-napthylphthalamic acid and 2,3,5-triiodobenzoic acid; and on the phytohormone ABA (Figure 1). In addition, the delayed germination, lateral root defects, aberrant vascularization, and serrated leaves were rescued by wild-type IBR5 supplied from a transgene (Figures 1C, 1D, 2C, 2D, 3A, and 3B). Together, these analyses confirm that we have identified the IBR5 gene and that this single lesion is responsible for the pleiotropic ibr5 phenotypes.

To identify a second ibr5 allele, we obtained a mutant with a T-DNA insertion 161 bp upstream of the IBR5 start codon (27 bp upstream of the 5’ untranslated region; Figure 4B) from the Salk Institute Genomic Analysis Laboratory collection (Alonso et al., 2003). ibr5-2 has a similar but slightly weaker phenotype than ibr5-1 (Figures 1, 2, and 3), consistent with the potentially leaky nature of the ibr5-2 mutation (see below).

To determine the splicing pattern of the IBR5 gene and the amino acid sequence of the encoded protein, we cloned and sequenced a full-length IBR5 cDNA (see Methods). This analysis revealed that IBR5 has five exons separated by four introns (Figure 4B). Comparison of this cDNA with At2g04550 revealed that the computer-generated annotation failed to recognize the fourth intron, resulting in 26 extra amino acids predicted to include a transmembrane domain in the computer-annotated version.

IBR5 encodes a 257–amino acid protein similar to characterized dual-specificity phosphatases from Arabidopsis, human, mouse, and rat (Figures 4E and 4F). The IBR5 dual-specificity phosphatase catalytic domain (amino acids 49 to 182; Figure 4D) is ~35% identical to the catalytic domain of known dual-specificity MAPK phosphatases, including human MKP-1 and PAC-1 (Sun et al., 1993; Farooq et al., 2003). Within this catalytic domain is the highly conserved dual-specificity phosphatase active-site motif VxVxHxGxSxRxAYLM (Figures 4D and 4E). The premature stop codon in ibr5-1 allows translation...
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to glutathione phatase activity. Although we could express soluble IBR5 fused protein, we sought to determine if IBR5 possesses phosphatase activity. Although we could express soluble IBR5 fused protein, we sought to determine if IBR5 possesses phosphatase activity. It may be necessary to identify the in vivo substrates of IBR5 before enzymatic characterization can be completed.

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Because IBR5 encodes a dual-specificity phosphatase-like protein, we sought to determine if IBR5 possesses phosphatase activity. Although we could express soluble IBR5 fused to glutathione S-transferase (GST) in E. coli and purify the recombinant protein (see Methods), we did not detect the dephosphorylation of the generic substrate p-nitrophenyl phosphate by this recombinant protein, even after cleavage of the GST tag (data not shown). The only Arabidopsis dual-specificity phosphatase with demonstrated in vitro activity after heterologous expression is AtDpPTP1, which nonetheless has very low specific activity with p-nitrophenyl phosphate (Gupta et al., 1998). Interestingly, certain mammalian MAPK phosphatases require the binding of their substrate MAPK for in vitro activity (Camps et al., 1998; Hutter et al., 2000). It may be necessary to identify the in vivo substrates of IBR5 before enzymatic characterization can be completed.

IBR5 Accumulates throughout the Plant

IBR5 transcript levels are not altered in 4-week-old wild-type plants treated with ABA (Hoht et al., 2002) or in 7-day-old wild-type plants treated with auxin (data not shown). Comparison of IBR5 mRNA levels in ibr5-1 and the wild type revealed an ~2.5-fold decrease in ibr5 message in the ibr5-1 mutant (data not shown), consistent with the nonsense-mediated decay of the mutant mRNA (Wagner and Lykke-Andersen, 2002).

To determine in which tissues IBR5 is expressed, we transformed wild-type plants with an IBR5 promoter–GUS fusion. We found that IBR5-GUS was expressed in root tips, root vasculature, cotyledons, and true leaves, including the vasculature and hydathodes; in the sepals, anther filaments, and carpels of flowers; and in siliques (Figures 5A to 5E and 5G). In addition, IBR5-GUS was expressed in dark-grown seedlings in both hypocotyls and cotyledons (Figure 5F). Thus, IBR5-GUS was expressed widely throughout development, including in most of the tissues in which the ibr5 mutant displayed phenotypes. However, it is possible that this reporter is not sufficiently sensitive to detect all tissues in which IBR5 plays important roles. For example, we detected no IBR5-GUS expression in lateral root primordia or newly emerged lateral roots (Figure 5B), but we did detect reduced expression of the auxin-responsive DHR5-GUS promoter in these tissues in the ibr5 mutant (Figure 3D).

We developed a polyclonal IBR5 antibody to examine IBR5 levels in our ibr5 alleles and to determine the tissues in which IBR5 protein accumulates. This antibody detects two proteins (~30 and ~25 kD) in wild-type Arabidopsis; the predicted size for the IBR5 open reading frame is 28.7 kD. The ~30-kD protein was undetectable in the ibr5-1 mutant, which was disrupted early by a nonsense mutation, suggesting that the ~30-kD protein is IBR5 (Figures 5H to 5J). The ibr5-2 mutant, which contains a T-DNA insertion upstream of the IBR5 5’ untranslated region, accumulated a reduced but detectable amount of IBR5 protein (Figure 5I). The presence of some residual IBR5 protein in this second allele probably accounts for the generally weaker phenotype of ibr5-2 compared with the ibr5-1 presumed null allele (Figures 1 to 3).

Consistent with the IBR5 promoter-reporter gene expression patterns, we found that IBR5 protein accumulated throughout wild-type plants, including in rosette and cauline leaves, flowers, stems, siliques, and seeds (Figure 5H). During germination, IBR5 levels declined slightly at 2 days after imbibition (Figure 5J).

Overexpression of IBR5

To determine whether IBR5 levels are normally limiting for auxin responsiveness, we transformed wild-type plants with a vector expressing the IBR5 cDNA driven by the strong 35S promoter of Cauliflower mosaic virus (35S-IBR5). We isolated several lines with increased IBR5 protein accumulation, as judged by immunoblot analysis (Figure 6A), and we assayed auxin responsiveness in these lines. We found that IBR5 overexpression did not dramatically alter auxin sensitivity (Figure 6B). Moreover, these lines appeared morphologically similar to the wild-type line (data not shown). The observation that these overexpressing lines did not have significantly shorter roots or

![Figure 2.](image-url)
longer hypocotyls than wild-type plants (data not shown) suggests that IBR5 is not normally limiting for auxin responsiveness.

**DISCUSSION**

**IBR5 Encodes an Apparent Dual-Specificity Phosphatase**

IBR5 encodes an apparent phosphatase containing the highly conserved dual-specificity phosphatase active-site motif VxVHCx2GxSRSx5AYLM. Phosphatases have been most studied in animal and fungal systems, in which dual-specificity phosphatases often regulate MAPK pathways. MAPK kinases have dual activity, phosphorylating both Tyr and Thr residues on target MAPKs. MAPKs require this dual phosphorylation for full activation. Conversely, phosphatases can dephosphorylate MAPKs at either or both residues. Protein Tyr phosphatases dephosphorylate phosphotyrosine residues, whereas Ser/Thr protein phosphatases dephosphorylate phosphothreonine residues. In addition, a number of phosphatases that dephosphorylate both phosphotyrosine and phosphothreonine residues of MAPKs, termed dual-specificity or MAPK phosphatases, have been identified (reviewed by Keyse, 1995; Camps et al., 2000). Dual-specificity phosphatases have a highly conserved active-

**Figure 3. ibr5 Morphological and Auxin-Related Phenotypes.**

(A) ibr5 displays aberrant vascular patterning. Cleared cotyledons of 10-day-old Col-0 (wild type), ibr5-1, ibr5-2, ibr5-1 transformed with pBINIBR5 (rescue), and axr1-12 seedlings are shown.

(B) ibr5 has increased leaf serration. Cauline (top) and rosette (bottom) leaves of 6-week-old Col-0 (wild type), ibr5-1, ibr5-2, and ibr5-1 transformed with 35S-ibr5 plants grown under short-day conditions are shown.

(C) Adult ibr5 plants are smaller than wild-type Col-0 plants. Five-week-old plants grown in continuous light are shown.

(D) ibr5 shows decreased levels of an auxin-inducible reporter. Shoot apex (top) and lateral root (bottom) staining of homozygous wild-type and ibr5-1 seedlings expressing DR5-GUS are shown. Seedlings were grown for 10 days in the light on unsupplemented medium before staining.
Figure 4. Map-Based Positional Cloning of IBR5 Reveals Similarity to Dual-Specificity Phosphatases.

(A) Recombination mapping of ibr5-1. Mapping with the PCR-based markers RGA1 (Silverstone et al., 1998), AIR3 (Silverstone et al., 1998), T1O3.9, F7D11, and F5G3 (see Methods) localized the defect to a region spanned by BACs T1O3 and F7D11.

(B) IBR5 contains five exons (thick boxes) separated by four introns (lines). The position of the stop codon in ibr5-1 is indicated with an asterisk, and the position of the T-DNA insertion in ibr5-2 is indicated with a triangle.

(C) ibr5-1 has a C-to-T mutation at position 123 (where 1 is the A of the initiator ATG) that converts a Gln (Q) residue at position 42 to a premature stop codon and destroys an AciI restriction site.

(D) Alignment of Arabidopsis (At) IBR5 and its plant homologs. Sequences were aligned with the MegAlign program (DNAStar, Madison, WI) using the CLUSTAL W method. Residues identical in at least four sequences are shaded in black, similar residues are shaded in gray, and dashes indicate gaps introduced to maximize alignment. The gray oval overlies the dual-specificity phosphatase catalytic domain, circles mark residues defining the dual-specificity phosphatase active-site motif, and the open circle marks the catalytic Cys nucleophile. Sequences from soybean (Gm), Medicago truncatula (Mt), wheat (Ta), and Chlamydomonas reinhardtii (Cr) are from plant genome projects assembled in the TIGR Gene Index Database (http://www.tigr.org/tdb/tgi) (Quackenbush et al., 2001), and the maize (Zm) sequences are from GenBank accession numbers AY105390 and AY108971. The Chlamydomonas cDNA sequence was truncated at the 3' end, so only the N-terminal region is shown.

(E) Alignment, as in (D), of part of the phosphatase catalytic domains of the proteins shown in (D) with the corresponding catalytic domains of dual-specificity phosphatases from rice (Os), human (Hs), and Xenopus laevis (Xl). α-Helices (black rectangles), β-sheets (arrows), and loops (brackets) ob...
The Arabidopsis genome encodes 20 likely MAPKs (Ichimura et al., 2002) and 18 genes in the dual-specificity phosphatase family (Kerk et al., 2002). Of these 18, only 5 (IBR5/At2g04550, AtDsPTP1/At3g23610, At5g23720, At3g06110, and AtMKP1/At3g55270) have the complete dual-specificity phosphatase active-site motif VxVHxGxSRSxAYLM. Two of these five apparent phosphatases have been characterized to date: AtDsPTP1 (Gupta et al., 1998) and AtMKP1 (Ulm et al., 2001, 2002). AtDsPTP1 (26% identical to IBR5) and the uncharacterized At3g06110 protein (29% identical) are the closest IBR5 relatives in the Arabidopsis genome (Figures 4E and 4F). A dsptp1 mutant has not been reported; however, AtDsPTP1 can dephosphorylate and inactivate the MAPK AtMPK4 in vitro (Gupta et al., 1998). AtMKP1 has not been characterized in vitro, but genetic analysis indicates that AtMKP1 negatively regulates an ~49-kD MAPK. This MAPK has increased activity in mkp1 plants exposed to UV light, suggesting a role for AtMPK1 in UV resistance (Ulm et al., 2001). Although IBR5 has ~35% sequence identity to AtDsPTP1 within the catalytic domain (Figure 4E; 26% overall), the N and C termini are quite dissimilar. IBR5 also is diverged from AtMKP1 (19% identical), which has an extended C-terminal region.

Although IBR5 lacks close relatives within the Arabidopsis genome, examination of assembled cDNAs from various plant genome projects (TIGR Gene Index Databases; http://www.tigr.org/tdb/tgi) (Quackenbush et al., 2001) revealed that IBR5 does have close relatives in monocots, dicots, and the unicellular green alga Chlamydomonas reinhardtii (Figures 4D to 4F). These proteins represent a unique clade of similarly sized proteins (257 to 275 amino acids; Figure 4F), of which IBR5 is the first characterized member. Unlike the similarity between IBR5 and other characterized phosphatases, which is limited to the catalytic domain, the similarity among these IBR5-like proteins extends throughout the proteins (Figure 4D). For example, IBR5 is 74% identical throughout its length to the wheat protein encoded by TC90531 (Figure 4D). Moreover, proteins in the IBR5 clade share a deletion of part of the general acid loop (Figure 4E) observed in structural studies of the human phosphatases VHR (Yuvaniyama et al., 1996) and PAC-1 (Faroq et al., 2003). Interestingly, the 30 N-terminal residues of the proteins in the IBR5 clade are almost identical among the flowering plants within this clade (Figure 4D). Certain mammalian MAPK phosphatases use N-terminal docking modules to bind their substrate MAPks (Tanoue et al., 2002); it is possible that the IBR5-like proteins also use this region to dock substrates. If so, the extreme conservation of this region suggests that the substrate(s) of the IBR5-like phosphatases will be conserved as well. Unfortunately, this N-terminal region of IBR5 is responsible for the transcriptional activation seen when IBR5 is fused to a DNA binding domain in a yeast two-hybrid bait vector (data not shown), rendering the yeast two-hybrid system unsuitable for identifying IBR5 substrates.

**Models for the IBR5 Regulation of Phytohormone Signaling**

The phenotypes of plants lacking IBR5 suggest that IBR5 normally modulates auxin signaling. ibr5 has decreased sensitivity to root elongation inhibition by the application of natural auxins, synthetic auxins, and auxin transport inhibitors (Figure 1A and data not shown). Moreover, examination of the mutant on medium lacking hormones revealed that ibr5 may be less sensitive to endogenous auxin as well. Endogenous auxin promotes lateral root development (Casimiro et al., 2003) and hypocotyl elongation in the light (Romano et al., 1995; Gray et al., 1998; Jensen et al., 1998). Whereas auxin may be necessary for primary root elongation as well, the concentration of auxin in wild-type Arabidopsis roots is thought to be supraoptimal for elongation (Hobbie and Estelle, 1995). Consequently, mutants that overproduce auxin have longer hypocotyls, shorter primary roots, and more lateral roots than the wild type when grown in the light (Delarue et al., 1998; Zhao et al., 2001). Conversely, certain mutants with auxin-response defects, including axr1 and axr4, have short hypocotyls and long roots with fewer lateral roots compared with the wild type when grown on unsupplemented medium (Figure 2) (Estelle and Somerville, 1987; Lincoln et al., 1990; Hobbie and Estelle, 1995). Like these auxin-response mutants, both ibr5 alleles have shorter hypocotyls and longer primary roots with slightly fewer lateral roots on unsupplemented medium compared with the wild type (Figure 2), consistent with reduced responsiveness to endogenous auxin. Auxin also directs vascular patterning in leaves (reviewed by Berleth and Mattsson, 2000), and the discontinuous veins and spurs seen in ibr5 vasculature (Figure 3A) further suggest auxin-response defects. Because ibr5 appears to be less responsive to endogenous and exogenous auxin, and because ibr5-1 is a recessive loss-of-function mutation, these results indicate that IBR5 may normally promote auxin responsiveness, either directly or indirectly. In support of this idea, the auxin-responsive DR5-GUS reporter displays decreased expression in the shoot apex, roots, hydathodes, true leaves, and sepals in the ibr5-1 mutant (Figure 3D and data not shown).

Interestingly, IBR5 also appears to modulate certain aspects of ABA signaling. ibr5 is less sensitive than the wild type to the...
inhibitory effects of ABA on root elongation (Figure 1B) and germination (Figure 1D). In addition, ibr5 appears to germinate earlier than the wild type even on unsupplemented medium (Figure 1C), suggesting that ibr5 may be less sensitive to both endogenous and exogenous ABA. However, not all ABA responses are disrupted in ibr5. For example, ibr5 does not have a wilty phenotype, and detached ibr5 rosettes lose water at a similar rate as wild-type rosettes (data not shown), suggesting that ABA regulation of stomatal closure is functioning normally. In addition, ibr5 responds normally to high levels of exogenous sugar (data not shown), whereas several previously described ABA-response mutants were defective in both ABA and sugar signaling (reviewed by Fedoroff, 2002; Gazzarrini and McCourt, 2003).

Links between auxin and ABA have been noted previously. For example, auxin can enhance the inhibitory effects of ABA on germination (Brady et al., 2003), and certain mutants originally identified as auxin insensitive, such as axr1 and axr2, also display decreased ABA sensitivity during germination or in root elongation inhibition (Figure 1B) (Wilson et al., 1990; Tiryaki and Staswick, 2002). In addition, mutants defective in the RCN1 protein phosphatase 2A regulatory subunit, which influences

Figure 5. IBR5 Expression and Protein Accumulation in Wild-Type Plants throughout Development.

(A) to (C) Staining of 10-day-old seedlings revealed IBR5-GUS expression in the primary root tip (A); in lateral root tips of longer (arrowhead) but not shorter (arrow) lateral roots and primary root vasculature (B); and in the shoot (C).

(D) to (G) Staining of mature plants revealed IBR5-GUS expression in the hydathodes of cauline leaves (D); in the sepals, anther filaments, and carpels of flowers (E); and in green siliques (G). Staining of 6-day-old dark-grown seedlings revealed expression throughout the hypocotyl and cotyledons (F).

(H) Immunoblot analysis with an anti-IBR5 antibody of protein prepared from seeds, rosette (r.) and cauline (c.) leaves, flowers, stems, and siliques of wild-type plants and from rosette leaves of ibr5-1 and ibr5-2 mutants.

(I) Overexposure of the last three lanes of (H). No IBR5 protein is detected in rosette leaves of ibr5-1, and reduced amounts of IBR5 protein are detected in rosette leaves of ibr5-2. wt, wild type.

(J) Immunoblot analysis with an anti-IBR5 antibody of protein prepared from seedlings at 1 to 8 days after imbibition. The positions of IBR5 and an unidentified cross-reacting protein (asterisk) are indicated at right in (H) to (J), and the positions of molecular mass markers (in kD) are indicated at left.
auxin transport and gravity responses (Garbers et al., 1996; Rashotte et al., 2001), have decreased sensitivity to ABA (Kwak et al., 2002). Moreover, ABA inhibits DRS-GUS expression in emerging lateral roots (De Smet et al., 2003), and the abi3 mutant, isolated originally as ABA insensitive during germination (Koornneef et al., 1984), shows decreased sensitivity to the emerging lateral roots (Brady et al., 2003). ABI3 expression is induced by auxin in roots (Brady et al., 2003), and when the maize ABI3 homolog VP1 is expressed in Arabidopsis, ABA is able to suppress auxin-induced lateral root formation (Suzuki et al., 2001), further supporting a role for ABI3 in auxin–ABA interactions.

Because ibr5 has decreased sensitivity to auxin and ABA and is defective in an apparent dual-specificity phosphatase, we can envision several possible roles for ibr5. One possibility is that IBR5 dephosphorylates a single MAPK acting in a signaling pathway. Because MAPK phosphatases inactivate MAPKs and the loss-of-function ibr5 mutants are less responsive to auxin and ABA, this putative MAPK may normally inhibit both auxin and ABA responses. The integration of auxin and ABA responses could be either a direct or an indirect result of this signaling. It also is possible that IBR5 has more than one MAPK substrate, explaining the pleiotropic nature of the ibr5 mutant phenotypes. Finally, IBR5 may dephosphorylate a protein or proteins not involved in a canonical signaling pathway, and the loss of this dephosphorylation reduces sensitivity to auxin and ABA.

Previous evidence has implicated MAPK pathways in both auxin and ABA signaling. Studies with the Arabidopsis MAP-KKK ANP1 and the tobacco homolog NPK1, which are involved in oxidative stress pathways, show that transient expression in protoplasts of constitutively active ANP1 or NPK1 correlates with the activation of the AtMPK3 and AtMPK6 MAPKs and with a decrease in auxin-responsive gene transcription (Kovtun et al., 1998, 2000). AtMPK3 is activated by ABA, and AtMPK3 overexpression increases Arabidopsis ABA sensitivity (Lu et al., 2002). Coexpression of the mouse MAPK phosphatase MKP1 in protoplasts abolishes NPK1’s effects on MAPK activation and restores auxin-responsive transcription (Kovtun et al., 1998). Therefore, it is unlikely that IBR5 functions to inactivate AtMPK3 or AtMPK6, because failure to inactivate these MAPKs is expected to increase, rather than decrease, ABA sensitivity.

In addition, Mockaitis and Howell (2000) demonstrated auxin-induced activation of an ∼44-kD MAPK in Arabidopsis. The activation of this MAPK correlates with the induction of auxin responses, and the reduced activation of this MAPK is seen in the axr4 auxin-response mutant (Hobbie, 1998; Mockaitis and Howell, 2000). Failure to inactivate this auxin-activated MAPK presumably would heighten rather than reduce auxin responsiveness, making it unlikely that IBR5 inactivates this MAPK. Thus, it is likely that IBR5 acts on a MAPK not currently known to influence ABA or auxin signaling or that IBR5 acts on substrates that are not MAPKs.

The identification of IBR5 provides a new tool with which to dissect auxin responsiveness and the interaction between auxin and ABA signaling pathways. The presence of IBR5 homologs throughout the plant kingdom implies that this subfamily of phosphatase-like proteins may play plant-specific signal-
newly developed PCR-based markers. For the marker T103.9, PCR amplification with the primers 5’-CACAACTCATAAACCATCTCTAGGTTGAC-3’ and 5’-CACGCGAGAAATGGAACCTCCATTGCTCT-3’ yielded a 1.3-kb product with two Tail sites in Col-0 and one site in Ws. For the marker F7D11, PCR amplification with the primers 5’-GAGCTAGC- CATCATCATCTTCCATTCAAGG-3’ and 5’-GGATTGAGGATTTAGGAA- AAAATTGGT-3’ yielded a 1.1-kb product with two EcoRV sites in Col-0 and no sites in Ws. For the marker F5G3, PCR amplification with the primers 5’-GGACACACTAGCTAAAACAGTTAGAC-3’ and 5’-GCA- TTATGATCTCTACAAGG-3’ yielded a 976-bp product with one DpnII site in Col-0 and no sites in Ws.

A candidate gene (IBRS; A12g04550) within the mapping interval was examined for changes in the mutant. Genomic DNA extracted from ibr5-1 mutant plants was amplified using two pairs of oligonucleotides (T103.4-1 [5’-CTTAATTTCTTCTGCTGTGAATCAAGGG-3’] and T103.4-2 [5’-GCA-GGCCCAAGTTGATGAGAGTAAGAG-3’]; T103.4-3 [5’-CAAGCG- GTCCTATGTGCGCAAGAATTAC-3’] and T103.4-4 [5’-CAAACTCTC- TCAAAGGTGGACAAACTCC-3’]) with a program of 40 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s. The resulting overlapping fragments were ~900 bp each and covered the gene from 90 bp upstream of the putative translation start site to 115 bp downstream of the stop codon. Amplification products were purified by ethanol precipitation followed by gel purification using a Matrix Gel Extraction kit (Marligen Bio-sciences, Ijamsville, MD) and sequenced directly using an automated DNA sequencer (Seqwright, Houston, TX; LoneStar Labs, Houston, TX) and the primers used for amplification. Subsequent ibr5-1 genotyping was performed by amplifying genomic DNA with the primers T103.4-1 and T103.4-6 [5’-CAAGCCAAACCTCAACTAACAAAG-3’], which yielded a 463-bp product with one AciI site in Col-0 and no sites in ibr5-1.

ibr5-2 (SALK_032185) contains a T-DNA insertion generated by the Salk Institute Genomic Analysis Laboratory, La Jolla, CA (Alonso et al., 2003). Segregating seeds from the ABRC were plated on 12 g/mL kanamycin. The T-DNA insertions were selected with 7.5 g/mL kanamycin after 10 days under white light. Phenotypic analyses were performed using seeds from homzygous progeny of BASTA-resistant transfectants.

**Reporters Gene Analysis**

A 2.0-kb IBRS promoter fragment (~2005 to ~30 from the IBRS initiator ATG) was excised from pKS/IBRSg with BglII and ligated into BamHI-cut pBI101.1 (Jefferson et al., 1987), forming an IBRS promoter–GUS fusion (IBRS-GUS), which was transformed into Col-0 plants. β-Glucuronidase activity was localized histochemically by staining for 2 days with 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid as described (Bartel and Fink, 1994). Three independent lines showed similar staining patterns with variable intensity.

Backcrossed ibr5-1 was crossed to Col-0 carrying DR5-GUS (Guiltinoy, 1999), and lines homozygous for both DR5-GUS and ibr5-1 were selected on 12 µg/mL kanamycin and by PCR genotyping the ibr5-1 mutation. For histochemical localization, homzygous seedlings or plant parts were stained for 2 days with 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as described (Bartel and Fink, 1994).

**IBRS Antibody Production and Purification**

A BglII-NotI fragment from pKS/IBRSc was ligated into the protein expression vector pGEX-KTO (Davies et al., 1999) cut with BamHI and NotI to make pGEX-IBRS, which expresses a GST-IBRS fusion protein in Escherichia coli. Protein expression and purification using glutathione-agarose were performed as described previously (Davies et al., 1999). After purification, the GST was removed from IBRS using a Thrombin Cleavage kit (Novagen, Madison, WI) for 1 h at room temperature. The released IBRS was separated from GST by SDS-PAGE, detected using Coomassie Brilliant Blue R250 staining (Ausubel et al., 1999), and excised from the gel.

To obtain an IBRS polyclonal antibody, gel fragments containing IBRS protein were used to immunize two rabbits at Cocalico Biologicals (Reamstown, PA) with a primary injection (100 µg) followed by three boosts (50 µg each). IBRS antibody was selected from the resulting serum by incubating for 1 h on IBRS-GST-soaked nitrocellulose membrane, removing the unbound serum, rinsing the membrane two times for 5 min with TBS-T (Ausubel et al., 1999), and eluting bound IBRS antibodies by incubating for 10 min with 100 mM glycine, pH 2.5. Eluted affinity-purified antibodies were neutralized immediately by adding 0.1 volume of 1 M Tris-HCl, pH 8.0, and stored at ~80°C until use.

**IBRS Protein Analysis**

Protein was extracted from ibr5-1 and Col-0 by grinding frozen tissue with a pestle, adding 1 volume of buffer (0.1 M Tris-HCl, pH 6.8, 20% glycerol, and 4% SDS), vortexing, removing debris by brief centrifugation, and heating to 100°C for 5 min. Protein extracts were separated by SDS-PAGE beside Cruz markers (Santa Cruz Biotechnology, Santa Cruz, CA) using NuPAGE 10% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred for 40 min at 24 V to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using NuPAGE transfer buffer. After blocking for 1 h in 5% milk in TBS-T, the membrane was incubated overnight at 4°C with affinity-purified IBRS antibody diluted 1:100 or 1:25 in TBS-T, incubated with a 1:500 or 1:300 dilution of horseshad peroxidase–conjugated goat anti-

**cDNA Isolation**

An IBRS cDNA was isolated by hybridizing a cDNA library (Minet et al., 1992) with a 32P-labeled probe made by PCR amplifying genomic DNA with T103.4-3 and T103.4-5 (5’-CAAGTACGCTCAACAACAGCCGTTGTT-3’). The 1138-bp NotI insert from the hybridizing clone was subcloned into pBluescript KS+ to give pKS/IBRSc, which was sequenced. 3SS-IBRS was made by ligating the NotI insert of the IBRS cDNA into NotI-cut 3SSpBARN (LeClere and Bartel, 2001), a plant transformation vector with the 3SS promoter of Cauliflower mosaic virus and the nos terminator. The 35S-IBRS plasmid was electroporated (Ausubel et al., 1999) into A. tumefaciens, which was used to transform wild-type Col-0 and ibr5-1. Transformants were identified on PNS medium supplemented with 7.5 µg/mL BASTA after 10 days under white light. Phenotypic analyses were performed using seeds from homzygous progeny of BASTA-resistant transfectants.
rabbit IgG (Santa Cruz Biotechnology) for 1 h at room temperature, and then visualized using Lumiglo reagent (Cell Signaling, Beverly, MA).

**Microarray Analysis**

Total RNA was extracted using RNeasy Mini Kits (Qiagen, Valencia, CA) from triplicate samples of 7-day-old ibr5-1 and wild-type (Col-0) seedlings grown on filter paper overlaid on PNS under white light. Thirty to 40 μg of total RNA from each sample was sent to the laboratory of Thomas McKnight at Texas A&M University (College Station), where ibr5-1 and wild-type mRNA were converted to cDNA, amplified to yield biotin-labeled cRNA, and hybridized to Affymetrix (Santa Clara, CA) ATH1 Arabidopsis whole-genome microarray chips containing ~22,000 genes per chip. The 13,169 mRNAs that were statistically present (Microarray Suite 5.0; Affymetrix) on all six chips were analyzed further. Fold changes were calculated for each ibr5-1 chip compared with each Col-0 chip individually. The fold changes from all nine comparisons were averaged. A >2.5-fold change in message level observed in all nine comparisons was defined as significant.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Bonnie Bartel, bartel@rice.edu.

**Accession Number**

The GenBank accession number for IBR5 is AY337455.

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**REFERENCES**


