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Arabidopsis *iba response5* Suppressors Separate Responses to Various Hormones

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ABSTRACT

Auxin controls numerous plant growth processes by directing cell division and expansion. Auxin-response mutants, including *iba response5* (*ibr5*), exhibit a long root and decreased lateral root production in response to exogenous auxins. *ibr5* also displays resistance to the phytohormone abscisic acid (ABA). We found that the *sar3 suppressor of auxin resistant1* (*axr1*) mutant does not suppress *ibr5* auxin-response defects, suggesting that screening for *ibr5* suppressors might reveal new components important for phytohormone responsiveness. We identified two classes of *Arabidopsis thaliana* mutants that suppressed *ibr5* resistance to indole-3-butyric acid (IBA): those with restored responses to both the auxin precursor IBA and the active auxin indole-3-acetic acid (IAA) and those with restored response to IBA but not IAA. Restored IAA sensitivity was accompanied by restored ABA responsiveness, whereas suppressors that remained IAA resistant also remained ABA resistant. Some suppressors restored sensitivity to both natural and synthetic auxins; others restored responsiveness only to auxin precursors. We used positional information to determine that one *ibr5* suppressor carried a mutation in *PLEIOTROPIC DRUG RESISTANCE9* (*PDR9*/*ABCG37*/*At3g53480*), which encodes an ATP-binding cassette transporter previously implicated in cellular efflux of the synthetic auxin 2,4-dichlorophenoxyacetic acid.

AUXIN is an essential plant hormone controlling root elongation, lateral root initiation, stem elongation, embryo patterning, and leaf expansion through its effects on cell division and expansion (reviewed in Davies 2004; Woodward and Bartel 2005). Auxin signaling requires auxin recognition by TIR1/ABF receptor proteins, which are components of $SCF^{TIR1/ABF}$ ubiquitin-protein ligases that promote degradation of Aux/IAA transcriptional repressors by the 26S proteasome (reviewed in Parry and Estelle 2006). Aux/IAA protein degradation is thought to allow auxin-responsive transcription by relieving repression of the activating class of AUXIN RESPONSE FACTOR (ARF) proteins. Loss-of-function mutations in genes encoding or modulating the $SCF^{TIR1/ABF}$ complex (reviewed in Woodward and Bartel 2005) and gain-of-function stabilizing mutations in certain Aux/IAA proteins (reviewed in Reed 2001) can confer resistance to applied and endogenous auxin.

The phytohormone abscisic acid (ABA) controls diverse processes including shoot and root growth, stomatal closure, seed storage protein synthesis, and seed dormancy (reviewed in Davies 2004). Although responses to auxin and ABA are distinct, sensitivity to auxin appears to correlate with ABA sensitivity. For example, mutations in *AUX1*, *AXR1*, *AXR2*, *IBR5*, and *TIR1*, which were all isolated in mutant screens for reduced auxin sensitivity (Lincoln *et al.* 1990; Wilson *et al.* 1990; Bennett *et al.* 1996; Ruegger *et al.* 1998; Monroe-Augustus *et al.* 2003), also confer decreased ABA sensitivity (Wilson *et al.* 1990; Tiryaki and Staswick 2002; Monroe-Augustus *et al.* 2003; Strader *et al.* 2008). Although connections have been made between auxin and ABA signaling, the molecular nature of the relationship between these two phytohormones remains largely undefined.

The Arabidopsis *iba response5* (*ibr5*) mutant was originally isolated in a screen for resistance to the auxin indole-3-butyric acid (IBA; Zolman *et al.* 2000) and is defective in a putative dual-specificity protein phosphatase (Monroe-Augustus *et al.* 2003). In addition to IBA resistance, loss-of-function *ibr5* mutants are resistant to natural and synthetic auxins as well as to ABA (Monroe-Augustus *et al.* 2003). *ibr5* exhibits decreased basal and auxin-induced expression of the auxin-responsive transcriptional reporter DR5-GUS (Monroe-Augustus *et al.* 2003; Strader *et al.* 2008), but, unlike other characterized auxin-response mutants, Aux/IAA proteins are not stabilized in *ibr5* (Strader *et al.* 2008). These results suggest that ARF functions can be regulated by means in addition to modulation of Aux/IAA repressor protein stability.

Genetic modifiers can be useful for uncovering additional components in signaling pathways. Previous screens

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for suppressors of auxin-resistant mutants have identified *pax1*, which partially suppresses *axr3-1* (Tanimoto *et al.* 2007), and *sar1* and *sar3*, which partially restore auxin response to *axr1* (Cernac *et al.* 1997; Parry *et al.* 2006). *SAR1* and *SAR3* encode nucleoporins; these mutants may suppress *axr1* by altering Aux/IAA protein transport into the nucleus (Parry *et al.* 2006). We found that *sar3* fails to suppress *tir1* or *ibr5* auxin resistance. To better understand IBR5 function, we isolated extragenic suppressors that restored *ibr5* responsiveness to IBA. We found that these suppressors fell into two classes: those that restored *ibr5* sensitivity to both IBA and indole-3-acetic acid (IAA) (class 1) and those that restored sensitivity to IBA but not to IAA (class 2). Suppressors that restored *ibr5* IAA sensitivity also restored ABA sensitivity, whereas those that remained IAA resistant retained ABA resistance. We mapped four *ibr5*-suppressing mutations to four distinct loci and used recombination mapping to clone the gene defective in one class 2 suppressor. This suppressor restored *ibr5* responses to a subset of auxins, but not to ABA, and carries a mutation in *PDR9/ABCG37*, which encodes an ATP-binding cassette (ABC) transporter previously reported to transport the auxinic compound 2,4-dichlorophenoxyacetic acid (2,4-D) out of cells (Ito and Gray 2006). Our results suggest that PDR9 may also facilitate IBA efflux.

MATERIALS AND METHODS

Plant materials and growth conditions: *Arabidopsis thaliana* accession Colombia (Col-0) was used as wild type for all experiments. Surface-sterilized (Last and Fink 1988) seeds were plated on plant nutrient medium (PN) Haughn and Somerville 1986) supplemented with 0.5% (w/v) sucrose (PNS), solidified with 0.6% (w/v) agar. Hormone stocks were dissolved in ethanol at 0.1, 1.0, or 100 mm and ethanol-supplemented media were used as controls with all treatments normalized to the same ethanol content (<0.2 μl ethanol/ml medium). Seedlings were grown at 22° under continuous illumination through yellow long-pass filters to slow indolic compound breakdown (Stasinopoulos and Hangarter 1990) unless otherwise indicated.

Mutant isolation and nomenclature: *ibr5-1* seeds (Monroe-Augustus *et al.* 2003) were mutagenized with ethyl methanesulfonate (EMS; Normanly *et al.* 1997). M_2 seeds were surface sterilized (Last and Fink 1988) and plated on PNS supplemented with 8 μm IBA at ∼1000 seeds/150-mm plate. After 8 days, putative modifier mutants with short roots were selected, transferred to unsupplemented medium to recover for several days, moved to soil, genotyped for the *ibr5-1* mutation (Monroe-Augustus *et al.* 2003), and allowed to self-fertilize. M_3 progeny lines were retested by comparing root lengths of seedlings grown on mock- and 8 μm IBA-supplemented media. Lines displaying IBA-responsive root elongation inhibition similar to wild type were retained as *ibr5* suppressors.

Suppressor lines used for the initial IBA retests were analyzed as the progeny of the original isolates. Most mutant lines (MS34, MS72, MS115, MS182, MS252, MS339) used in subsequent phenotypic analyses were from the first backcross to the parental *ibr5-1* line. Other mutants (MS5, MS109, MS371) were analyzed as the progeny of the original isolates.

Phenotypic assays: All assays were conducted at least twice with similar results. For auxin-responsive root elongation assays, seedlings were grown for 8 days on the indicated auxin concentrations and primary root lengths were measured. For 1-aminocyclopropane-1-carboxylic acid (ACC)-responsive root elongation assays, seedlings were grown for 10 days on medium supplemented with either ethanol or 100 nm ACC under white light and primary root lengths were measured. For ABA-responsive root elongation assays, imbibed seeds were incubated at 4° for 4 days in the dark and then plated on unsupplemented medium. Plates were incubated in the light at 22° for an additional 4 days to allow efficient germination. Seedlings then were transferred to medium supplemented with either ethanol or 10 μm ABA and primary root lengths were measured after an additional 4 days of growth in the light.

For lateral root assays, 4-day-old seedlings grown on unsupplemented medium were transferred to medium supplemented with either ethanol or 10 μm IBA and grown for an additional 4 days. Lateral roots were counted under a dissecting microscope; primordia emerging from the primary root were counted as lateral roots.

Double-mutant isolation: The *ibr5-1* mutant (Monroe-Augustus *et al.* 2003) was crossed to *sar3-3* (Parry *et al.* 2006), *pdr9-1*, and *pdr9-2* (Ito and Gray 2006), all in the Col-0 accession. The *tir1-1* mutant (Ruegger *et al.* 1998) was crossed to *sar3-3* (Parry *et al.* 2006); *tir1-1 pdr9-1* was a gift from William Gray (Ito and Gray 2006). The *axr1-3* mutant (Lincoln *et al.* 1990) was crossed to *sar3-3*. Double mutants were identified by PCR analysis of DNA prepared from the F_2 plants. Amplification of *SAR3* with SAR3-1 (5′-AACATAACTCCTTGGCT TCC-3′) and SAR3-2 (5′-ACTTGGGCTGTGTTGTCATC-3′) yields a 400-bp product in wild type and no product in *sar3-3*. *SAR3* amplification with SAR3-2 and LB1-SALK (5′-CAAAC CAGCGTGGACCGCTTGCTGCAACTC-3′) yields a 333-bp product in *sar3-3* and no product in wild type. *PDR9* amplification with PDR9-13 (5′-GCTTTCCCCTCTGTTGCTTGGTTC-3′) and PDR9-16 (5′-ATCTCACCGTAACTCAAAGG-3′) yields a 390-bp product with two *Msp*I restriction sites in wild type and one in *pdr9-72*. *PDR9* amplification with the derived cleaved amplified polymorphic sequence (dCAPS; Michaels and Amasino 1998; Neff *et al.* 1998) primers PDR9-HinPI (5′-TGGATGAGCCAACGACGGGGCTAGGC-3′; underlined nucleotide indicates an introduced mutation for dCAPS) and PDR9-17 (5′-TGTAGATCATGCGACCACCTC-3′) yields a 270-bp product with one *Hin*PI restriction site in wild type and none in *pdr9-1*. *PDR9* amplification with PDR9-1 (5′-CAACGTTTTCTCTGATTACAC-3′) and PDR9-2 (5′-GCTAC CAACGCCCTGACAACGAG-3′) yields a 1472-bp product in wild type and no product in *pdr9-2*. *PDR9* amplification with PDR9-1 and LB1-SALK yields an ∼1-kbp product in *pdr9-2* and no product in wild type. PCR-based identification of *axr1-3* (Strader *et al.* 2008), *ibr5-1* (Monroe-Augustus *et al.* 2003), and *tir1-1* (Strader *et al.* 2008) alleles was as described previously.

Genetic analysis: The *ibr5-1* mutation, originally in the Col-0 background, was introgressed into the Wassilewskija (Ws-2) accession by crossing *ibr5-1* to Ws-2 three times. Outcrossing was monitored using genetic markers (Konieczny and Ausubel 1993; Bell and Ecker 1994) polymorphic between Col-0 and Ws-2. Ws-2-introgressed *ibr5-1* was homozygous for Ws-2 DNA at markers nga59, nga63, nga280, nga111, RGA1, nga168, nga172, nga112, SC5, nga249, GA3, and MBK-5.

Several *ibr5-1* suppressors (in the Col-0 background) were outcrossed to Ws-introgressed *ibr5-1* for mapping. F_2 seedlings from the MS34 and MS115 outcrosses were screened on 10 μm IBA, and F_2 seedlings from the MS72 and MS182 outcrosses were screened on 2 μm 2,4-dichlorophenoxybutyric acid (2,4-DB). DNA from sensitive individuals was isolated (Celenza

TABLE 1

New markers used in *ibr5* suppressor mapping

Marker	Nearest gene	Enzyme	Size of products (bp)			Oligonucleotides[a]
			Col-0	L*er*-0	Ws-2	
LCS104	*At1g53645*	*Eco*NI	185	165, 20	165, 20	CAAAGTAGGCCACCATCTCCTCTTG AGGCTCACACTCAATCTGCAAACCAAAATAG
SNP3	*At1g60950*	*Hin*fI	190	160, 30	160, 30	AGTCAACTTCTAATGGCCTTTCAGTACATG ATCAACCGATGTAGATGGTCTCATACTCGACT
LCS301	*At3g52910*	*Aci*I	385	365, 20	365, 20	AGTAGATTTGGTTAATTACAAAC TGTGTTAATAAGAGGAAGTGGTTGC
LCS302	*At3g54050*	*Eci*I	462	ND	440	ATCAGGCCCAACTCTTTATTATC CTCGCCGCCGTTTTCGTCTC
LCS320	*At3g53400*	*Fok*I	190, 30	220	220	GGTAGACAACAAAAAAATGGATCTTTGGAT CAACACCTCAAAGCCCATAGTAG
LCS304	*At3g51530*	*Hin*fI	168, 130	ND	298	GACGGCGATATGACTAGAGAAGAAC TCCACGGTTGACTGAGAAGAG
GLL340	*At3g52510*	*Apo*I	399, 295, 47	399, 342	399, 342	AAAAGGAGAAAGAGGAAGAAGATACTACTG CATTTTACTTTTAGGCGTTGAGGTGAC
T8M16	*At3g56770*	*Apo*I	309, 96	405	405	CCCGACAAAGTGATTATCAGCTTCAGAG CATATTCTTCAGTACTCGTCTAAACATGC

ND, not determined.

[a] Underlined nucleotide is the introduced mutation for this dCAPS marker (Michaels and Amasino 1998; Neff *et al.* 1998).

et al. 1995) for mapping using published genetic markers (Konieczny and Ausubel 1993; Bell and Ecker 1994) and newly developed PCR-based markers (Table 1). New markers were identified by PCR amplifying and sequencing ~1.6-kbp genomic DNA fragments from Ws-2 and identifying polymorphisms that altered restriction enzyme recognition sites. To ensure that those individuals in the mapping population that exhibited a short root on IBA or 2,4-DB had restored sensitivity, rather than merely delayed germination or general growth defects, progeny from mapping plants were retested on PNS with and without 10 μm IBA or 2 μm 2,4-DB.

Identification of the *pdr9-72* mutation: A candidate gene (*PDR9/ABCG37/At3g53480*) within the MS72 mapping interval was examined for defects in the mutant. Genomic DNA extracted from MS72 mutant plants was amplified using six oligonucleotide pairs [PDR9-1 (5′-CAACGTTTTCTCTGAT TACAC-3′) and PDR9-2 (5′-GCTACCAACGCCCTGACAAC GAG-3′); PDR9-3 (5′-AAAGCCAGGAAGGTTAGTAGTTG-3′) and PDR9-4 (5′-CATAGGATTCTGGGGCGGGTTG-3′); PDR9-5 (5′-TCAACCCGCCCCAGAATCCTATG-3′) and PDR9-6 (5′-TG AAGAGCACAGTGAAACCCAACAAG-3′); PDR9-7 (5′-ACTGG GTATCATTATGTGCCTTGTTGG-3′) and PDR9-8 (5′-CTCT TGCGTCTAGCCCCGTCGTTG-3′); PDR9-9 (5′-CCGTCGATT ATATTTATGGATGAGC-3′) and PDR9-10 (5′-ATGAAGTTTGG CGTGATGGAGAC-3′); PDR9-11 (5′-ATCGGTTTCTATCCTTC AGCCTAC-3′) and PDR9-12 (5′-AGTTAACTATTGCCCATTTT TCTTGATTTG-3′)]. The resulting overlapping fragments covered the gene from 282 bp upstream of the putative translation start site to 358 bp downstream of the stop codon. Amplification products were purified using a QIAquick PCR purification kit (QIAGEN) and sequenced directly (Lone Star Labs, Houston) with the primers used for amplification.

Auxin accumulation assays: Primary root tips (5 mm) from 8-day-old light-grown Col-0, *aux1-7*, *pdr9-1*, *pdr9-2*, and *pdr9-72* seedlings were excised and incubated in 40 μl uptake buffer (20 mm 2-[*N*-morpholino]ethanesulfonic acid, 10 mm sucrose, 0.5 mm $CaSO_4$, pH 5.6) for 10 min at room temperature. An additional 40 μl uptake buffer containing radiolabeled auxins was added to a final concentration of 25 nm [^{3}H]-indole-3-acetic acid (20 Ci/mmol; American Radiolabeled Chemicals, St. Louis) or 25 nm [^{3}H]-indole-3-butyric acid (25 Ci/mmol; American Radiolabeled Chemicals) and incubated at room temperature for 1 hr. Root tips were briefly rinsed with three changes of uptake buffer and placed in a fourth change of uptake buffer. After 20 min, root tips were removed from the buffer, placed in 3 ml Cytoscint scintillation cocktail (Fisher Scientific), and analyzed by scintillation counting.

RESULTS

***sar3* fails to suppress *ibr5* phenotypes:** Both *sar1* (Cernac *et al.* 1997) and *sar3* (Parry *et al.* 2006) were isolated as *axr1* suppressors and display pleiotropic phenotypes. An additional *sar3* allele (*mos3*) was isolated in a screen for suppressors of a mutant that displays constitutive pathogenesis phenotypes (Zhang and Li 2005), and another *sar1* allele (*atnup160-1*) was isolated in a screen for mutants with impaired cold responsiveness (Dong *et al.* 2006). *SAR1* (*At1g33410*) and *SAR3* (*At1g80680*) encode nucleoporins related to human NUP160 and NUP96, respectively, and may suppress *axr1* phenotypes by excluding the Aux/IAA transcriptional repressors, which are stabilized in *axr1* (Gray *et al.* 2001; Zenser *et al.* 2001), from the nucleus (Parry *et al.* 2006). Because *ibr5* differs from *axr1* in that it appears to affect auxin responses without stabilizing Aux/IAA proteins (Strader *et al.* 2008), we were interested in determining whether loss of *SAR3* could suppress *ibr5* phenotypes. We crossed *ibr5-1* to the *sar3-3* T-DNA disruption allele and isolated the double mutant. For controls, we crossed *axr1-3* and *tir1-1* to *sar3-3* and isolated the corresponding double mutants. As previously reported for *sar3-1* (Parry *et al.* 2006), we

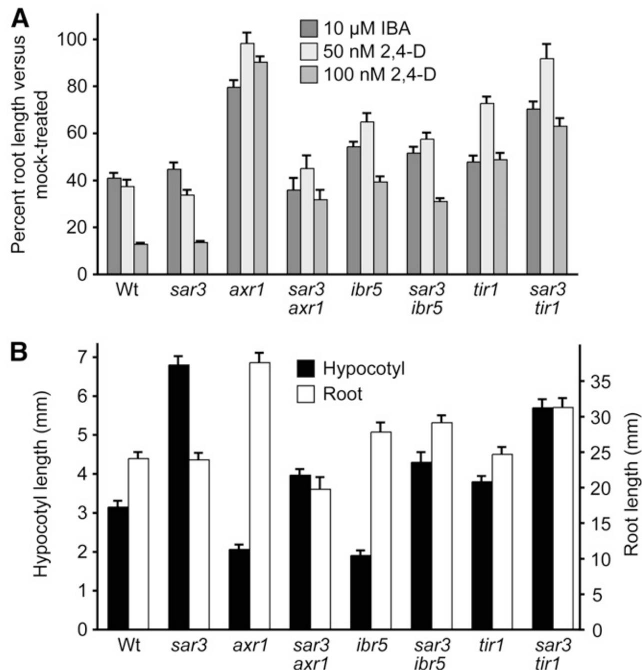

FIGURE 1.—*sar3 ibr5* auxin response. (A) Normalized primary root lengths of 8-day-old Col-0 (Wt), *sar3-3*, *axr1-3*, *sar3-3 axr1-3*, *ibr5-1*, *sar3-3 ibr5-1*, *tir1-1*, and *sar3-3 tir1-1* seedlings grown under yellow-filtered light at 22° on medium supplemented with the indicated concentrations of IBA or 2,4-D. Data were normalized by comparing auxin-treated root lengths to the mean root length on mock-supplemented media ($n \geq 13$). (B) Hypocotyl and root lengths of seedlings grown at 22° under continuous yellow-filtered light on unsupplemented medium ($n \geq 15$). Error bars represent standard errors of the means.

found that the *sar3-3* allele restored *axr1-3* 2,4-D responsiveness (Figure 1A). Similarly, *sar3-3* restored *axr1-3* IBA responsiveness (Figure 1A). In contrast, *sar3-3* did not fully rescue the reduced responses of *ibr5* to the inhibitory effects of 2,4-D or IBA on root elongation (Figure 1A). Unexpectedly, *sar3* appeared to enhance, rather than suppress, *tir1* auxin-response defects (Figure 1A).

Light-grown *axr1* (Lincoln *et al.* 1990), *ibr5* (Monroe-Augustus *et al.* 2003), and *tir1* (Ruegger *et al.* 1998) have long primary roots in the absence of exogenous hormone. We found that *sar3* suppressed the *axr1* long primary root but did not decrease *ibr5* or *tir1* root lengths (Figure 1B). Light-grown *sar3-3* exhibits a long hypocotyl (Parry *et al.* 2006), whereas *axr1* (Lincoln *et al.* 1990) and *ibr5* (Monroe-Augustus *et al.* 2003) have short hypocotyls in the light, and *tir1* hypocotyls are similar in length to wild type when grown at 20° (Ruegger *et al.* 1998). We found that the *sar3 axr1*, *sar3 ibr5*, and *sar3 tir1* double-mutant hypocotyl lengths were intermediate compared to their respective parents (Figure 1B). In contrast, *sar3* early flowering was not suppressed by *ibr5* or *tir1* (data not shown).

Because *sar3* did not restore *ibr5* or *tir1* auxin responsiveness, we concluded that the defects resulting from disruption of the SAR3 nucleoporin were likely to specifically affect AXR1 function rather than generally affect all mutants with decreased auxin responsiveness. The failure of the *axr1* suppressor *sar3-3* to suppress *ibr5* auxin-response defects is consistent with IBR5 acting downstream of Aux/IAA repressor degradation (Strader *et al.* 2008) and suggested that a mutant screen for *ibr5* suppressors might reveal novel factors involved in auxin responses in general and the IBR5 pathway in particular.

Isolation of *ibr5* suppressors with restored IBA responsiveness: IBA inhibits primary root elongation in Arabidopsis (Zolman *et al.* 2000), and *ibr5* mutants exhibit a long root on exogenous IBA (Zolman *et al.* 2000; Monroe-Augustus *et al.* 2003). To isolate suppressors of *ibr5* IBA-resistant root growth, we generated 32 pools of EMS-mutagenized *ibr5-1* seed and screened ~48,000 of the resultant M_2 progeny for seedlings with restored IBA responsiveness. We selected 371 putative suppressor mutants exhibiting a short root on IBA. Of these, 62 died, 32 were infertile, and 23 were wild-type contaminants. Progeny of the 254 remaining putative mutants were rescreened for restored sensitivity to IBA; 212 of those mutants had notably short roots with or without auxin and displayed a percentage of root elongation on IBA *vs.* unsupplemented medium similar to *ibr5*. These mutants were discarded, as mutations in these lines may have affected general seedling growth rather than auxin responsiveness. The 42 mutants that displayed a percentage of root elongation on IBA-supplemented *vs.* unsupplemented medium similar to wild type were retained as *ibr5* suppressors. Some of these mutants displayed partial defects in root elongation even without auxin. Nine of the IBA-sensitive suppressor lines were characterized in detail (Figures 2 and 3). Because these mutants came from eight different M_2 seed pools (Table 2), the mutants represent at least eight independent mutagenic events. All 42 suppressors retained the original *ibr5-1* lesion and were thus expected to be extragenic, as the *ibr5-1* parent contains an early stop codon (Monroe-Augustus *et al.* 2003).

Additional auxin phenotypes of *ibr5* suppressors: The basis of the suppressor screen was restored sensitivity to the inhibitory effect of IBA on root elongation (Figure 2B). In addition to inhibiting primary root growth, IBA promotes lateral root production in Arabidopsis (Zolman *et al.* 2000), and *ibr5* produces fewer lateral roots than wild type with and without auxin treatment (Monroe-Augustus *et al.* 2003). All nine suppressors restored IBA-responsive lateral root production to *ibr5*, and one suppressor (MS5) appeared to be more sensitive than wild type to IBA-promoted lateral root production (Figure 2C).

In Arabidopsis, genetic evidence suggests that the auxin activity of IBA requires carboxyl sidechain shortening to IAA in peroxisomes (Zolman *et al.* 2000, 2007). *ibr5* is resistant to inhibition of root elongation caused by either IBA or IAA, reflecting general auxin resistance

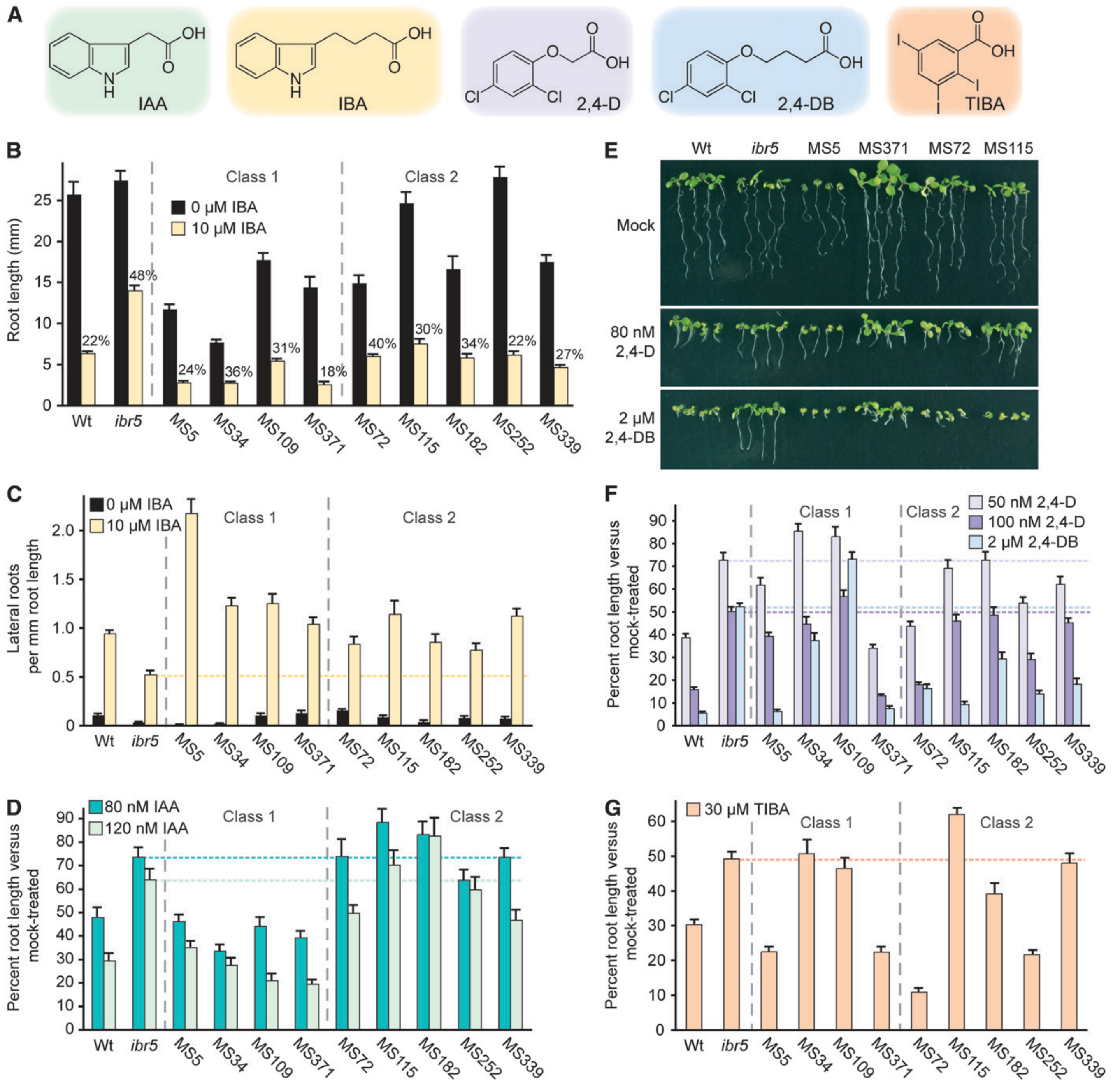

Figure 2.—Auxin responses of *ibr5* suppressors. (A) Compounds used to monitor auxin responses. IAA is a naturally occurring auxin, IBA is a naturally occurring IAA precursor, 2,4-D is a synthetic auxin, 2,4-DB is a 2,4-D precursor, and TIBA is a synthetic auxin transport inhibitor. (B) Primary root lengths of 8-day-old Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings grown under yellow-filtered light at 22° on medium supplemented with ethanol (0 μm IBA) or 10 μm IBA ($n \geq 17$). Numbers above bars represent the percentage of root length on IBA compared to the control. (C) Number of lateral roots per millimeter of root length 4 days after transfer of 4-day-old seedlings to medium supplemented with either ethanol (0 μm IBA) or 10 μm IBA ($n \geq 13$). (D) Primary root lengths of 8-day-old Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings grown under yellow-filtered light at 22° on medium supplemented with 80 or 100 nm IAA shown normalized to the mean root length of each genotype on medium lacking IAA ($n \geq 11$). (E) Photograph of 8-day-old Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings grown under white light at 22° on medium supplemented with ethanol (mock), 80 nm 2,4-D, or 2 μm 2,4-DB. (F) Normalized primary root lengths of 8-day-old Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings grown under yellow-filtered light at 22° on medium supplemented with 50 nm 2,4-D, 100 nm 2,4-D, or 2 μm 2,4-DB ($n \geq 16$). (G) Normalized primary root lengths of 8-day-old Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings grown under yellow-filtered light at 22° on medium supplemented with 30 μm TIBA ($n \geq 17$). Error bars represent standard errors of the means.

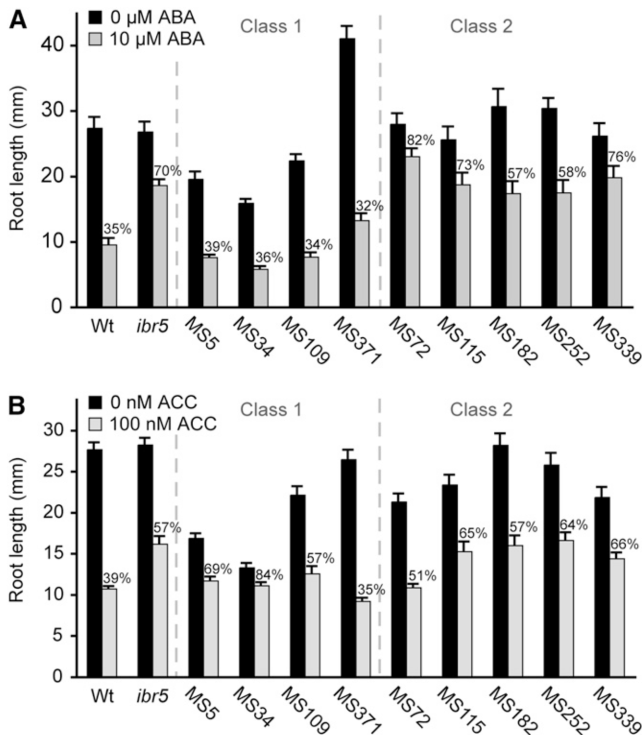

FIGURE 3.—ABA and ACC response of *ibr5* suppressors. (A) Primary root lengths of Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings 4 days after transfer of 4-day-old seedlings to medium supplemented with either ethanol (0 μM ABA) or 10 μM ABA ($n \geq 13$). The percentage of root length of seedlings transferred to ABA compared to control seedlings is indicated above the bars. (B) Primary root lengths of 10-day-old Col-0 (Wt), *ibr5-1*, and various *ibr5* suppressor (MS lines) seedlings grown under white light at 22° on medium supplemented with either ethanol (0 nM ACC) or 100 nM ACC ($n \geq 16$). Error bars represent standard errors of the means.

(MONROE-AUGUSTUS *et al.* 2003). We examined the *ibr5* suppressors on IAA and found that MS5, MS34, MS109, and MS371 exhibited restored IAA-responsive root elongation inhibition, while the remaining five mutants remained IAA resistant (Figure 2D) despite displaying restored IBA responsiveness (Figure 2, B and C). We designated the mutants exhibiting restored response to both IBA and IAA as class 1 mutants and those exhibiting restored response to IBA but not to IAA as class 2 mutants (Figure 2, B–D; Table 2).

We also examined the suppressor responses to the auxinic compounds 2,4-D and 2,4-DB (Figure 2A). As with IBA conversion to IAA, 2,4-DB requires chain shortening to 2,4-D for auxin activity (HAYASHI *et al.* 1998). We found that MS371, MS72, and MS252 displayed nearly wild-type 2,4-D responsiveness, whereas the remaining suppressors displayed 2,4-D resistance similar to *ibr5* (Figure 2, E and F). In contrast, most suppressors restored *ibr5* 2,4-DB responsiveness (Figure 2, E and F); only MS34 and MS109 displayed 2,4-DB resistance similar to *ibr5*, and MS182 showed intermediate 2,4-DB responsiveness (Figure 2F).

In addition to resistance to the effects of auxin and auxinic compounds, *ibr5* is resistant to the effects of the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA; MONROE-AUGUSTUS *et al.* 2003). We tested the *ibr5* suppressors on TIBA and found that MS5, MS371, MS72, and MS252 had restored responsiveness to 30 μM TIBA, whereas MS34, MS109, MS115, and MS339 remained resistant, and MS182 displayed an intermediate phenotype (Figure 2G).

ABA responsiveness of *ibr5* suppressors: Mutations in *IBR5* confer resistance to the inhibitory effects of the phytohormone ABA on root elongation (MONROE-AUGUSTUS *et al.* 2003). We examined the nine IBA-sensitive *ibr5* suppressors to determine if they also restored ABA responses and found that all four class 1 mutants (MS5, MS34, MS109, and MS371) exhibited restored ABA-induced root elongation inhibition whereas all five class 2 mutants remained resistant to the inhibitory effects of ABA on root elongation, although comparison of the percentage of elongation on ABA *vs.* on unsupplemented medium revealed that some mutants in the latter class were no longer as dramatically ABA resistant as the *ibr5* parent (Figure 3A).

Ethylene responsiveness of *ibr5* suppressors: Like several other auxin-resistant mutants (STEPANOVA *et al.* 2007), *ibr5* is weakly resistant to the inhibitory effects of the ethylene precursor ACC on root elongation (Figure 3B). We examined the effects of ACC on the nine *ibr5* suppressors and found that only one line, MS371, restored wild-type ACC responsiveness to *ibr5* (Figure 3B). The other eight suppressors remained resistant to the inhibitory effects of ACC on root elongation (Figure 3B).

A mutation in the gene encoding the PDR9/ABCG37 2,4-D transporter suppresses a subset of *ibr5* phenotypes: MS72 was isolated as an *ibr5* suppressor on IBA (Figure 2B), but subsequent testing revealed that the suppression of *ibr5* auxin-resistant root elongation was more apparent on 2,4-DB and 2,4-D than on IBA or IAA (Figure 2). We used restored 2,4-DB responsiveness to map the recessive *ibr5*-suppressing lesion in MS72 to a 215-kbp region on the lower arm of chromosome 3 between LCS320 and LCS302 (Figures 4 and 5A). This region contains *PLEIOTROPIC DRUG RESISTANCE9* (*PDR9/ABCG37*), a dominant mutation of which has been identified as *eta4* in a *tir1* enhancer screen (ITO and GRAY 2006). In contrast to the gain-of-function *eta4/pdr9-1* mutation, which enhances *tir1* 2,4-D resistance, loss of PDR9 function in the *pdr9-2* T-DNA insertion allele results in 2,4-D hypersensitivity (ITO and GRAY 2006), making *pdr9* a reasonable candidate for an *ibr5* suppressor. We PCR amplified and sequenced *PDR9* from MS72 genomic DNA and identified a G-to-A base change at position 3072 (where the A of the ATG is at position 1) that causes a Gly-to-Asp missense mutation in a conserved amino acid (Figure 5, B and D). Because the identified nucleotide change destroys an *Msp*I site, we confirmed the mutation by amplifying and digesting

TABLE 2

Classification of *ibr5* suppressors

Class[a]	Isolate	M_2 pool	Hormone response in root elongation[b]						
			IBA	IAA	2,4-D	2,4-DB	TIBA	ABA	ACC
—	Wild type	—	S	S	S	S	S	S	S
—	*ibr5-1*	—	R	R	R	R	R	R	R
1	MS5	3	S	S	R	S	S	S	R
1	MS34	4	S	S	R	R	R	S	R
1	MS109	6	S	S	R	R	R	S	R
1	MS371	19	S	S	S	S	S	S	S
2	MS72	5	I	R	S	S	S	R	R
2	MS115	6	S	R	R	S	R	R	R
2	MS182	12	S	R	R	I	I	R	R
2	MS252	16	S	R	S	S	S	R	R
2	MS339	18	S	R	R	S	R	R	R

[a] Class 1 suppressors restore IBA-, IAA-, and ABA-responsive root elongation inhibition to *ibr5*; class 2 suppressors restore IBA responses but remain IAA and ABA resistant.

[b] S indicates that the line was sensitive (similar to wild type); R indicates that the line was resistant (similar to *ibr5-1*); I indicates that the line displayed intermediate resistance between wild type and *ibr5-1*. Data are summarized from Figures 2 and 3.

this region of *PDR9* from wild-type and mutant genomic DNA. We named the identified mutation in MS72 *pdr9-72*.

To test whether the observed MS72 phenotypes were caused by the *pdr9-72* lesion, we crossed MS72 to wild type and isolated the homozygous *pdr9-72/pdr9-72* mutant in a wild-type *IBR5/IBR5* background. We then crossed *pdr9-72* to the previously described loss-of-function *pdr9-2* allele (Ito and Gray 2006) and tested 2,4-D responsiveness in the *pdr9-2/pdr9-72* F_1 progeny. We found that *pdr9-72* failed to complement the *pdr9-2* hypersensitivity to root growth inhibition by 2,4-D (Figure 5E). Because both *pdr9-2* (Ito and Gray 2006) and *pdr9-72* (data not shown) are recessive, this lack of complementation indicates that the lesion that we identified in *pdr9-72* confers a *PDR9* loss of function.

To verify that loss of *PDR9* could suppress *ibr5* auxin resistance in MS72, we crossed the *pdr9-2* loss-of-function mutant (Ito and Gray 2006) to *ibr5-1* and compared *ibr5 pdr9-2* auxin responses to those of *ibr5 pdr9-72* and the single mutants. We found that the *pdr9-72* and *pdr9-2* single mutants were similarly hypersensitive to the inhibitory effects of 2,4-D, 2,4-DB, and TIBA on primary root elongation and that *pdr9-2* and *pdr9-72*

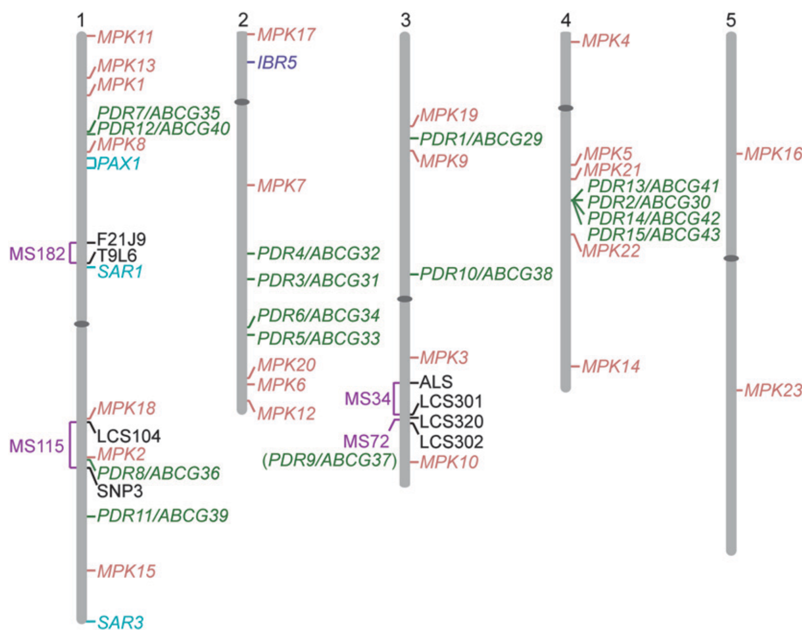

Figure 4.—Map positions of IBA-sensitive *ibr5* suppressors. Approximate map positions of molecular markers (in black type), *IBR5* (in dark purple type; Monroe-Augustus *et al.* 2003), the 23 *MPK* genes (in tan type; Tena *et al.* 2001), and the 15 *PDR/ABCG* genes (in green type; Verrier *et al.* 2008) are shown to the right of each chromosome. Map positions of the previously isolated suppressors *SAR1* (Parry *et al.* 2006), *SAR3* (Parry *et al.* 2006), and the *pax1* mapping interval (Tanimoto *et al.* 2007) are in aqua type. The interval to which each *ibr5*-suppressing mutation maps is shown to the left of the chromosomes in light purple type. The *ibr5*-suppressing mutation in MS182 maps to chromosome 1 south of F21J9 (Leclere *et al.* 2004) and north of T9L6 (Magidin 2002) with 5/120 and 1/120 recombinants, respectively. The recessive *ibr5*-suppressing mutation in MS115 maps to chromosome 1 south of LCS104 and north of SNP3 with 16/482 and 5/270 recombinants, respectively. The recessive *ibr5*-suppressing mutation in MS34 maps to chromosome 3 south of ALS (http://www.arabidopsis.org) and north of LCS301 with 8/76 and 5/76 recombinants, respectively. And the recessive *ibr5*-suppressing mutation in MS72 (*pdr9-72*) maps south of LCS320 and north of LCS302 (Figure 5).

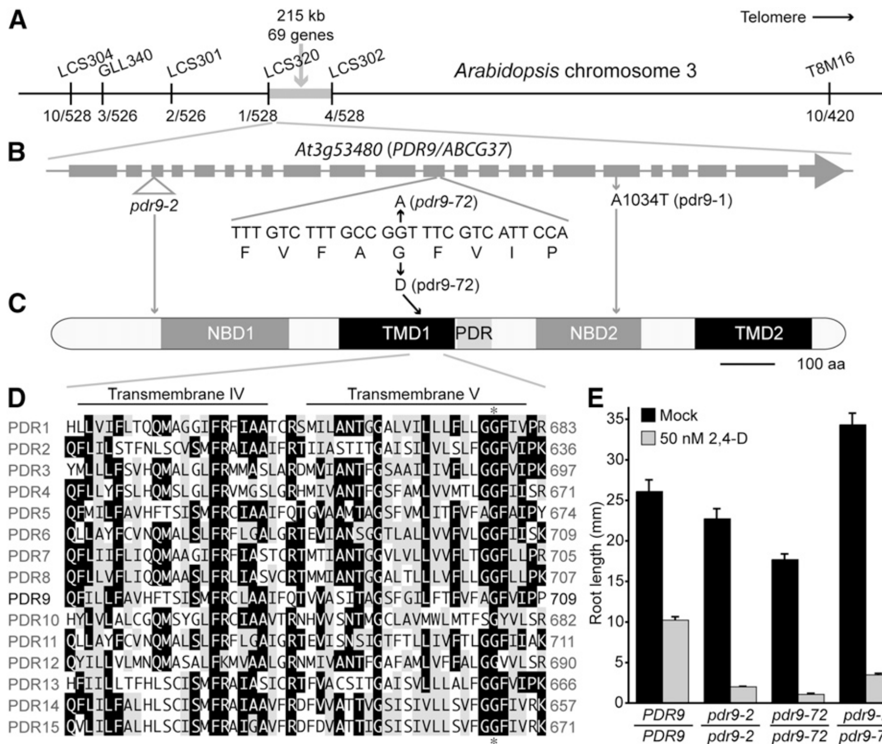

FIGURE 5.—Positional cloning of *PDR9/ABCG37*. (A) Recombination mapping with PCR-based markers T8M16, LCS304, GLL340, LCS301, LCS320, and LCS302 (Table 1) localized the *ibr5*-suppressing mutation in MS72 between LCS320 and LCS302 with 1/526 north and 4/528 south recombinants. (B) Examination of the *PDR9* (*At3g53480*) gene in this region revealed a G-to-A mutation at position 3072 in MS72 DNA that destroys an *Msp*I site and results in a Gly704-to-Asp substitution. *pdr9-2* carries a T-DNA insert in the third exon of *PDR9* (ITO and GRAY 2006). *pdr9-1* results in an Ala1034-to-Thr substitution (ITO and GRAY 2006). (C) PDR9 schematic based on output from the domain-predicting program SMART (SCHULTZ *et al.* 1998). PDR9 contains two NBDs, two TMDs each containing six transmembrane spans, and a PDR signature motif. (D) The *pdr9-72* mutation disrupts a conserved glycine in the fifth predicted transmembrane span of TMD1. The alignment shows the fourth and fifth predicted transmembrane spans of the 15 Arabidopsis PDR family members. Sequences were aligned using the MegAlign program (DNAStar, Madison, WI). Amino acid residues identical in at least eight sequences are against a solid background; chemically similar residues in at least eight sequences are shaded. The position of the *pdr9-72* mutation is indicated with an asterisk. (E) Complementation test showing primary root lengths of 8-day-old Col-0 wild-type (*PRD9/PDR9*), *pdr9-2/pdr9-2*, *pdr9-72/pdr72-2*, and *pdr9-2/pdr9-72* seedlings grown under yellow-filtered light at 22° on medium supplemented with ethanol (mock) or 50 nM 2,4-D. Error bars represent standard errors of the means ($n \geq 13$).

restored 2,4-D, 2,4-DB, and TIBA responsiveness to *ibr5* to a similar extent (Figure 6, A and B). As previously reported (ITO and GRAY 2006), *pdr9-2* responded similarly to wild type to the inhibitory effects of IBA on root elongation (Figure 6C). Moreover, we found that *pdr9-2* failed to restore *ibr5* root elongation inhibition in response to IBA (Figure 6C). However, both *pdr9-72* and *pdr9-2* appeared to be more sensitive than wild type to IBA-promoted lateral root production and at least partially restored *ibr5* IBA-induced lateral root induction (Figure 6D). Because *pdr9-72* responded similarly to the *pdr9-2* likely null allele (ITO and GRAY 2006) in these assays and because both alleles similarly restored *ibr5* responsiveness to 2,4-D, 2,4-DB, and TIBA (Figure 6, A and B), we concluded that the *pdr9-72* lesion reduced PDR9 function and was responsible for suppression of a subset of *ibr5* phenotypes in MS72.

To determine whether the gain-of-function *pdr9-1* allele (ITO and GRAY 2006) could enhance *ibr5* phenotypes, we crossed *pdr9-1* to *ibr5-1* and compared the resultant *ibr5 pdr9-1* double mutant to the single mutants and the previously described (ITO and GRAY 2006) *tir1 pdr9-1* mutant. As previously reported (ITO and GRAY 2006), the *pdr9-1* single mutant was resistant to the inhibitory effects of 2,4-D on primary root elongation (Figure 7A). In addition, we found that *pdr9-1* was resistant to 2,4-DB (Figure 7B) and slightly resistant to the auxin precursor IBA in both root elongation inhibition and lateral root promotion (Figure 7, C and D). In contrast to the heightened TIBA sensitivity of the *pdr9* loss-of-function alleles (Figure 6B), *pdr9-1* resembled wild type in sensitivity to the auxin transport inhibitor TIBA (Figure 7B). In the double mutants, we found that *pdr9-1* enhanced *ibr5-1* resistance to root elongation inhibition by 2,4-D (Figure 7A), conferring similar 2,4-D resistance as the *tir1 pdr9-1* double mutant. In addition to enhancing 2,4-D resistance, we found that *pdr9-1* enhanced *tir1* and *ibr5* resistance to 2,4-DB (Figure 7B) and IBA (Figure 7C) in root elongation inhibition. However, *pdr9-1* failed to enhance *tir1* or *ibr5* resistance to IBA in lateral root initiation (Figure 7D) or to TIBA in root elongation inhibition (Figure 7B) in the conditions tested.

Using an excised root-tip auxin transport assay, ITO and GRAY (2006) demonstrated that *pdr9-1* root tips accumulate less [^{14}C]-2,4-D than wild type, whereas *pdr9-2* root tips accumulate more [^{14}C]-2,4-D than wild type, consistent with a role for PDR9 in 2,4-D efflux that is supported by the root elongation phenotypes of the *pdr9* alleles on 2,4-D-supplemented media. Because we found that *pdr9* alleles also display altered IBA responsiveness (Figure 6D and Figure 7, C and D), we sought to determine whether PDR9 also might play a role in IBA efflux. We assessed [^{3}H]-IAA and [^{3}H]-IBA accumulation

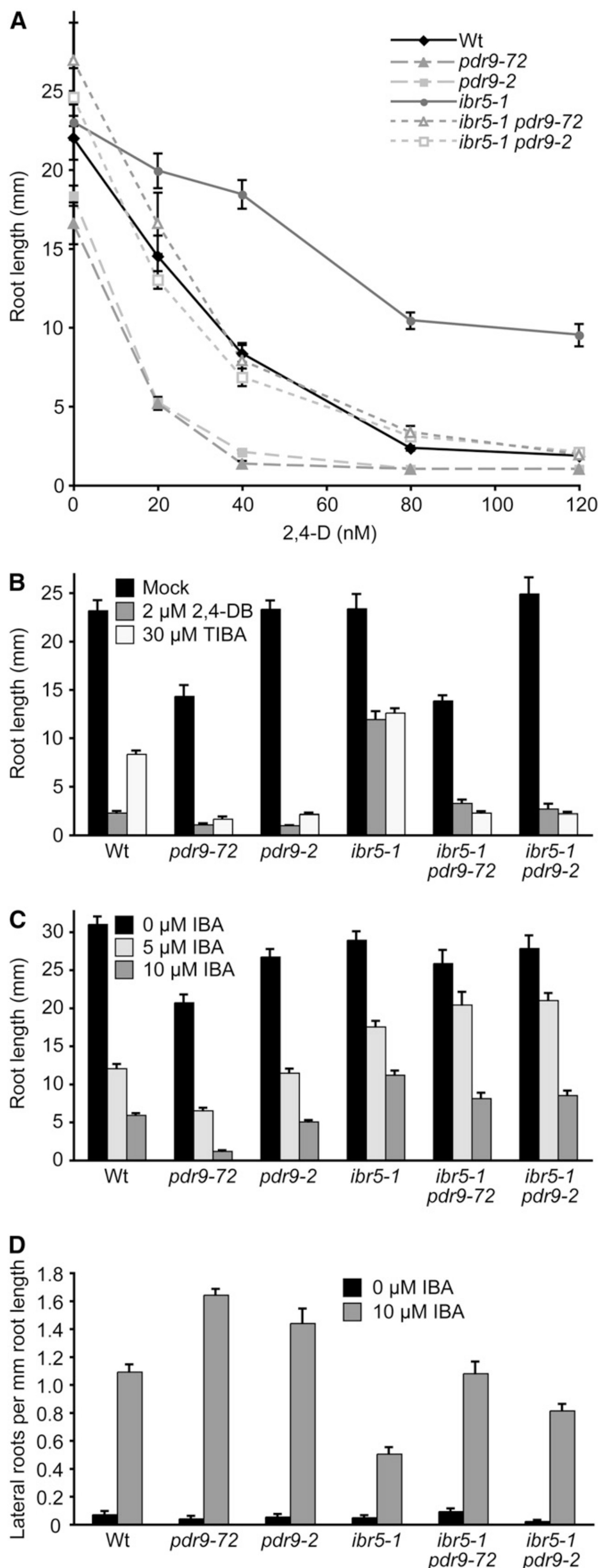

Figure 6.—*ibr5 pdr9-72* and *tir1 pdr9-2* auxin response. (A) Primary root lengths of 8-day-old Col-0 (Wt), *pdr9-72*, *pdr9-2*, *ibr5-1*, *ibr5-1 pdr9-72* (MS72), and *ibr5-1 pdr9-2* seedlings grown under yellow-filtered light at 22° on medium supplemented with ethanol (mock) or various concentrations of 2,4-D ($n \geq 9$). (B) Primary root lengths of 8-day-old seedlings grown under yellow-filtered light at 22° on medium supplemented with ethanol (mock), 2 μM 2,4-DB, or 30 μM TIBA ($n = 15$). (C) Primary root lengths of 8-day-old seedlings grown on medium supplemented with ethanol (0 μM IBA) or IBA ($n = 15$). (D) Lateral roots were counted 4 days after transfer of 4-day-old seedlings to medium supplemented with either 0 (ethanol control) or 10 μM IBA ($n = 12$). Error bars represent standard errors of the means.

in excised root tips from 8-day-old seedlings. As previously reported (Ito and Gray 2006), we found that *pdr9* mutants displayed wild-type [^{3}H]-IAA accumulation in this assay (Figure 8A). We included the *aux1* IAA influx mutant as a control and found reduced [^{3}H]-IAA accumulation in *aux1* root tips (Figure 8A), as expected. In addition, we found that *aux1* mutant root tips displayed wild-type [^{3}H]-IBA accumulation (Figure 8B), consistent with the normal [^{3}H]-IBA transport reported in *aux1* roots (Rashotte *et al.* 2003) and the inability of excess IBA to compete with [^{3}H]-IAA uptake by AUX1 expressed in Xenopus oocytes (Yang *et al.* 2006). In contrast to *aux1*, we found that root tips of both *pdr9-2* and *pdr9-72* clearly hyperaccumulated [^{3}H]-IBA (Figure 8B). Moreover, we observed a small but statistically significant reduction in [^{3}H]-IBA accumulation in *pdr9-1* root tips (Figure 8B). These results are consistent with the possibility that PDR9 facilitates IBA efflux from root cells.

Mapping second-site mutations in additional *ibr5* suppressors: In addition to MS72, we used recombination mapping with PCR-based markers to localize three additional recessive *ibr5*-suppressing mutations (MS34, MS115, and MS182) to three distinct chromosomal regions. None of the mapped suppressors appeared to be allelic, as none mapped to the same interval (Figure 4). In addition, none of the mapping intervals include the previously isolated auxin-resistance-suppressing mutations *pax1* (Tanimoto *et al.* 2007), *sar1*, or *sar3* (Parry *et al.* 2006), suggesting that additional novel *ibr5*-suppressing pathways remain to be identified. Map-based cloning of the defective genes in these *ibr5*-suppressing mutants is ongoing.

DISCUSSION

***PDR9* role in auxin response:** PDR subfamily members of ABC transporters are found only in fungi and plants and, like other full-sized ABC transporters, contain two apparent nucleotide-binding domains (NBD) and two transmembrane domains (TMD) consisting of six membrane-spanning sequences each (reviewed in Crouzet *et al.* 2006; Verrier *et al.* 2008). Fifteen *PDR/ABCG* genes have been identified in Arabidopsis (Sanchez-Fernandez *et al.* 2001; Martinoia

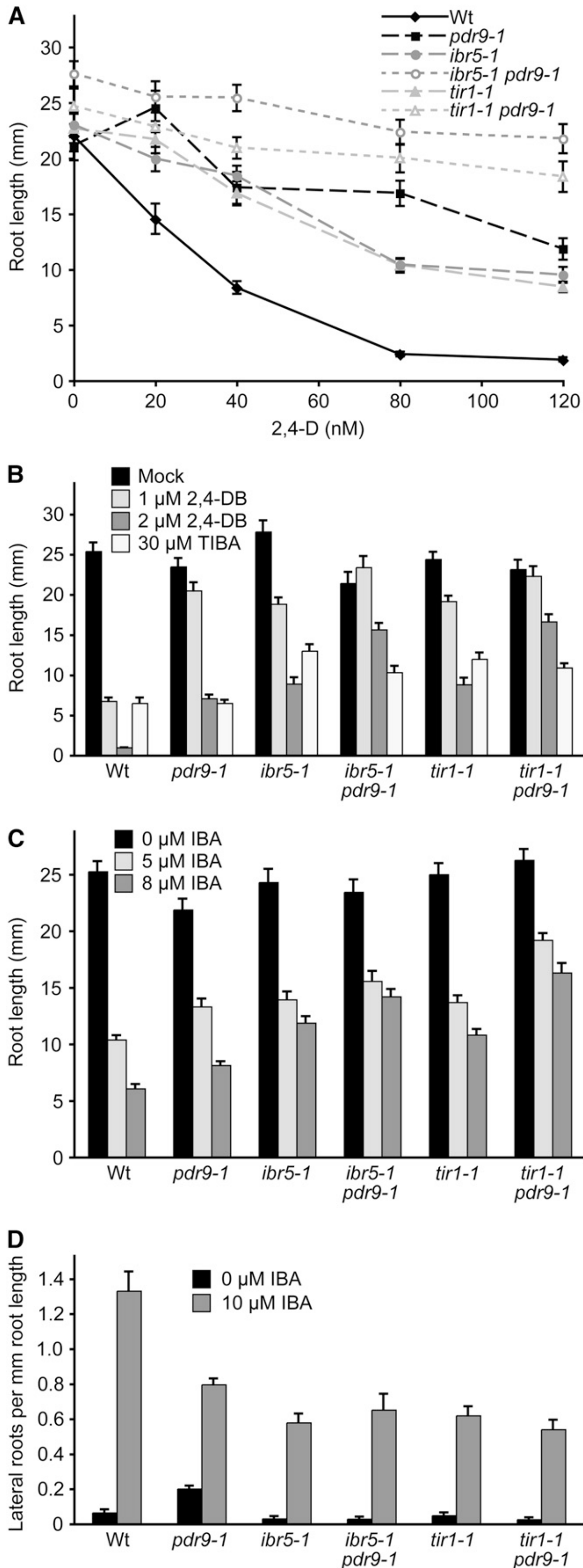

Figure 7.—*ibr5 pdr9-1* and *tir1 pdr9-1* auxin response. (A) Primary root lengths of 8-day-old Col-0 (Wt), *pdr9-1, ibr5-1, ibr5-1 pdr9-1, tir1-1,* and *tir1-1 pdr9-1* seedlings grown under yellow-filtered light at 22° on medium supplemented with ethanol (mock) or various concentrations of 2,4-D ($n = 12$). (B) Primary root lengths of 8-day-old seedlings grown under yellow-filtered light at 22° on medium supplemented with ethanol (mock) or various concentrations of 2,4-DB or 30 μm TIBA ($n \geq 12$). (C) Primary root lengths of 8-day-old seedlings grown on medium supplemented with ethanol (0 μm IBA) or various concentrations of IBA ($n \geq 12$). (D) Lateral roots were counted 4 days after transfer of 4-day-old seedlings to medium supplemented with either 0 (ethanol control) or 10 μm IBA ($n = 12$). Error bars represent standard errors of the means.

et al. 2002; van den Brule and Smart 2002; Verrier *et al.* 2008), but only a few have been functionally characterized in genetic studies. PDR9/ABCG37 is a 2,4-D efflux facilitator localized in the plasma membrane (Ito and Gray 2006); the gain-of-function *pdr9-1* mutant is 2,4-D resistant and hypoaccumulates 2,4-D, whereas the loss-of-function *pdr9-2* mutant displays increased 2,4-D sensitivity and hyperaccumulates 2,4-D (Ito and Gray 2006).

We isolated the *pdr9-72* mutant as a class 2 *ibr5* suppressor (Figure 2; Table 2). To our knowledge, this is the first example of a mutation in an auxin transporter suppressing the phenotype of an auxin-resistant mutant. The identical 2,4-D, 2,4-DB, and TIBA hypersensitivity of the *pdr9-2* likely null allele (Ito and Gray 2006) and the *pdr9-72* allele that we isolated as an *ibr5* suppressor (Figure 6, A and B) suggests that the pdr9-72 Gly704-to-Asp change abolishes PDR9 function.

Although *ibr5* is 2,4-D resistant, it is not completely 2,4-D insensitive, as it responds to high 2,4-D concentrations (Monroe-Augustus *et al.* 2003; Strader *et al.* 2008). *pdr9* may counteract *ibr5* 2,4-D resistance by allowing 2,4-D to accumulate to higher levels within cells. We envision that when PDR9 is disrupted, applied 2,4-D is less efficiently removed from cells (Ito and Gray 2006), and the consequent 2,4-D hyperaccumulation allows the *ibr5 pdr9* double mutant to respond to 2,4-D similarly to wild type (Figure 6A).

We found that *pdr9* TIBA responses resembled previously reported responses of *pdr9* alleles to the auxin transport inhibitor 1-*N*-naphthylphthalamic acid (NPA). The loss-of-function *pdr9-2* is hypersensitive to NPA (Ito and Gray 2006) and TIBA (Figure 6B), whereas the gain-of-function *pdr9-1* responds similarly to wild type to NPA (Ito and Gray 2006) and TIBA (Figure 7B). Because *pdr9-2* and *pdr9-72* mutants are TIBA hypersensitive and completely abolish the TIBA resistance of *ibr5* (Figure 6B), it is possible that the PDR9 transporter may efflux TIBA in addition to 2,4-D. Why the gain-of-function *pdr9-1* allele is resistant to some (2,4-D and 2,4-DB) but not all compounds to which the loss-of-function *pdr9-2* allele is hypersensitive (2,4-D, 2,4-DB, TIBA, and NPA) remains unexplained.

Loss of PDR9 does not restore all *ibr5* defects. Although *pdr9-72* restores *ibr5* responsiveness to 2,4-D, 2,4-

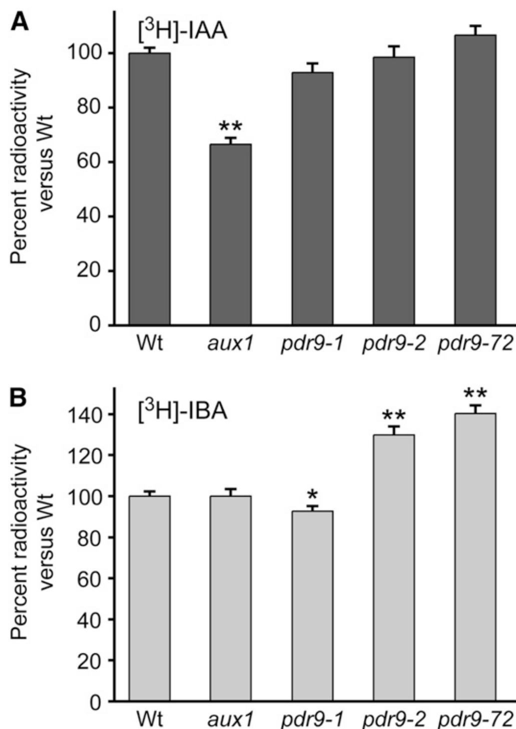

FIGURE 8.—[^{3}H]-IAA and [^{3}H]-IBA accumulation in *pdr9* mutants. Root tips of 8-day-old Col-0 (Wt), *aux1-7*, *pdr9-1*, *pdr9-2*, and *pdr9-72* seedlings were incubated for 1 hr in buffer containing 25 nM [^{3}H]-IAA (A) or 25 nM [^{3}H]-IBA (B), rinsed three times, and incubated for an additional 20 min in buffer. Root tips were then removed and analyzed by scintillation counting. Data were averaged from two (A) or four (B) independent experiments, each with eight replicates of five root tips of each genotype. Data were normalized by comparison to the mean radioactivity of wild-type samples, which ranged from 13,083 to 15,406 cpm for the [^{3}H]-IAA experiments (A) and from 17,144 to 22,167 cpm for the [^{3}H]-IBA experiments (B). Error bars represent standard errors of the means, and asterisks indicate significant differences from wild type in two-tailed *t*-tests assuming equal variance (*$P \leq 0.01$; **$P \leq 0.001$).

DB, and TIBA, responses to IAA, ABA, and ACC appear largely unaffected (Figures 2 and 3). The IBA response of *pdr9* is more complex. We isolated MS72 (*ibr5-1 pdr9-72*) in a screen for *ibr5* suppressors displaying a short root when grown on IBA, but subsequent analyses revealed that our initial MS72 line displayed a short root even on unsupplemented medium, which undoubtedly contributed to MS72 isolation. In contrast, the *pdr9-2* root elongates normally and is not hypersensitive to IBA-induced root elongation inhibition (Figure 6; ITO and GRAY 2006). Moreover, *pdr9-2/pdr9-72* seedlings were 2,4-D hypersensitive but did not display root elongation defects on unsupplemented medium (Figure 5E), indicating that the short root of the initial MS72 line was likely caused by extraneous recessive mutations. Interestingly, however, both *pdr9-2* and *pdr9-72* mutants displayed heightened sensitivity to IBA in lateral root induction and partially restored *ibr5* lateral rooting defects (Figure 6D), and the *pdr9-1* mutant displayed slight resistance to IBA in root elongation inhibition under our conditions (Figure 7C), consistent with the possibility that PDR9 effluxes substrates in addition to 2,4-D. Indeed, we found that root tips of both *pdr9-2* and *pdr9-72* hyperaccumulated [^{3}H]-IBA and the gain-of-function *pdr9-1* allele displayed slightly reduced [^{3}H]-IBA accumulation in an auxin transport assay, suggesting that PDR9 may promote IBA efflux.

***ibr5* suppressors restore distinct subsets of *ibr5* phenotypes:** We identified and characterized *ibr5* suppressors with the anticipation that analysis of the genes defective in these suppressors will help elucidate the role of IBR5 in auxin, ABA, and ethylene responses. IBR5 is a putative MAP kinase phosphatase (MONROE-AUGUSTUS *et al.* 2003), and IBR5 phosphatase activity appears to be required for full auxin and ABA responsiveness (STRADER *et al.* 2008). Although we expect that mutation of a substrate MAP kinase might suppress some *ibr5* defects, *MPK2* and *MPK18* are the only *MPK* genes in or near our current *ibr5* suppressor mapping intervals (Figure 4), and these genes are not mutated in MS115 (data not shown), demonstrating that there are means to restore *ibr5* hormone responsiveness that do not involve *MPK* mutations. Although additional backcrossing will be needed to ensure that all of the phenotypes observed in this initial analysis result from disruptions in single loci, the diversity of *ibr5* suppressor phenotypes (Table 2) suggests that several mechanisms can restore IBA responsiveness to *ibr5*.

We found that all of the suppressor mutants that restored *ibr5* root elongation inhibition in response to IBA (Figure 2B) also restored *ibr5* defects in IBA-responsive lateral root production (Figure 2C). We classified the suppressors on the basis of the response to the natural auxin IAA (Table 2). The class 1 mutants (MS5, MS34, MS109, MS371) restored IAA-responsive root elongation inhibition to *ibr5*, whereas the class 2 mutants (MS72, MS115, MS182, MS252, MS339) remained IAA resistant.

Although the *ibr5* suppressors can be divided into two broad classes, mutants within each class have varied phenotypes, suggesting that they restore auxin responses differently from one another. All of the class 1 mutants regained the ability to respond to ABA, but only MS371 exhibited restored response to all hormones tested. Moreover, MS371 was the only suppressor that fully restored *ibr5* responses to the ethylene precursor ACC (Figure 3B). These data suggest that the gene disrupted in MS371 might act closely with *IBR5*.

MS109 and MS34 displayed restored response to naturally occurring auxins (IAA and IBA) but remained resistant to the synthetic compounds 2,4-D, 2,4-DB, and TIBA. This dichotomy suggests that these suppressors might impact a process that can differentiate between natural and synthetic auxins, such as transport or metabolism. For example, a mutant that reduces IAA efflux or inactivation might render plants more sensitive to

IAA (and IBA, which can be converted to IAA) without affecting responses to synthetic auxins.

The class 2 suppressors restored *ibr5* responses to IBA but not to IAA; none of these mutants restored ABA responses (Table 2). The class 2 mutants also displayed diverse phenotypes in auxin response assays. Like MS72 (*ibr5 pdr9-72*), MS252 regained sensitivity to 2,4-D and 2,4-DB, but not to IAA or ABA. However, MS252 can be distinguished from MS72: although MS252 restored *ibr5* TIBA sensitivity, MS252 does not appear to be more TIBA sensitive than wild type, as are both *ibr5 pdr9-72* and *ibr5 pdr9-2* (Figures 2G and 6B).

The MS115 and MS339 class 2 suppressors specifically increase sensitivity to IBA and 2,4-DB, which are four-carbon side-chain auxins (Figure 2A) that require peroxisomal chain shortening for auxin activity (Hayashi *et al.* 1998; Zolman *et al.* 2000). MS115 and MS339 might increase the efficiency of IBA-to-IAA and 2,4-DB-to-2,4-D conversion and thereby restore IBA and 2,4-DB responses to *ibr5* to near wild-type levels without restoring IAA, 2,4-D, TIBA, or ABA responses. One mechanism to increase the efficiency of IBA-to-IAA conversion might be to block IBA efflux, and it is interesting that a *PDR/ABCG* gene is found in the MS115 mapping interval (Figure 4).

Intriguingly, the examined suppressors exhibiting restored IAA response (class 1) also displayed restored ABA response, whereas the suppressors that remained IAA resistant (class 2) also remained ABA resistant (Figure 2A). Indeed, all previously examined IAA-resistant mutants also exhibit ABA resistance (Wilson *et al.* 1990; Tiryaki and Staswick 2002; Monroe-Augustus *et al.* 2003; Strader *et al.* 2008). Because IAA is an active form of auxin in the plant, this correlation suggests that response to endogenous IAA is necessary for root elongation inhibition in response to exogenous ABA.

Disruption of many genes can restore *ibr5* auxin responsiveness: Previous genetic screens for suppressors of auxin-resistant mutants have yielded the *axr3* suppressor *pax1* (Tanimoto *et al.* 2007) and the *axr1* suppressors *sar1* and *sar3* (Cernac *et al.* 1997; Parry *et al.* 2006). Although *PAX1* has not been cloned, both *SAR1* and *SAR3* encode nucleoporins (Parry *et al.* 2006). We found that *sar3* fails to suppress the auxin resistance of *ibr5* or *tir1* (Figure 1A), suggesting that the means of restoring auxin responsiveness may not be the same for every mutant.

Our screen for *ibr5* suppressors with restored response to IBA has identified 42 confirmed mutants, and the 9 mutants that we describe here comprise at least four distinct loci (Figure 4). The disparate phenotypes and distinct mapping positions of the characterized mutants suggest that we have not identified many alleles of any particular gene. Our data are consistent with the possibility that lesions in various genes can restore distinct subsets of *ibr5* defects. Strikingly, all but one of the suppressors restored *ibr5* responses to only some hormones (Table 2). In particular, many of the suppressors remained resistant to 2,4-D, a commonly used synthetic auxin, and thus would not have been identified had we used 2,4-D in our primary screen. It is possible that similarly screening for restored responsiveness to other auxins, auxin precursors, ABA, or ACC might yield additional novel *ibr5* suppression pathways. Moreover, characterizing the ability of *ibr5* suppressors to restore auxin responsiveness to other mutants, such as *tir1* or *axr1*, may illuminate different auxin-signaling mechanisms. Future cloning and characterization of the genes defective in the *ibr5* suppressors identified here will contribute to our understanding of auxin metabolism, transport, and interactions with other hormones and also may allow identification of IBR5 substrates that contribute to the pleiotropic phenotypes of *ibr5*.

We are grateful to William Gray for *pdr9-1*, *pdr9-2*, and *pdr9-1 tir1-1* seeds; Mark Estelle for *axr1-3*; the Arabidopsis Biological Resource Center at Ohio State University for *sar3-3* (SALK_109959) and *tir1-1*; Bhavika Kaul for technical assistance; A. Raquel Adham for developing the T8M16 marker; Arthur Millius for developing the SNP3 marker; and Matthew Lingard, Naxhiely Martinez, Dereth Phillips, Sarah Ratzel, and Andrew Woodward for critical comments on the manuscript. This research was supported by the National Science Foundation (NSF; IBN-0315596 and MCB-0745122 to B.B.), the Robert A. Welch Foundation (C-1309 to B.B.), and the National Institutes of Health (NIH; F32-GM075689 to L.C.S.). M.M.-A. was supported in part by NIH Training grant T32-GM08362, K.C.R. was supported in part by the Rice-Houston Alliance for Graduate Education and the Professoriate Program (NSF HRD-0450363), and G.L.L. was supported in part by the Beckman Scholars Program funded by the Arnold and Mabel Beckman Foundation.

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