Genetic Analysis of Indole-3-butyric Acid Responses in *Arabidopsis thaliana* Reveals Four Mutant Classes

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ABSTRACT

Indole-3-butyric acid (IBA) is widely used in agriculture because it induces rooting. To better understand the *in vivo* role of this endogenous auxin, we have identified 14 Arabidopsis mutants that are resistant to the inhibitory effects of IBA on root elongation, but that remain sensitive to the more abundant auxin indole-3-acetic acid (IAA). These mutants have defects in various IBA-mediated responses, which allowed us to group them into four phenotypic classes. Developmental defects in the absence of exogenous sucrose suggest that some of these mutants are impaired in peroxisomal fatty acid chain shortening, implying that the conversion of IBA to IAA is also disrupted. Other mutants appear to have normal peroxisomal function; some of these may be defective in IBA transport, signaling, or response. Recombination mapping indicates that these mutants represent at least nine novel loci in Arabidopsis. The gene defective in one of the mutants was identified using a positional approach and encodes PEX5, which acts in the import of most peroxisomal matrix proteins. These results indicate that in *Arabidopsis thaliana*, IBA acts, at least in part, via its conversion to IAA.

AUXIN is an essential plant hormone that influences numerous aspects of growth and development, including vascular development, lateral root initiation, apical dominance, phototropism, and gravitropism (Davies 1995). Indole-3-acetic acid (IAA) is the most abundant endogenous auxin and its *in vivo* role has been examined extensively. Indole-3-butyric acid (IBA) was long regarded as a synthetic auxin, but recently has been shown to occur naturally in several plant species (Schneider *et al.* 1985; Epstein *et al.* 1991; Ludwig-Müller and Epstein 1991; Nordstrom *et al.* 1991; Sutter and Cohen 1992), including Arabidopsis (Ludwig-Müller *et al.* 1993). Although plants have slightly less IBA than IAA *in vivo* (Ludwig-Müller and Epstein 1993; Ludwig-Müller *et al.* 1993), IBA is often more effective than IAA in root initiation, making it important in commercial and agricultural settings (Hartmann *et al.* 1990; de Klerk *et al.* 1999). In Arabidopsis, IBA induces adventitious roots (King and Stimart 1998) and was widely used to induce roots on shoot explants following Agrobacterium-mediated transformations (Schmidt and Willmitzer 1988; Chaudhury and Signer 1989; Márton and Browse 1991).

Several hypotheses have been advanced to explain the rooting efficacy of IBA. IBA is more stable than IAA under various light and temperature conditions, both in solution and *in vivo* (Nissen and Sutter 1990; Nordstrom *et al.* 1991). Differences in transport, uptake, or metabolism might also contribute to the superior activity (reviewed in Epstein and Ludwig-Müller 1993). Alternatively, a specific IBA to IAA ratio may be important for development, and the application of exogenous IBA might shift the balance to promote root development (reviewed in Epstein and Ludwig-Müller 1993).

How IBA acts at a molecular level is also unknown. IBA may be a *bona fide* auxin, with its own receptor and signal transduction pathway. Alternatively, IBA may function solely through its conversion to IAA, acting as a "slow release" form of IAA (van der Krieken *et al.* 1997) in a manner similar to certain IAA conjugates (Hangarter and Good 1981; Bartel 1997). Previous work in several plant species has supported each hypothesis, suggesting that both mechanisms are important *in vivo*. For example, the conversion of exogenous IBA to IAA has been observed in pea (Nordstrom *et al.* 1991), apple (van der Krieken *et al.* 1992), pear (Baraldi *et al.* 1993), grape, and olive (Epstein and Lavee 1984). Although application of radiolabeled IBA to apple cuttings elevates both IBA and IAA levels, adding IBA increases internal free IAA levels more than a similar IAA application (van der Krieken *et al.* 1992). Interestingly, when concentrations of exogenous IBA and IAA are used that produce equivalent levels of free internal IAA, IBA induces more lateral roots (van der Krieken *et al.* 1992). These results suggest that even though IBA is converted to IAA *in vivo*, it also can have independent or synergistic effects with IAA.

Previous work has demonstrated that IBA and other

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even chain-length derivatives of IAA have auxin effects, and wheat and pea extracts shorten these derivatives in two carbon steps (Fawcett *et al.* 1960). IBA was consequently proposed to be converted to IAA in a process resembling fatty acid β-oxidation. Plants catalyze these reactions (and probably those that convert IBA to IAA) in peroxisomes, which are small organelles bounded by a single lipid bilayer. Peroxisomes contain oxidases that produce hydrogen peroxide, catalases that inactivate reactive molecules, and all of the enzymes necessary for fatty acid β-oxidation (reviewed in Gerhardt 1992; Kindl 1993; Olsen 1998). In seedlings and senescent tissues, specialized peroxisomes called glyoxysomes also contain the enzymes of the glyoxylate cycle, which converts the acetyl-CoA generated during fatty acid degradation to succinate. Leaf peroxisomes lack the glyoxylate cycle, but contain some enzymes involved in photorespiration. Because peroxisomes do not contain DNA, matrix proteins enter peroxisomes post-translationally using one of two *p*eroxisomal *t*argeting *s*ignals (PTS). Proteins with a PTS1 consensus sequence (the tripeptide SKL or a conserved variant; Gould *et al.* 1989) at the extreme C terminus are bound by the PEX5 receptor, which facilitates peroxisomal protein translocation (reviewed in Olsen 1998). Proteins with a PTS2 signal have a nine-amino-acid consensus sequence near the N terminus, which is removed following import. *PEX7* encodes the PTS2 receptor (Schumann *et al.* 1999).

Several Arabidopsis mutants have been identified with defects in fatty acid β-oxidation. *Pe*roxisome *d*eficient (*ped*) mutants were isolated in a screen for plants that elongated roots on inhibitory concentrations of 2,4-dichlorophenoxybutyric acid (2,4-DB), but remained sensitive to the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D; Hayashi *et al.* 1998b). This screen was based on the observation that 2,4-DB is β-oxidized to 2,4-D, which inhibits root elongation (Wain and Wightman 1954). The 2,4-DB-resistant mutants then were assayed for the ability to develop without exogenous sucrose. Because oilseed plants, like Arabidopsis, β-oxidize storage fatty acids during germination to provide energy, plants with β-oxidation defects require supplemental sugar. Three such 2,4-DB-resistant mutants were further examined (Hayashi *et al.* 1998b). *ped1* has a missense mutation in a gene encoding thiolase, which acts in the final step of β-oxidation (Hayashi *et al.* 1998b). The defects in *ped2* and *ped3* have not been reported. A fourth 2,4-DB resistant mutant, *aim1* (*a*bnormal *i*nflorescence *m*eristem), has a T-DNA insertion in a gene encoding a multifunctional protein acting in fatty acid β-oxidation (Richmond and Bleecker 1999).

Despite the importance of IBA in commercial applications and its presence in a wide range of plant species, little genetic analysis of IBA function has been conducted. To understand auxin action, the functional significance of the various auxins found in plants must be determined. We are using a genetic approach to elucidate the *in vivo* role of IBA in the model organism *Arabidopsis thaliana*. We have identified IBA-response mutants that retain IAA sensitivity and have phenotypically characterized these mutants in several auxin-related and peroxisomal assays. We have mapped 11 of these mutants, which represent nine loci, and used positional information to clone one of the defective genes, which encodes the PTS1 peroxisomal import protein PEX5. Our results indicate an important role for IBA *in vivo* and begin to define the mechanism of IBA action in Arabidopsis.

MATERIALS AND METHODS

Plant materials and growth conditions: *A. thaliana* ecotypes Columbia (Col-0), Landsberg *erecta tt4* (L*er*), and Wassilewskija (Ws) were used. Plants were grown in soil (Metromix 200; Scotts, Marysville, OH) at 22–25° under continuous illumination by Sylvania Cool White fluorescent bulbs, except for *aim1* plants, which were grown in short days (8 hr light) to allow seed set (Richmond and Bleecker 1999). Plants grown aseptically were plated on PNS (plant nutrient media with 0.5% sucrose; Haughn and Somerville 1986) solidified with 0.6% agar, either alone or supplemented with hormones (from 1 or 100 mm stocks in ethanol). Plates were wrapped with gas-permeable Leukopor surgical tape (Beiersdorf Inc., Norwalk, CT) and grown at 22° under continuous yellow-filtered light to prevent breakdown of indolic compounds (Stasinopoulos and Hangarter 1990). Genetic crosses were performed as described (Koornneef *et al.* 1998).

Mutant isolation and nomenclature: Col-0 seeds were mutagenized with ethyl methanesulfonate (EMS; Normanly *et al.* 1997). M_2 seeds were surface sterilized (Last and Fink 1988) and plated on PNS containing 20 μm IBA at ~1000 seeds per 150-mm plate. After 8 days, putative mutants with elongated roots were selected, transferred to soil, and allowed to self-fertilize. Resulting M_3 seeds were screened separately for resistance to 5 and 20 μm IBA and wild-type sensitivity to 50 and 500 nm IAA and backcrossed to Col-0 to remove any unlinked mutations. T-DNA lines (Feldmann 1991; Campisi *et al.* 1999) from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH) were similarly screened. Mutant lines used in phenotypic assays were homozygous plants from the first (*ibr1-1*, *ibr2*, *ibr3*, B17, B29, *ibr6*) or second (*ibr1-2*, *pex5-1*) backcross to the parental (Col-0) line (*ibr*, *IB*A *r*esponse). A few mutants were analyzed as selfed progeny of the original isolates (*ibr4*, *ibr5*, B7, B11, B40, and B52), except in Figure 1 and Table 2, where the B7 and B11 lines were from the first backcross to Col-0. B52 and *ibr4* are mutants from T-DNA lines in the Ws background (Feldmann 1991) and *ibr6* is from a Col-0 T-DNA line (Campisi *et al.* 1999).

The β-oxidation defective mutants (Class 1) are usually referred to by their isolation number, because we have determined by mapping in only five cases (*pex5*, B11, B17, B29, B52) that they are not allelic to the previously isolated *ped* (Hayashi *et al.* 1998b) or *aim1* (Richmond and Bleecker 1999) mutants. These mutants will be named in the future based on the nature of the defective genes, which are expected to be similar to genes of known function. Other mutants were given *ibr* designations because we have determined by mapping or complementation analysis that they represent unique loci.

Phenotypic assays: Seeds were surface sterilized and plated on PNS with the indicated concentration of hormone. Seed-

TABLE 1

New CAPS markers used in the *PEX5* cloning

Marker	Enzyme	Size of products (bp)		Oligonucleotides
		Col-0	L*er*	
PDC2	*Bam*HI	360, 65	425	CAGTGGATCACTCCCAAGACGCCTC GCACTCAACTTATATATATTTCAG
MDA7	*Alu*I	931, 312	931, 170, 142	GACCTAATGATGCTTGACCCATG GCAGAATCTAGGGTTACGTGGC

PDC2 and MDA7 are CAPS markers (Konieczny and Ausubel 1993) that reveal polymorphisms when cut with the indicated restriction enzymes following amplification. PCR conditions for *PDC2* were 40 cycles of 15 sec at 94°, 15 sec at 55°, and 30 sec at 72°; PCR conditions for MDA7 were 40 cycles of 30 sec at 94°, 30 sec at 55°, and 3 min at 72°. Products were visualized following electrophoresis on 4% agarose gels. In MDA7, Ws products are the same as L*er*; amplification of Ws DNA was not observed with the *PDC2* marker.

lings were grown for the indicated number of days, removed from the agar, and the length of the hypocotyl or longest root was measured. At least 12 plants were measured for each data point. Data is expressed as percentage elongation on supplemented *vs.* unsupplemented media. In the lateral root assays (Figures 1 and 4), seeds were germinated and grown on PNS for 4 days, then transferred to media containing IBA or IAA and grown for 4 additional days, when the number of lateral roots was counted under a dissecting microscope. In the hypocotyl elongation assay (Figure 3), seeds were plated on PN (without sucrose) or PNS and incubated for 24 hr under white light before being transferred to the dark for 5 or 8 additional days.

Gas chromatography (GC) analysis of seed storage lipids: Seeds were surface sterilized and grown on filter paper on agar-solidified PNS media under continuous white light. Fatty acids were isolated and esterified by heating 75 5-day-old-seedlings in methanolic HCl as previously described (Browse *et al.* 1986), except that 1.2 μg of docosanoic acid (Sigma, St. Louis) was added to each sample as an internal standard, and samples were extracted in methanolic HCl at 80° for 3 hr rather than 1 hr. Samples were chromatographed on a Hewlett Packard gas chromatograph (HP6890 equipped with a 30 m × 0.25 mm Rtx-5 column). The oven was held at 100° for 1 min and warmed 25°/min to 160° and 10°/min to 200°, where it remained for 7 min. A 10°/min increase to 220° was followed by 6 min at 220°. Specific methyl esters were identified by comparing elution times to those of reference compounds (Sigma).

Genetic analysis: The EMS-induced IBA-resistant mutants, which are in a Col-0 background, were outcrossed to Ws or L*er* for mapping. The resulting F_2 seeds were plated on 15 μM IBA, and DNA from resistant individuals was isolated (Celenza *et al.* 1995). Mutants were mapped using published simple sequence length polymorphisms (Bell and Ecker 1994) and *d*eaved *a*mplified *p*olymorphic *s*equences (CAPS; Konieczny and Ausubel 1993) markers and by developing additional PCR-based markers (Table 1). New markers were identified by PCR amplifying and sequencing ~1.3-kbp genomic DNA fragments from different ecotypes and identifying polymorphisms that altered fragment sizes or restriction enzyme recognition sites.

Identification of the *pex5-1* mutation: A candidate gene (*PEX5*, GenBank no. AF074843) within the mapping interval was examined for defects in the B44 mutant. Genomic DNA extracted from B44 mutant plants was amplified using 5 pairs of oligonucleotides (PEX5-1: GTCGTTGGCTGAATATTTTG TTCGGC and PEX5-2: GTGGCAAGTAAGACCCTAAAGTGA AC; PEX5-3: GACGCATCACCCTGCATCTAAC and PEX5-4: GACCACAAGTATCCATATATGTGAAC; PEX5-5: CGGTCA GTGCATGTTATCTAGC and PEX5-6: GACGTAACGTCTTT GTAAGATTTTC; PEX5-7: CTACATCTAACCGTTTTGTATC CGGG and PEX5-8: CCATACTGATAGATTCAACGACGGTG; PEX5-9: CCTATTTCTCCTGAATGACACGTTG and PEX5-10: GTTGCAGCTACAGAACACTATCAG) using 40 cycles of 95° for 30 sec, 56° for 30 sec, and 72° for 3 min. The resultant overlapping fragments covered the gene from 60 bp upstream of the putative translation start site to 75 bp downstream of the stop codon. Amplification products were purified by sequential ethanol, polyethylene glycol, and ethanol precipitations (Ausubel *et al.* 1999) and sequenced directly using an automated DNA sequencer (Lone Star Labs, Houston, TX) with the primers used for amplification.

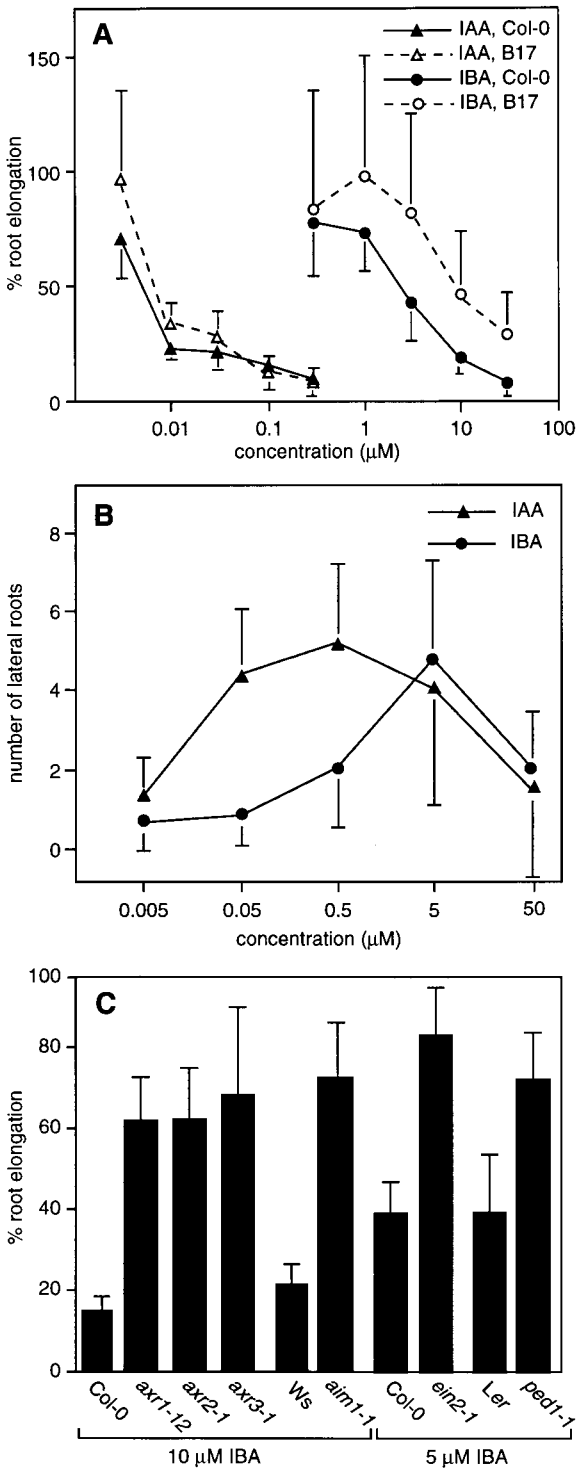
The *pex5-1* mutation was confirmed by amplifying genomic DNA with primers PEX5-1 and PEX5-2, which amplify a 1.2-kbp PCR product containing three and two *Eco*RI sites in Col-0 and *pex5-1*, respectively.

A full-length *PEX5* cDNA was isolated from a plasmid-based cDNA library (Minet *et al.* 1992) by colony hybridization with a 920-bp probe (made by amplifying genomic DNA with primers PEX5-7 and PEX5-8), subcloned into the *Not*I site of pBluescript II KS+ (Stratagene, La Jolla, CA), and sequenced with vector-derived primers. The cDNA was then subcloned in the sense orientation into the *Not*I site of the 35SpBARN plant transformation vector, a derivative of p1'barbi (Mengiste *et al.* 1997) modified to contain the Cauliflower Mosaic Virus 35S promoter and *nos* terminator flanking a multiple cloning site (S. LeClere and B. Bartel, unpublished data). This plasmid was electroporated (Ausubel *et al.* 1999) into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell 1986), which was used to transform *pex5-1* (B44) mutant plants by the floral dip method (Clough and Bent 1998). Transformants were identified by spraying soil-grown plants with Finale herbicide (AgrEvo Environmental Health, Montvale, NJ) diluted in water to a concentration of 0.26 mg/ml glufosinate-ammonium at ~1 and 2 wk after germination.

RESULTS

Auxin effects of IBA in *A. thaliana*: IBA has auxin activity in several bioassays. We compared the responses of Arabidopsis seedlings to IAA and IBA in two tests, inhibition of root elongation and promotion of lateral root initiation. Wild-type seeds were germinated on a range of IBA and IAA concentrations, and root elonga-

tion was measured after 8 days (Figure 1A). IBA inhibited primary root elongation similarly to IAA, but required a >100-fold higher concentration. To quantify lateral root promotion by IBA, we transferred 4-day-old seedlings to IAA- or IBA-containing media and counted lateral roots 4 days after transfer (Figure 1B). As expected, IBA induced lateral roots similarly to IAA. In this case, however, the optimum concentration of IBA was only 10-fold more than that of IAA. Therefore, IBA maximally promotes lateral roots at concentrations that only moderately affect elongation, whereas IAA promotes lateral roots at concentrations that severely inhibit elongation. These results confirm that IBA acts as an auxin in Arabidopsis and provide the basis for the mutant screen described below.



We also examined IBA responses of known auxin-resistant mutants. Several IAA-resistant mutants, including *axr1* (Estelle and Somerville 1987; Leyser *et al.* 1993), *axr2* (Timpte *et al.* 1994), *axr3* (Leyser *et al.* 1996; Rouse *et al.* 1998), *shy2* (Tian and Reed 1999), and *iaa28* (L. E. Rogg, J. Lasswell and B. Bartel, unpublished results), elongate roots more than wild type on inhibitory concentrations of IBA (Figure 1C and data not shown). The IBA resistance of these auxin response mutants is consistent with IBA acting as an auxin or auxin precursor in Arabidopsis.

Several components that mediate polar IAA transport have been identified in Arabidopsis (reviewed in Estelle 1998). The *aux1* mutant (Pickett *et al.* 1990), which is defective in an auxin influx carrier (Bennett *et al.* 1996; Marchant *et al.* 1999), is resistant to the inhibitory effects of IBA on root elongation (Figure 2). This suggests that IBA and IAA enter root cells through the same carrier, as previously indicated by the competition of labeled IBA uptake by excess IAA in Arabidopsis seedlings (Ludwig-Müller and Hilgenberg 1995). A defect in auxin efflux causes the roots of the *eir1-1* (= *agr1* = *pin2*) mutant (Chen *et al.* 1998; Luschnig *et al.* 1998; Müller *et al.* 1998; Utsuno *et al.* 1998) to bend into media containing IAA or 1-naphthaleneacetic acid (NAA) when grown vertically (Utsuno *et al.* 1998). Interestingly, we found that *eir1-1* mutant roots fail to bend and enter agar containing IBA under similar conditions (data not shown), suggesting that IBA is not an EIR1 substrate.

In contrast to *eir1*, we found that other ethylene mu-

Figure 1.—Auxin effects of IBA. (A) Root elongation on IBA and IAA. Eight-day-old Col-0 seedlings grown on the indicated concentrations of IBA and IAA under yellow-filtered light were removed from the agar and the length of the longest root was measured (solid symbols). The percentage elongation of plants grown on hormone *vs.* hormone-free media is shown. Error bars indicate the standard deviations of the means ($n \geq 12$). Also shown is the B17 mutant, which is resistant to IBA and sensitive to IAA (open symbols). (B) Lateral root initiation on IBA and IAA. Four-day-old Col-0 seedlings were transferred from hormone-free media to media containing the indicated concentration of IBA or IAA. After 4 additional days, plants were removed from the agar and the number of lateral roots were counted. Error bars indicate the standard deviations of the means ($n \geq 12$). (C) Root elongation of previously characterized mutants on IBA. Roots of 8-day-old seedlings grown on 5 or 10 μM IBA were measured as described above ($n \geq 12$). *axr1-12*, *axr2-1*, *axr3-1*, and *ein2-1* are in the Col-0 background; *aim1-1* is in the Ws background; and *ped1-1* is in the L*er* background.

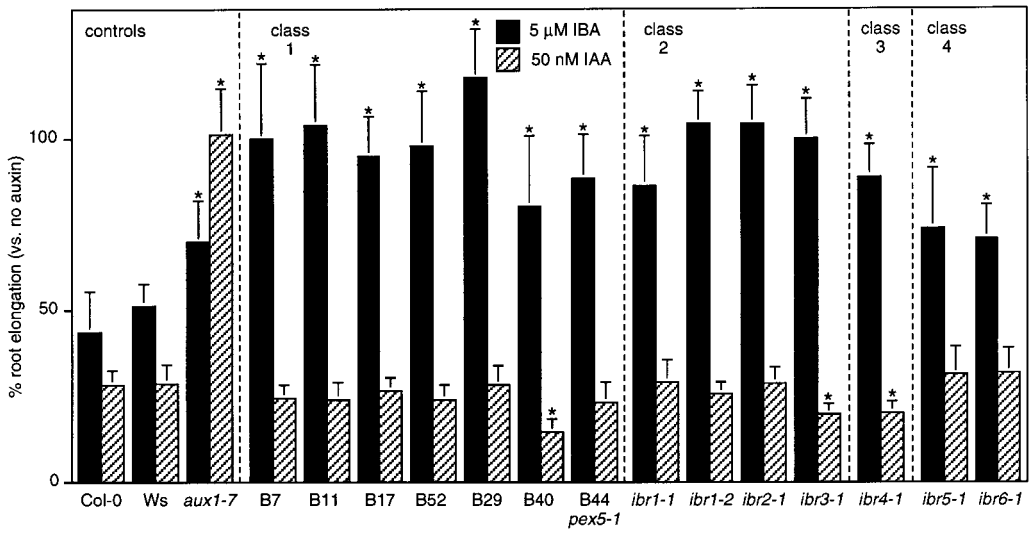


FIGURE 2.—Root elongation on IBA and IAA. Roots of 8-day-old seedlings grown on 5 μM IBA or 50 nM IAA were measured as described in the legend of Figure 1A. All mutants are in the Col-0 background except for *ibr4* and B52, which are in the Ws background. Error bars indicate the standard deviations of the means ($n \geq 14$), and asterisks indicate measurements significantly different from the corresponding wild type (Student's *t*-test, $P < 0.0001$).

tants have altered IBA responses. *ein2* alleles have been identified in ethylene (GUZMÁN and ECKER 1990; ALONSO *et al.* 1999), cytokinin (CARY *et al.* 1995), abscisic acid (ALONSO *et al.* 1999), and auxin transport inhibitor screens (FUJITA and SYONO 1996). We found that *ein2-1* is resistant to the inhibitory effects of IBA (Figure 1C) and 2,4-D on root elongation, but has responses similar to wild type when grown on IAA or NAA (data not shown). Similarly, the ethylene-resistant mutant *etr1* mutant (BLEECKER *et al.* 1988; HUA and MEYEROWITZ 1998) also has an IBA-resistant root, but *ctr1* (KIEBER *et al.* 1993), which has a constitutive ethylene response, has a wild-type reaction to IBA (data not shown).

New mutants with altered IBA responses: We screened for IBA-resistant mutants as a first step in elucidating the molecular mechanisms of IBA function. On the basis of the observation that IBA inhibits primary root elongation in Arabidopsis (Figure 1A), we searched for plants that elongated roots when grown on inhibitory concentrations of IBA. A total of 36,000 EMS-mutagenized M_2 seeds and 15,000 T-DNA lines were examined on 20 μM IBA (see MATERIALS AND METHODS). Seeds from putative mutants were rescreened separately on IBA and IAA, and 14 IBA-resistant, IAA-sensitive mutants were retained for further analysis. Because the 3 mutants from the T-DNA lines are from independent pools and the 11 EMS-induced mutants come from 10 different M_2 pools, the mutants isolated represent at least 13 independent mutagenic events.

The root elongation phenotypes of these mutants on IBA and IAA are shown in Figure 2. The mutants show varying degrees of resistance to IBA, but all are sensitive to IAA. Whereas all of the mutants are significantly resistant to 5 μM IBA (Figure 2), assays at higher IBA concentrations indicate that none of the mutants are completely IBA insensitive (data not shown).

Peroxisomal function in IBA-response mutants: IBA is thought to be converted to IAA in a process similar to fatty acid β-oxidation (FAWCETT *et al.* 1960), which is peroxisomal in plants (reviewed in OLSEN 1998). We therefore expected that mutations disrupting peroxisome biogenesis or β-oxidation could cause an IBA-resistant, IAA-sensitive phenotype. Because germinating Arabidopsis plants derive sugar from stored long-chain fatty acids until photosynthesis begins, the ability to germinate and develop on media lacking sugar reflects fatty acid β-oxidation efficiency (HAYASHI *et al.* 1998b). To determine if our IBA-resistant mutants could β-oxidize fatty acids, we examined growth without exogenous sucrose. To eliminate complications from photosynthesis, we compared hypocotyl elongation of plants grown with and without sucrose after 5 or 8 days in the dark (Figure 3). Whereas all of the mutants developed normally with sucrose, some of the IBA-resistant mutants (B7, B11, B17, B29, B40, B44, and B52) failed to germinate or grew more slowly in the dark without exogenous sucrose. This suggests that these plants were unable to break down stored fatty acids and may have mutations in proteins acting in peroxisomal biogenesis or β-oxidation. The remaining mutants did not require sucrose for growth in the dark, suggesting functional peroxisomes. These mutants may be specifically blocked in the conversion of IBA to IAA (and not fatty acid β-oxidation) or may affect other aspects of IBA function.

To directly measure the rate of fatty acid β-oxidation in the mutant plants, we examined the relative levels of a specific fatty acid during germination. Eicosenoic acid (20:1) is an abundant Arabidopsis seed storage lipid that is present only at low levels in other tissues (LEMIEUX *et al.* 1990), so its rate of decline during germination should reflect β-oxidation of seed storage fatty acids. We extracted lipids from dry seeds and from 5-, 7-, and 10-day-old seedlings and measured 20:1 levels using GC (BROWSE *et al.* 1986). In dry seeds, all of the mutants had fatty acid profiles similar to wild-type plants (data not shown), with ~15% of fatty acid present as eicosen-

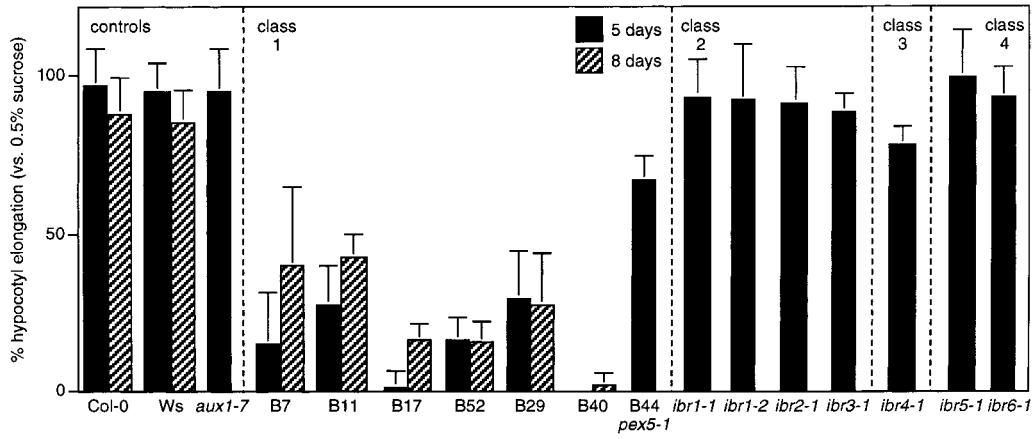


FIGURE 3.—Hypocotyl elongation in the dark in the absence of sucrose. Five-day-old seedlings grown on sucrose-free media in darkness were removed from the agar and the length of the hypocotyl was measured and compared to plants grown on 0.5% sucrose. Mutants with short hypocotyls without sucrose also were measured after 8 days. The percent elongation compared to media containing sucrose is shown. Error bars indicate the standard deviations of the means ($n \geq 12$).

oic acid. However, 5 and 7 days after germination, wild-type plants retained little eicosenoic acid (Table 2 and data not shown), indicating that they metabolized it efficiently. After 10 days, eicosenoic acid was virtually undetectable in the wild type (data not shown). In each putative β-oxidation mutant, 20:1 levels decreased more slowly than in wild type (Table 2), indicating that these mutants did not utilize fatty acids as efficiently as wild-type plants. These mutants also grow more slowly than wild type even when the media is supplemented with 0.6% sucrose, as evidenced by the reduced fresh weight of some of the mutant seedlings used in the GC experiment (Table 2). We cannot conclude from this experiment whether the reduced rate of fatty acid β-oxidation in these mutants reflects or causes the reduced growth rate. However, it is clear that a reduced ability to elongate hypocotyls in the dark in the absence of sucrose (Figure 3) correlates with reduced rates of fatty acid β-oxidation (Table 2).

This pleiotropic group represents the first class of IBA-response mutants, which are likely to have a reduced conversion of IBA to IAA. These mutants may have defects in β-oxidation enzymes or in proteins acting in peroxisomal biogenesis or maintenance. We have not yet named most of these mutants because the responsible genes have probably already been identified in Arabidopsis or other organisms. We have designated the mutants with apparently normal fatty acid β-oxida-

TABLE 2

Eicosenoic acid (20:1) levels in 5-day-old seedlings

Class	Plant	% 20:1 *vs.* total fatty acids	Amount of 20:1 (pg/seedling)	Fresh weight (mg/seedling)
Wild type	Col-0	1.8 ± 0.4	50 ± 16	0.99 ± 0.09
Wild type	Ws	5.7 ± 3.1	150 ± 91	0.71 ± 0.09
1	B7	12.5 ± 3.5	680 ± 430	0.59 ± 0.02
1	B11	8.1 ± 0.8	270 ± 40	0.58 ± 0.03
1	B17	14.0 ± 3.5	690 ± 540	0.48 ± 0.02
1	B52	15.1 ± 3.3	650 ± 340	0.53 ± 0.04
1	B29	8.6 ± 2.8	230 ± 100	0.49 ± 0.05
1	B40	11.8 ± 2.5	530 ± 220	0.42 ± 0.07
1	B44 (*pex5-1*)	8.8 ± 1.3	270 ± 53	0.55 ± 0.06
2	*ibr1-1*	3.7 ± 1.5	99 ± 43	0.71 ± 0.06
2	*ibr1-2*	6.7 ± 2.7	210 ± 130	0.60 ± 0.05
2	*ibr2*	4.8 ± 1.3	130 ± 40	0.72 ± 0.04
2	*ibr3*	4.2 ± 0.8	110 ± 40	0.63 ± 0.06
3	*ibr4*	6.5 ± 2.5	140 ± 60	0.67 ± 0.08
4	*ibr5*	1.6 ± 0.01	33 ± 2	0.76 ± 0.05
4	*ibr6*	3.3 ± 1.8	94 ± 63	0.90 ± 0.10

Values represent the mean ± standard deviation of 3 samples of 75 seedlings. All mutants are in the Col-0 background except for B52 and *ibr4*, which are in the Ws background. The percent eicosenoic acid (20:1) was calculated by dividing the area of the 20:1 GC peak by the total area of fatty acid peaks. The amount of 20:1 was calculated by multiplying the area of the 20:1 peak by the amount of standard added and dividing by the area of the standard peak. Fresh weights were measured immediately before processing.

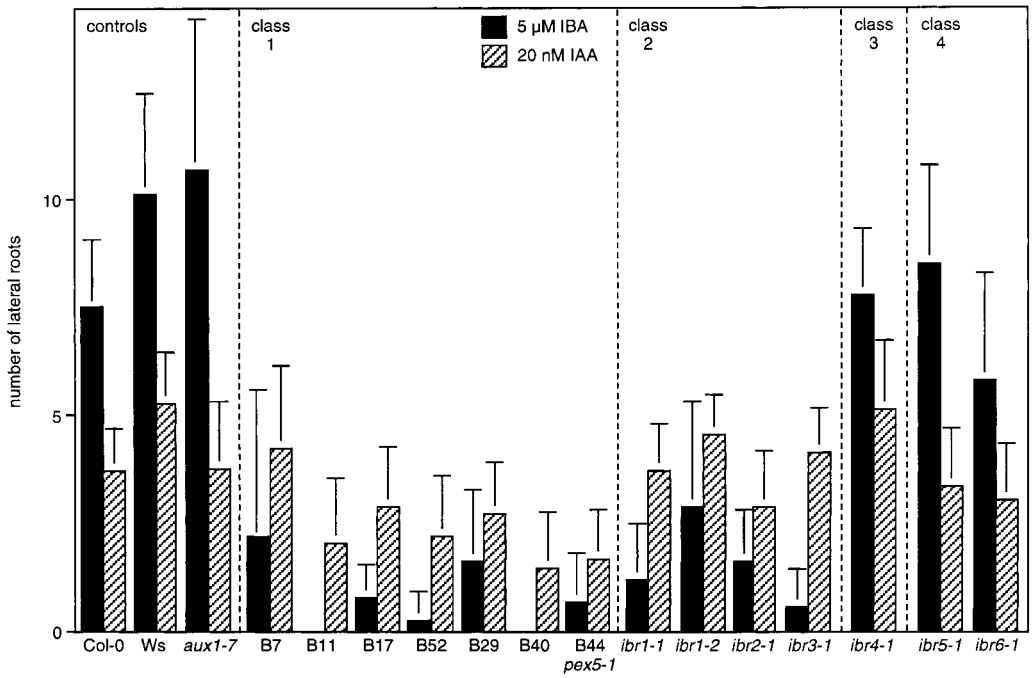


FIGURE 4.—Lateral root initiation on IBA and IAA. Plants were transferred to 5 μM IBA or 20 nM IAA and the number of lateral roots was counted as described in the legend of Figure 1B. Error bars indicate the standard deviations of the means ($n \geq 12$).

tion that we have shown by mapping or complementation to be unique *ibr* mutants.

Auxin phenotypes of IBA-response mutants: IBA promotes lateral root initiation in Arabidopsis (Figure 1B). To assay this response in the mutants, we transferred 4-day-old seedlings to media containing 5 μM IBA or 20 nM IAA and grew them for 4 additional days (Figure 4). In wild-type plants, these concentrations give similar root elongation inhibition (Figure 1A), but IBA induces more lateral roots than IAA (Figure 1B). Many of the mutants, including all of the class 1 putative peroxisomal mutants, induce wild-type numbers of lateral roots on IAA, but not on IBA. Several of the nonperoxisomal IBA-response mutants also are resistant to the effects of IBA on lateral root initiation (*ibr1*, *ibr2*, and *ibr3*), and we designated these as class 2 mutants. In contrast, three of the mutants (*ibr4*, *ibr5*, and *ibr6*) induce lateral roots in response to both IBA and IAA similarly to wild-type plants, indicating that they are resistant to the effects of IBA on root elongation inhibition, but not on lateral root formation.

As shown in Figure 5, all of the IBA-response mutants also are resistant to the IBA analog 2,4-DB, which is β-oxidized to the synthetic auxin 2,4-D in a mechanism similar to the IBA to IAA conversion (WAIN and WIGHTMAN 1954; HAYASHI *et al.* 1998b). Whereas all of these mutants remain sensitive to IAA (Figure 2), two of the mutants, *ibr5* and *ibr6*, are resistant to the inhibitory effects of the synthetic auxin 2,4-D on root elongation (Figure 5). Interestingly, *ibr5* and *ibr6* are two of the three mutants that remain sensitive to the promotion of lateral roots by IBA (Figure 4). We therefore divided the mutants that respond to IBA in the lateral root assay into two categories, class 3 mutants (*ibr4*), which are 2,4-D sensitive, and class 4 mutants (*ibr5* and *ibr6*), which are 2,4-D resistant.

Because two of the IBA response mutants are 2,4-D resistant and neither IBA (data not shown) nor 2,4-D (UTSUNO *et al.* 1998) appear to be substrates of the EIR1 efflux carrier, we tested the response of the IBA-resistant mutants to polar auxin transport inhibitors. Whereas most of the mutants have wild-type responses to these inhibitors, the class 4 (2,4-D resistant) mutants are resistant to the auxin transport inhibitors 1-napthylphthalamic acid (NPA; Figure 5), 9-hydroxyfluorene-9-carboxylic acid (HFCA; data not shown), and 2,3,5-triiodobenzoic acid (TIBA; data not shown). Therefore, these mutants may be defective in IBA transport. In contrast to the *aux1* and *eir1* mutants, which are defective in IAA transport (reviewed in ESTELLE 1998), the class 4 mutants respond similarly to wild type when grown on the ethylene precursor 1-aminocyclopropane-1-carboxylic acid either in the light or in the dark (data not shown).

Mapping IBA-response mutant loci: We used recombination mapping with PCR-based markers to localize 11 of the IBA-response mutants to nine chromosomal positions, which are shown in Figure 6. The *ibr1-1* and *ibr1-2* mutants are allelic; they map to the same interval on the top of chromosome 4 and do not complement each other. B17 and B52 also are allelic and map to the same interval on the bottom of chromosome 5. Although we have not mapped *ibr2*, it complements all of the other class 2 mutants (data not shown) and therefore is likely to represent a novel locus.

None of the mapped mutants occupy positions of the previously isolated peroxisome-defective *ped* or *aim1* mutants (HAYASHI *et al.* 1998b; RICHMOND and BLEECKER

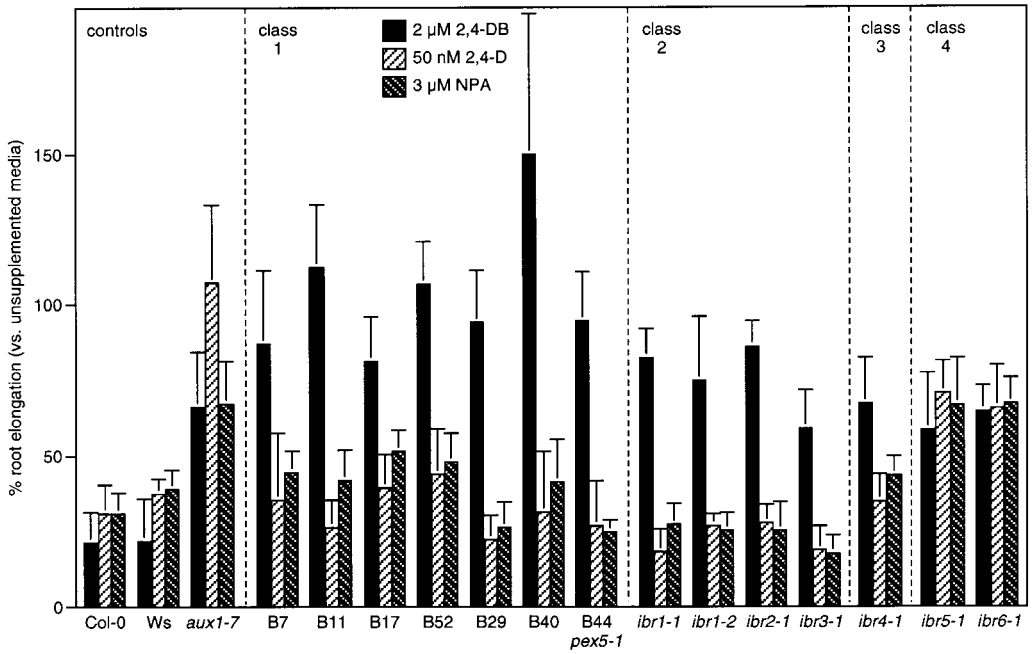


FIGURE 5.—Root elongation on 2,4-DB, 2,4-D, and NPA. Root elongation of plants grown on 2 μM 2,4-DB, 50 nM 2,4-D, or 3 μM NPA was measured as described in the legend to Figure 1A. Error bars indicate the standard deviations of the means ($n \geq 12$).

1999). In addition, the auxin-transport inhibitor resistant *ibr5* mutant maps to an interval on chromosome 2 that does not contain any of the previously characterized *tir* (*t*ransport *i*nhibitor *r*esponse) mutants (RUEGGER *et al.* 1997). Although *ibr6* maps to the same region on chromosome 3 as *tir1*, it is unlikely to be allelic because *tir1*, unlike *ibr6*, is resistant to the inhibitory effects of IAA (RUEGGER *et al.* 1998).

A mutant gene encoding the PEX5 peroxisome receptor protein: We mapped the class 1 B44 mutant to an ~200-kbp region on the bottom of chromosome 5 (Figure 7) between MDA7 (KANEKO *et al.* 1998) and *ILL2* (DAVIES *et al.* 1999). The Kazusa DNA Research Institute (www.kazusa.or.jp/arabi/) sequenced this region as a part of the Arabidopsis Genome Initiative. Because the B44 mutant appears to have peroxisomal defects (Figure 3; Table 2), we scanned the sequence in this interval for candidate genes that might be involved in peroxisomal biogenesis or function and found that the *PEX5* gene (BRICKNER *et al.* 1998), which encodes the PTS1 peroxisomal receptor, was located on the P1 clone MCD7 (SATO *et al.* 1998). To determine whether the B44 mu-

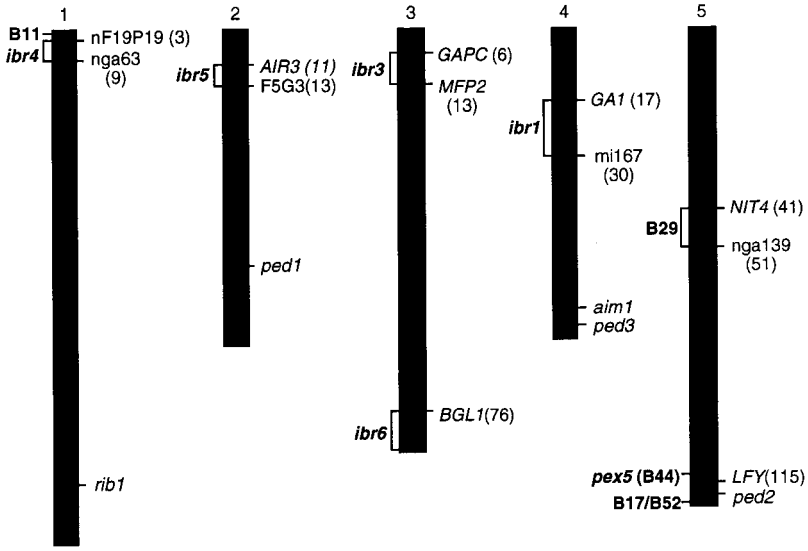


FIGURE 6.—Map positions of IBA-resistant mutants. Approximate map positions (in centimorgans) of molecular markers, *aim1* (RICHMOND and BLEECKER 1999) and *ped* (HAYASHI *et al.* 1998b) peroxisomal defective mutants, and the *rib1* IBA-resistant mutant (POUPART and WADDELL 2000) are shown to the right of each chromosome. The interval to which each IBA mutant maps is shown to the left of the chromosomes. B11 maps to chromosome 1 north of nF19P19 (http://genome.bio.upenn.edu/SSLP_info/SSLP.html) and *ibr4* maps between nF19P19 and nga63 (BELL and ECKER 1994), with 4/202 and 6/202 recombinants, respectively. *ibr5* maps between *AIR3* (SILVERSTONE *et al.* 1998) and F5G3 (LIN *et al.* 1999) with 15/742 and 11/744 recombinants, respectively. *ibr3* is located between *GAPC* (KONIECZNY and AUSUBEL 1993) and *MFP2* (RICHMOND and BLEECKER 1999) with 18/300 and 1/304 recombinants. *ibr6* maps south of *BGL1* (KONIECZNY and AUSUBEL 1993) with 2/62 north recombinants. *ibr1* maps near the middle of chromosome 4. *ibr1-1* has 2/184 north recombinants at *GA1* (KONIECZNY and AUSUBEL 1993) and 31/176 south recombinants at mi167 (THOMPSON *et al.* 1996), whereas *ibr1-2* has 12/232 recombinants at *GA1* and 19/198 recombinants at mi167. B29 has 6/250 recombinants at *NIT4* (BARTEL and FINK 1994) and 0/250 recombinants at nga139 (BELL and ECKER 1994). B17 and B52, which are allelic, map to chromosome 5 south of *ped2* (HAYASHI *et al.* 1998b), and B44 (*pex5*) maps just north of *LFY* (Figure 7).

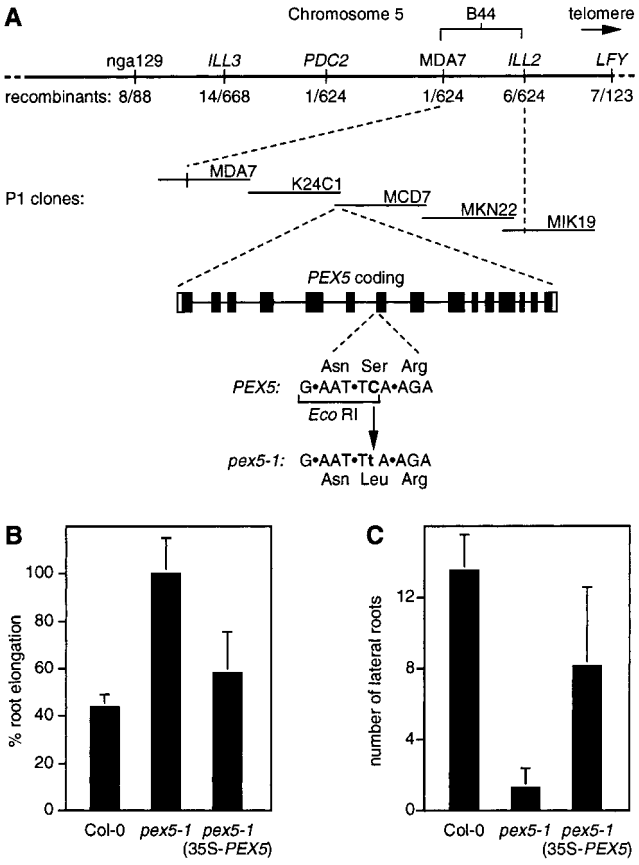


FIGURE 7.—Positional cloning of *PEX5*. (A) Recombination mapping with PCR-based markers nga129 (BELL and ECKER 1994), *ILL2* and *ILL3* (DAVIES *et al.* 1999), *PDC2* (Table 2), MDA7 (Table 2), and *LFY* (KONIECZNY and AUSUBEL 1993) localized B44 between MDA7 and *ILL2* with one north and six south recombinants. Examination of the sequenced P1 clones in this region (thin lines) revealed the *PEX5* gene on MCD7. This gene has a C-to-T mutation at position 2910 in the *pex5-1* mutant that destroys an *Eco*RI site. The first G of the sequence shown is the final residue of intron 6. (B and C) 35S-*PEX5* rescues *pex5* mutant phenotypes. Seeds from wild-type (Col-0), *pex5-1*, and *pex5-1* plants transformed with a 35S-*PEX5* construct were analyzed for root elongation on 5 μM IBA (B), as described in the legend to Figure 2, or for lateral root induction by 5 μM IBA (C), as described in the legend to Figure 4. Error bars indicate the standard deviations of the means ($n \geq 12$). The *pex5-1* (35S-*PEX5*) line shown is the progeny of a line hemizygous for the transgene and therefore includes ~25% untransformed plants.

tant had a mutation in *PEX5*, we amplified and sequenced this gene from B44 mutant DNA (see MATERIALS AND METHODS). Sequencing identified a C-to-T base change at position 2910 (where the A of the ATG is at position 1), which causes a Ser-to-Leu missense mutation. Figure 8 shows an alignment of Arabidopsis PEX5 and homologs from several other organisms and indicates the position of the mutation. Because this base-pair change destroys an *Eco*RI site in the mutant, we confirmed the mutation by amplifying and digesting the relevant region from wild-type and mutant genomic DNA. On the basis of this data, we named B44 *pex5-1*.

To confirm that the base change found in the *pex5-1* mutant causes the IBA-resistant phenotype, we tested a *PEX5* cDNA driven by the CaMV 35S promoter for complementation of the mutant phenotype (see MATERIALS AND METHODS). This clone restored wild-type IBA sensitivity to the *pex5-1* mutant in root elongation inhibition (Figure 7B) and lateral root initiation (Figure 7C) and also restored the ability of *pex5-1* hypocotyls to elongate in the dark without sucrose (data not shown), confirming that we have identified the gene responsible for the mutant phenotype. Because PEX5 acts to import most β-oxidation enzymes (DE HOOP and AB 1992), our finding that a mutation in this gene leads to IBA resistance implies that IBA acts via its conversion to IAA in Arabidopsis.

DISCUSSION

Auxin activity of IBA: IBA is an endogenous auxin in Arabidopsis (LUDWIG-MÜLLER *et al.* 1993) and numerous other plants, including maize (LUDWIG-MÜLLER and EPSTEIN 1991), tobacco (SUTTER and COHEN 1992), and apple (VAN DER KRIEKEN *et al.* 1992). IBA is used widely to induce rooting of vegetative cuttings (HARTMANN *et al.* 1990; DE KLERK *et al.* 1999). However, little is known regarding how IBA functions *in vivo*. Radiolabeling experiments have been conducted in several plant species to examine whether IBA functions via its conversion to IAA or if it acts on its own. However, the results are conflicting and do not exclude the possibility that both mechanisms are important *in vivo*.

We have confirmed that IBA acts as an auxin in *A. thaliana*, inhibiting root elongation (Figure 1A) and promoting lateral root formation (Figure 1B) in wild-type plants. At the high concentrations of IAA necessary for lateral root initiation, root elongation is severely inhibited (Figure 1). In contrast, IBA initiates lateral roots at a concentration where elongation is less inhibited (Figure 1). The *aux1* mutant (PICKETT *et al.* 1990) is resistant to the inhibitory effects of IBA on root elongation (Figure 2), suggesting that IBA enters cells through the AUX1 influx carrier, which also imports IAA and 2,4-D, but not NAA (YAMAMOTO and YAMAMOTO 1998; MARCHANT *et al.* 1999).

Four classes of IBA-response mutants: We have begun isolating and characterizing IBA-resistant mutants, anticipating that analysis of the defective genes will clarify whether IBA acts solely via its conversion to IAA or if it also functions directly as an auxin. Whereas all of the mutants are significantly resistant to the inhibitory effects of IBA on root elongation, they all remain sensitive to the more abundant auxin, IAA. Three of the mutants, B40, *ibr3*, and *ibr4*, appear in Figure 2 to be more sensitive than wild type to IAA. It will be interesting to determine whether this difference persists as these lines are backcrossed to remove unlinked mutations.

These IBA-response mutants fall into four classes. The

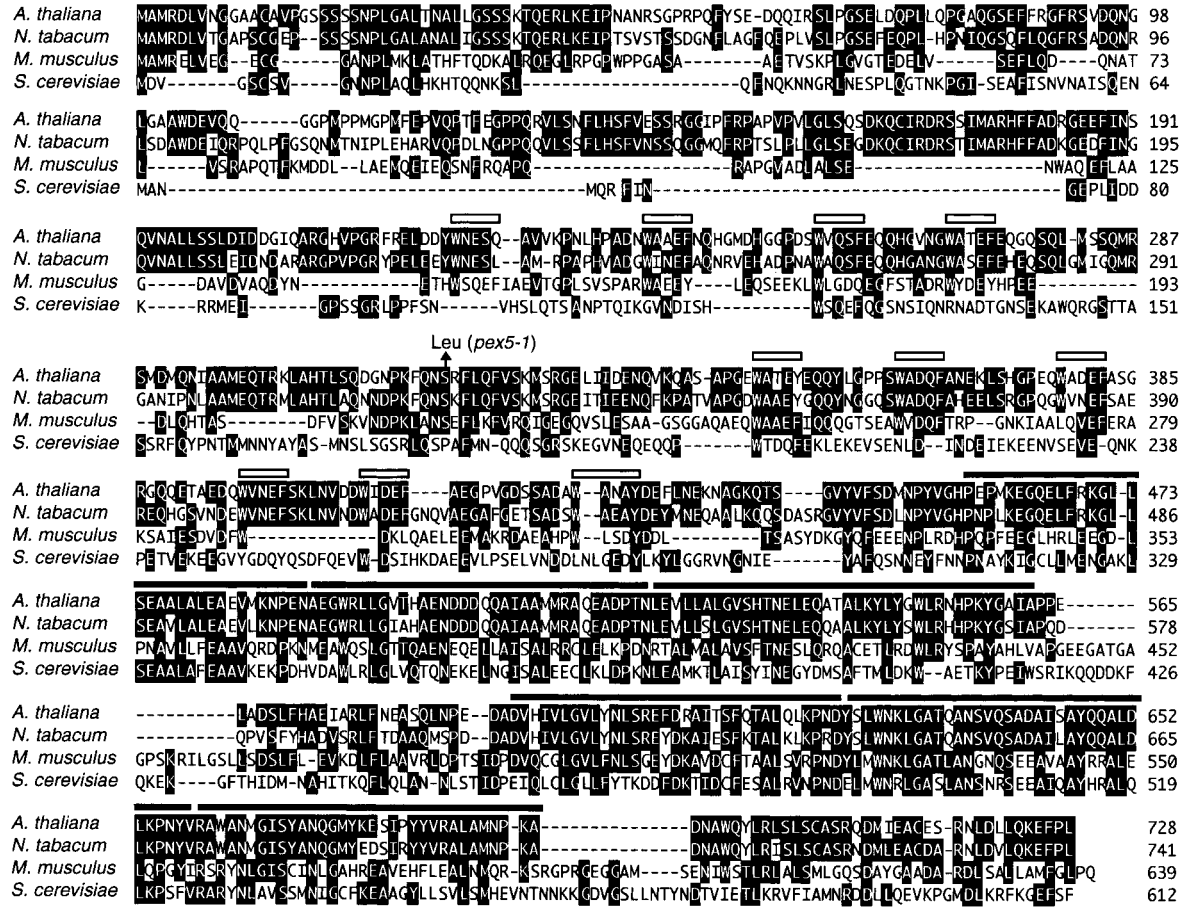


Figure 8.—Alignment of PEX5 from Arabidopsis with several homologs. Sequences were aligned with the MegAlign program (DNAStar, Madison, WI) using the Clustal method. Proteins shown are from Arabidopsis (Brickner *et al.* 1998), tobacco (Kragler *et al.* 1998), mouse (Baes *et al.* 1997), and yeast (Van der Leij *et al.* 1993). Amino acid residues identical in at least two of the sequences are in black boxes and hyphens indicate gaps introduced to maximize alignment. The site of the *pex5-1* mutation is indicated above the alignment. Pentapeptide repeat regions with the consensus sequence WXXX[F/Y] are indicated by open rectangles above the sequence. Solid lines indicate the seven TPR motifs, thought to act in binding the "SKL" targeting signal of PTS1 proteins (Brocard *et al.* 1994; Brickner *et al.* 1998).

class 1 mutants (*pex5-1*, B7, B11, B17/B52, B29, and B40) appear to be defective in fatty acid β-oxidation. These mutants are resistant to both the inhibitory effects of IBA on root elongation (Figure 2) and the stimulatory effects of IBA on lateral root induction (Figure 4). Two assays reveal defects in utilization of seed storage lipids: an indirect assay monitoring hypocotyl elongation of dark-grown plants without exogenous sugar (Figure 3) and a direct GC-based assay monitoring eicosenoic acid decline during germination (Table 2). We suggest that these mutants are defective in the conversion of IBA to IAA, which is, therefore, an important component of the auxin activity of IBA in Arabidopsis.

The other IBA-response mutants utilize stored fatty acids during germination normally (Figure 3, Table 2). Because these mutants are generally as resistant to IBA as the putative peroxisomal mutants (Figure 2), we do not believe that they are simply extremely leaky peroxisomal mutants. These nonperoxisomal mutants can be further subdivided into three classes. The class 2 mutants (*ibr1-1*, *ibr1-2*, *ibr2*, and *ibr3*) are resistant to the auxin effects of IBA on both root elongation and lateral root proliferation, but have wild-type responses to other auxins (IAA and 2,4-D) and auxin transport inhibitors (NPA, TIBA, and HFCA). The class 2 mutants could be defective in enzymes that convert IBA to IAA but are not necessary for the β-oxidation of seed storage lipids. Alternatively, these mutants may be defective in an IBA receptor, signaling pathway, or response factor. If these mutants have normal IBA to IAA conversion, it will suggest that IBA plays at least two roles in the promotion of lateral roots, one dependent on (disrupted in the class 1 mutants) and one independent of (disrupted in the class 2 mutants) its role as an IAA precursor. Identification and analysis of the genes defective in the IBA-resistant mutants with apparently normal peroxisomal function will enable us to determine whether IBA has a role independent of its conversion to IAA.

In contrast to the class 1 and 2 mutants, several nonperoxisomal mutants (*ibr4*, *ibr5*, and *ibr6*) still initiate

lateral roots in response to IBA. Interestingly, several mutants isolated on the basis of resistance to the inhibitory effects of other auxins (IAA and 2,4-D) on root elongation have a similar phenotype. For example, the *aux1* mutant (Pickett *et al.* 1990) remains sensitive to the promotion of lateral roots by IAA and IBA (Figure 4). Currently, the single representative of class 3 is the *ibr4* mutant, which has wild-type sensitivity to auxin transport inhibitors and synthetic auxins. This is an interesting class, as it separates two of the effects (inhibition of root elongation and initiation of lateral roots) of IBA on roots.

We also isolated two IBA-resistant mutants (class 4, *ibr5* and *ibr6*) that are resistant to the synthetic auxin 2,4-D and the auxin transport inhibitors NPA, TIBA, and HFCA (Figure 5 and data not shown). Auxin transport inhibitors block the action of the auxin efflux carrier (Lomax *et al.* 1995; Bennett *et al.* 1998). Root bending experiments (not shown) suggest that IBA is not a substrate of the EIR1 efflux carrier, which exports IAA and NAA but not 2,4-D (Utsuno *et al.* 1998). The observation that class 4 mutants are resistant to both 2,4-DB and 2,4-D indicates that the mechanism of IBA resistance in this class does not involve IBA to IAA conversion. The resistance to auxin transport inhibitors suggests that these two mutants may define components specific to IBA transport. Several Arabidopsis *tir* mutants have been described that are thought to be defective in auxin transport (Ruegger *et al.* 1997). Although the response of the *tir* mutants to IBA has not been reported, none map to the position of the *ibr5* mutant and only *tir1*, which is resistant to IAA, maps in the region of *ibr6* (Ruegger *et al.* 1998).

The *rib1* mutant (Poupart and Waddell 2000) has a similar phenotype to the class 4 *ibr* mutants (*r*esistant to *IB*A, 2,4-D, and auxin transport inhibitors; sensitive to IAA) and also may function in IBA transport. Because the map positions of *rib1*, *ibr5*, and *ibr6* are distinct (Figure 6), several specific components and regulatory proteins may be required to export IBA from plant cells, analogous to IAA and NAA efflux (Lomax *et al.* 1995; Bennett *et al.* 1998). One component involved in IBA efflux may be an uncharacterized member of the *EIR1/AGR1/PIN* gene family (Chen *et al.* 1998; Luschnig *et al.* 1998; Müller *et al.* 1998; Utsuno *et al.* 1998). It is also possible that the auxin transport inhibitor resistance observed in the class 4 mutants is an indirect effect not related to IBA transport.

IBA and ethylene: Although we have not yet isolated new alleles of known ethylene-resistant mutants (Johnson and Ecker 1998), these mutants share several phenotypes with class 4 IBA-response mutants, suggesting that IBA may interact with ethylene in the inhibition of root elongation. Whereas our class 4 mutants are not ethylene resistant in either roots or hypocotyls, the *ein2-1* and *etr1-1* mutants, like *ibr5* and *ibr6*, are resistant to the inhibitory effects of IBA (Figure 1C and data not shown), 2,4-D (data not shown), and auxin transport inhibitors (Fujita and Syono 1996; data not shown) on root elongation. Like the class 4 mutants, *ein2-1* remains sensitive to IAA and to the stimulatory effects of IBA on lateral root formation (data not shown). In contrast, the ethylene-insensitive *Never-ripe* tomato mutant is resistant to both the stimulatory effects of IBA on adventitious root formation and the inhibitory effects of IBA on root elongation (Clark *et al.* 1999), indicating that the precise relationship between ethylene and IBA may vary among species.

Numerous examples of interactions between auxin and ethylene signaling have been reported. Many Arabidopsis auxin-resistant mutants also have ethylene-resistant roots, including *aux1* (Pickett *et al.* 1990), *axr1* (Estelle and Somerville 1987; Leyser *et al.* 1993), *axr2* (Timpte *et al.* 1994), and *axr3* (Leyser *et al.* 1996; Rouse *et al.* 1998). Expression of the *ACC synthase 4* (*ACS4*) gene involved in ethylene biosynthesis is rapidly induced by IAA (Nakagawa *et al.* 1991; Abel *et al.* 1995) and IAA treatment increases ethylene levels in cotton (Morgan and Hall 1962). Certain auxin responses are thought to be mediated through these increases in ethylene, such as hypocotyl elongation in the light under nutrient limitation (Smalle *et al.* 1997). Our results suggest that ethylene responses previously ascribed to auxin may be differentially affected by the endogenous auxins IBA and IAA. Further analysis of IBA response mutants may allow a dissection of the contributions of IAA and IBA to ethylene responses.

A mutant PEX5: The *pex5-1* mutant is a member of the class 1 IBA-response mutants that are also defective in seed storage lipid utilization (Figure 3, Table 2). To our knowledge, this is the first plant peroxisomal import receptor mutant isolated, and it will be a valuable tool in the molecular dissection of peroxisome biogenesis in plants. The PEX5 protein has several domains, including C-terminal tetratricopeptide repeats (TPR) motifs (Figure 8), which act in binding the C-terminal "SKL" targeting signal of the PTS1 proteins (Brocard *et al.* 1994; Brickner *et al.* 1998). In addition, PEX5 contains 10 pentapeptide repeats (Figure 8), which also have been identified in the tobacco (Kragler *et al.* 1998) and watermelon proteins (Wimmer *et al.* 1998) and to a lesser extent in the human (seven copies; Schliebs *et al.* 1999) and yeast (one to three copies) proteins. The function of these repeats has not been determined, although it has been suggested that they may be a part of amphipathic α-helices that bind to peroxisomal membrane proteins necessary for protein import (Schliebs *et al.* 1999). PEX13 is a peroxisomal integral membrane protein that binds PEX5 via an SH3 domain and is required for PTS1 protein import (Urquhart *et al.* 2000). Deletion analysis of the *Pichia pastoris* PEX5 protein indicates that a 114-amino-acid region (Trp100 to Glu213), which includes the pentapeptide repeats, is necessary for PEX13 binding (Urquhart *et al.* 2000).

This N-terminal region of PEX5 also has been suggested to bind PEX14, a second integral membrane protein required in peroxisomal import (Schliebs *et al.* 1999). The *pex5-1* mutation alters a conserved Ser residue in the midst of the pentapeptide repeat region but upstream of the TPR domains, suggesting that it may disrupt some aspect of PEX5 function other than substrate binding, such as interaction with these or other peroxisomal membrane proteins (Schliebs *et al.* 1999).

The *pex5* mutant has a long root on inhibitory concentrations of IBA, implicating the PEX5 protein in IBA to IAA conversion. Because the majority of peroxisomal matrix proteins contain PTS1 signals and require PEX5 for import, it is likely that the role of PEX5 in the conversion of IBA to IAA is to ensure that the enzymes necessary for the conversion are efficiently imported into the peroxisomes. In the *pex5* mutant, IBA is probably converted to IAA at a much slower rate, due to the decreased import of required enzymes.

The *pex5* mutant defect suggests that the conversion of IBA to IAA is important for IBA function. In further support of this hypothesis, we found that two previously isolated and molecularly characterized fatty acid β-oxidation defective mutants, *aim1* (Richmond and Bleecker 1999) and *ped1* (Hayashi *et al.* 1998b), also are IBA-resistant class 1 mutants (Figure 1C and data not shown). Interestingly, AIM1 has a C-terminal PTS1 signal for peroxisomal import, indicating that it is among the proteins imported by PEX5 that act in IBA to IAA conversion (in addition to fatty acid β-oxidation).

Many genes can mutate to give IBA resistance: Our screen to identify IBA-resistant, IAA-sensitive mutants has been fruitful. Fourteen confirmed mutants have been identified, which comprise at least nine unique loci. The fact that we have not yet obtained more than two alleles of any gene indicates that defects in a large number of genes can lead to IBA resistance. In theory, this could include any proteins acting in IBA recognition, signal transduction, response, or transport. Because IBA acts, at least in part, via its conversion to IAA, other genes that may cause this phenotype if defective would include enzymes acting in β-oxidation and peroxisome biogenesis or maintenance. Multiple isozymes have been identified for each step of plant fatty acid β-oxidation, which may reflect chain-length specificity (Kirsch *et al.* 1986; Hooks *et al.* 1996; Hayashi *et al.* 1998a, 1999) or differences in expression. A potential pathway for the β-oxidation of IBA to IAA is proposed in Figure 9, which indicates the proteins likely to be involved in this conversion. Mutant genes in any of these steps could cause an IBA-resistant, IAA-sensitive phenotype. For example, the *aim1* (Richmond and Bleecker 1999) and *ped1* (Hayashi *et al.* 1998b) mutants would act in the conversion of IBA to IAA at steps 3/4 and 5, respectively (Figure 9).

Defects in proteins acting in peroxisome biogenesis (reviewed in Olsen 1998) should also result in an IBA-resistant and IAA-sensitive phenotype. Whereas at least 19 peroxins (encoded by *PEX* genes) are involved in yeast peroxisomal biogenesis (reviewed in Elgersma and Tabak 1996; Erdmann and Kunau 1992; Kunau 1998), few plant *PEX* genes have been characterized to date. The genome sequencing project and other directed efforts will undoubtedly reveal Arabidopsis homologs of many of these genes. IBA-resistance screens may represent an effective and nonbiased method to isolate plant *pex* mutants, which will be important in elucidating peroxisome function in plants.

In summary, analysis of IBA-resistant, IAA-sensitive mutants has revealed four phenotypic classes. Class 1

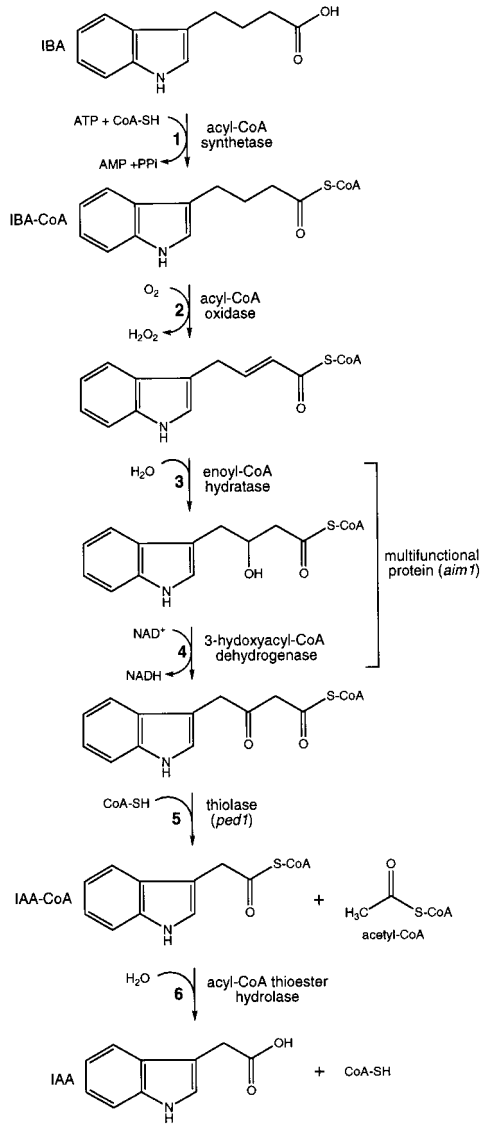


Figure 9.—A possible mechanism for the conversion of IBA to IAA in the peroxisome. Mutants in various steps of the pathway are indicated in parentheses. The *ped1* (Hayashi *et al.* 1998b) and *aim1* (Richmond and Bleecker 1999) mutants are defective at the corresponding steps in fatty acid β-oxidation, and their resistance to IBA suggests that they act in this pathway as well.

mutants are defective in peroxisomal β-oxidation, suggesting that at least part of IBA function is as an IAA precursor in Arabidopsis. Class 2 and 3 mutants may have defects in IBA-specific signaling or response factors, whereas the class 4 mutants may have compromised IBA transport. Continued molecular dissection of these IBA-response mutants, as well as the responses of other plant hormone mutants to IBA, may allow a better understanding of the mechanisms of IBA function in Arabidopsis and of how these two endogenous auxins interact.

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