The Arabidopsis *pxa1* Mutant Is Defective in an ATP-Binding Cassette Transporter-Like Protein Required for Peroxisomal Fatty Acid β-Oxidation[1]

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Peroxisomes are important organelles in plant metabolism, containing all the enzymes required for fatty acid β-oxidation. More than 20 proteins are required for peroxisomal biogenesis and maintenance. The Arabidopsis *pxa1* mutant, originally isolated because it is resistant to the auxin indole-3-butyric acid (IBA), developmentally arrests when germinated without supplemental sucrose, suggesting defects in fatty acid β-oxidation. Because IBA is converted to the more abundant auxin, indole-3-acetic acid (IAA), in a mechanism that parallels β-oxidation, the mutant is likely to be IBA resistant because it cannot convert IBA to IAA. Adult *pxa1* plants grow slowly compared with wild type, with smaller rosettes, fewer leaves, and shorter inflorescence stems, indicating that PXA1 is important throughout development. We identified the molecular defect in *pxa1* using a map-based positional approach. *PXA1* encodes a predicted peroxisomal ATP-binding cassette transporter that is 42% identical to the human adrenoleukodystrophy (ALD) protein, which is defective in patients with the demyelinating disorder X-linked ALD. Homology to ALD protein and other human and yeast peroxisomal transporters suggests that PXA1 imports coenzyme A esters of fatty acids and IBA into the peroxisome for β-oxidation. The *pxa1* mutant makes fewer lateral roots than wild type, both in response to IBA and without exogenous hormones, suggesting that the IAA derived from IBA during seedling development promotes lateral root formation.

Peroxisomes are small, ubiquitous organelles encased in a single lipid bilayer that contain hydrogen peroxide-producing oxidases and catalases to inactivate reactive molecules (for review, see Gerhardt, 1992; Kindl, 1993; Olsen, 1998; Tabak et al., 1999). Arabidopsis and other oilseed plants β-oxidize long-chain fatty acids (LCFAs) in peroxisomes to provide energy during germination. Plant peroxisomes also contain enzymes that act in photorespiration (Olsen, 1998) and the catabolism of branched-chain amino acids (Gerhardt, 1992; Zolman et al., 2001). In addition, seedlings and senescing tissues contain specialized peroxisomes called glyoxysomes that convert acetyl-coenzyme A (CoA) to succinate, which is transported to the mitochondria where it fuels the tricarboxylic acid cycle (Gerhardt, 1992; Olsen, 1998).

Mammals metabolize fatty acids in both mitochondria and peroxisomes, and each organelle shortens a distinct subset of fatty acids (Lazarow, 1993; Tabak et al., 1999). In contrast, plants and yeast catabolize fatty acids exclusively in peroxisomes (Gerhardt, 1992; Kindl, 1993). Because peroxisomes lack DNA, proteins required for β-oxidation and other peroxisomal processes are translated in the cytoplasm and then imported (Olsen, 1998; Subramani, 1998; Tabak et al., 1999). Peroxisomal matrix proteins contain one of two peroxisomal targeting signals (PTSs). The PTS1 is made up of the amino acids "SKL" (or a conserved variant) at the extreme C termini of peroxisomal matrix-bound proteins (Gould et al., 1989). The PEX5 receptor binds PTS1-containing proteins in the cytoplasm and translocates them into the peroxisome (Olsen, 1998; Subramani, 1998; Tabak et al., 1999). PEX7 imports proteins that have the nine-residue N-terminal PTS2 sequence (Olsen, 1998; Subramani, 1998; Tabak et al., 1999). Both the PEX5 and PEX7 matrix protein receptors have been identified in plants (Brickner et al., 1998; Kragler et al., 1998; Wimmer et al., 1998; Schumann et al., 1999).

In addition to matrix enzymes, peroxisomes must import the substrates and cofactors required in peroxisomal processes, such as fatty acids destined for catabolism. Fatty acids are synthesized and metabolized in different subcellular locations, and how they are transported between organelles is just beginning to be understood. Yeast and humans apparently transport LCFAs into peroxisomes via ATP-binding cassette (ABC)-containing ATPases in the peroxisomal membrane (Shani and Valle, 1998; for review, see Dubois-Dalcq et al., 1999; Holland and Blight, 1999). The transporter required for fatty acid uptake into peroxisomes has not yet been characterized in any plant species.

Yeast mutants defective in peroxisomal function or β-oxidation utilize alternative carbon sources poorly; screens for mutants with reduced growth on oleic acid have uncovered more than 20 proteins required for peroxisomal biogenesis, maintenance, and the im-

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port of enzymes and metabolites (for review, see Erdmann and Kunau, 1992; Lazarow, 1993; Olsen, 1998; Subramani, 1998; Tabak et al., 1999). In humans, mutations in peroxisomal proteins cause several life-threatening diseases, including Zellweger syndrome, X-linked adrenoleukodystrophy (X-ALD), and Refsum disease (for review, see Fujiki, 1997; Gärtner, 2000; Gould and Valle, 2000).

We are studying the phytohormone auxin, which influences virtually every aspect of plant growth and development, including root elongation, lateral root initiation, organ identity, and tropic responses (Davies, 1995). Two naturally occurring auxins found in plants are indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA). Although these compounds differ only in the two additional carbon atoms on the IBA side chain, they have different potencies in bioassays (Ludwig-Müller, 2000; Zolman et al., 2000; Bartel et al., 2001).

Experiments with labeled auxin indicate that several plant species can convert IBA to IAA (Epstein and Ludwig-Müller, 1993; Ludwig-Müller, 2000; Bartel et al., 2001). Because the conversion shortens the IBA side chain by two carbons, this process has been proposed to occur similarly to fatty acid β-oxidation (Wain and Wightman, 1954; Fawcett et al., 1960). Previously, we described a collection of Arabidopsis mutants that are resistant to the inhibitory effects of IBA on root elongation but that respond normally to IAA (Zolman et al., 2000). A subset of these mutants is distinguished by developmental defects in the absence of exogenous Suc and inefficient metabolism of LCFAs during germination (Zolman et al., 2000), suggesting defects in peroxisomal β-oxidation. Therefore, these IBA-response mutants probably have defects in the β-oxidation of both fatty acids and IBA, causing Suc-dependent seedling development and IBA-resistant root elongation. Some of these mutants are defective in proteins acting directly in β-oxidation. For example, enzymes defective in mutants resistant to IBA or the IBA analog 2,4-dichlorophenoxybutyric acid (2,4-DB) include an acyl-CoA oxidase (*acx3*; Eastmond et al., 2000), a multifunctional protein (*aim1*; Richmond and Bleecker, 1999), and a thiolase (*ped1*; Hayashi et al., 1998) that act in β-oxidation. In addition, because fatty acid β-oxidation is strictly peroxisomal in plants, mutations in peroxisomal biogenesis or maintenance proteins can disrupt β-oxidation. For example, a mutant defective in PEX5, the PTS1 peroxisomal matrix protein importer, is IBA resistant (Zolman et al., 2000) and the 2,4-DB-resistant *ped2* mutant is defective in the peroxisomal membrane protein PEX14 (Hayashi et al., 2000).

Here, we describe an IBA-response mutant that requires exogenous Suc for development and is resistant to IBA in both root elongation and lateral root initiation. We used a map-based positional approach to determine that the mutant is defective in *PXA1*, which encodes an ABC transporter similar to peroxisomal fatty acid transporters and the human protein disrupted in X-ALD. PXA1 appears to act in the peroxisomal import of fatty acids and IBA for β-oxidation.

RESULTS

pxa1 Has Altered Responses to IBA

We previously described the isolation of 14 Arabidopsis IBA-response mutants (Zolman et al., 2000). Here, we report the characterization and cloning of the *PXA1* gene, which is defective in one of the IBA-response mutants (see below). As shown in Figure 1, high levels of exogenous auxin inhibit wild-type root elongation. The *pxa1* mutant is resistant to the inhibition of root elongation by IBA over a range of concentrations (Fig. 1A) but remains sensitive to inhibition by IAA (Fig. 1B). *pxa1* also is resistant to the inhibitory effects of 2,4-DB but is sensitive to

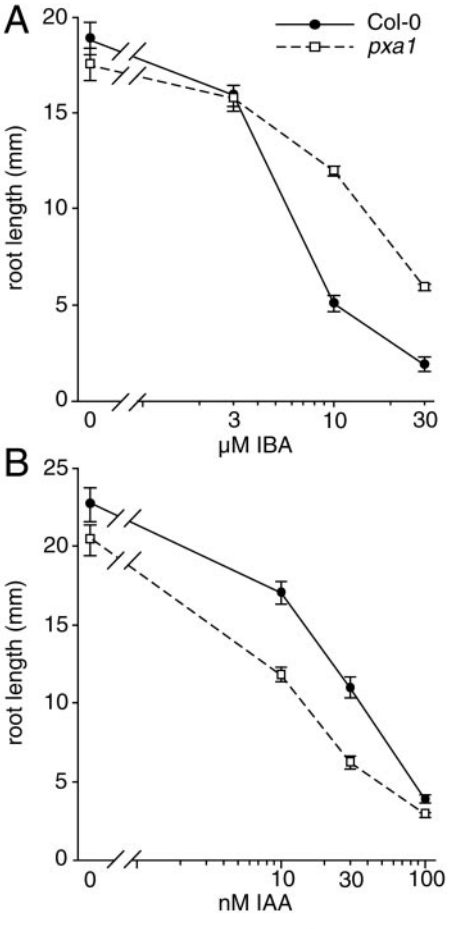

Figure 1. *pxa1* is IBA resistant. A, Root elongation on IBA. Eight-day-old wild-type and mutant seedlings grown on the indicated concentrations of IBA under yellow-filtered light were removed from the agar, and the length of the primary root was measured. Error bars indicate the SE values ($n > 9$). B, Root elongation on IAA. Primary root length on IAA was measured as described above. Error bars indicate the SE values ($n > 12$).

the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D; Zolman et al., 2000) and naphthalene-1-acetic acid (data not shown).

In addition, *pxa1* has defects in lateral root initiation. We examined root initiation by growing plants on unsupplemented medium for 4 d, transferring seedlings to IBA, IAA, or unsupplemented medium, and counting lateral roots after an additional 4 d. On unsupplemented medium, wild-type plants form few lateral roots (approximately one per plant; Fig. 2), but more lateral roots are induced when plants are transferred to either IBA (approximately seven per plant) or IAA (approximately five per plant; Fig. 2). *pxa1* has fewer lateral roots than wild type on unsupplemented medium and is completely unresponsive to IBA, making no lateral roots after 8 d (Fig. 2). However, the mutant does respond to the stimulatory effects of IAA (Fig. 2). To better quantify the defect, we extended the growth period for lateral root initiation to 6 d before and 6 d after transfer. Again, addition of IBA strongly initiates lateral roots in wild type (approximately 16 per plant) compared with plants on unsupplemented medium (approximately nine per plant; Fig. 2). Similar to the short experiment, *pxa1* has fewer lateral roots than wild type after 12 d and does not initiate additional lateral roots with IBA treatment (Fig. 2).

pxa1 Has Defects in Growth and Development

Mutants defective in peroxisomal β-oxidation have growth defects on minimal medium because oilseed plants (like Arabidopsis) use LCFAs as an energy source before photosynthesis begins (Hayashi et al., 1998). Plants that cannot catabolize fatty acids consequently cannot develop unless exogenous Suc is provided. *pxa1* and certain other IBA-response mutants catabolize seed storage fatty acids slowly and have growth defects on Suc-free medium, suggesting β-oxidation defects (Zolman et al., 2000). In fact, *pxa1* is among our most severe Suc-dependent mutants. To quantify the mutant defects, we examined germination (radicle emergence from the seed coat) and establishment (cotyledon expansion in light-grown plants; hypocotyl elongation in dark-grown plants) of wild-type and mutant seeds. Seeds were plated on medium either with or without Suc and grown in the light or in the dark. As shown in Table I, most (>80%) wild-type and mutant seeds germinated regardless of the growth conditions. Whereas Suc-grown *pxa1* seedlings developed normally, mutant seedlings grown in the absence of Suc arrested after germination, even in the light. This phenotype is consistent with a strong defect in peroxisomal β-oxidation. Germinated mutant seeds could reinitiate normal development when transferred to medium containing Suc (data not shown), indicating that they were developmentally arrested rather than dead.

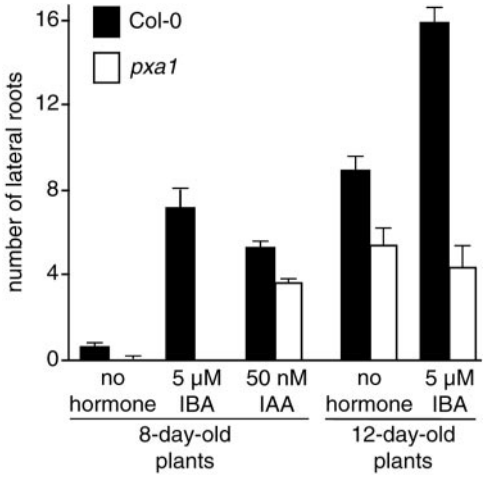

Figure 2. *pxa1* is defective in lateral root formation. Seedlings were grown for 4 or 6 d on hormone-free medium and transferred to medium with no hormone, 50 nM IAA, or 5 μM IBA. After 8 or 12 d, plants were removed from the agar, and the number of lateral roots was counted. Error bars indicate SE values ($n > 16$).

In addition to germination defects, *pxa1* mutants are smaller than wild type. *pxa1* has a slightly shorter root than wild type on unsupplemented medium, as seen at the "no hormone" data points in Figure 1. To further investigate the mutant growth defects, we examined wild-type and *pxa1* plants over time. The mutant has a smaller rosette (Fig. 3A) and fewer leaves (Fig. 3B) than wild type throughout adult development. The primary inflorescence also is consistently shorter in *pxa1* than wild type (Fig. 3C). Each of these characteristics suggests that *pxa1* plants grow more slowly than wild type. *pxa1* has a delayed time to flowering when measured by the number of days but flowers with a similar number of leaves as wild type (data not shown). These data indicate that *pxa1* is developmentally delayed, but the defect in this mutant does not affect flowering time pathways. Other than decreased size, mutant plants are morphologically similar to wild type (data not shown).

Positional Cloning Reveals a Defect in an Apparent ABC Transporter

The IBA-resistant root elongation in *pxa1* is dominant. As shown in Figure 4A, heterozygote plants can elongate roots similarly to the homozygous mutant on inhibitory concentrations of IBA. However, *pxa1* mutant development on Suc-free medium is recessive because the *PXA1/pxa1* heterozygote develops normally without Suc (Fig. 4B).

We used positional information to identify the molecular defect in the *pxa1* mutant. We outcrossed a mutant plant (Col-0 background) to Wassilewskija (Ws) and selected IBA-resistant F_2 progeny. Because the *pxa1* IBA-resistant root elongation phenotype is dominant (Fig. 4A), we tested the progeny from IBA-resistant F_2 plants on Suc-free medium to identify

Table I. *Seed germination and development*

Wild-type (Col-0) and *pxa1* mutant seeds were grown on medium in either the presence or absence of Suc for 6 d in the light or 1 d in the light and 5 d in the dark. At least 14 seeds were examined for each condition.

Genotype	Exogenous Suc	Light		Dark	
		Germinated[a]	Established[b]	Germinated	Established[c]
	mM	%			
pxa1	0	100	0	86	0
pxa1	15	100	95	88	88
Col-0	0	100	100	94	89
Col-0	15	100	93	82	82

[a] Radicle emergence from the seed coat. [b] Expansion of cotyledons. [c] Hypocotyl elongation.

lines that were homozygous at the mutant locus. We then used PCR-based polymorphic markers to localize the mutant defect to the bottom of chromosome 4, south of nga1139 (Fig. 5A; see "Materials and Methods"). Examination of the genes between this marker and the telomere identified a candidate gene on the T5J17 bacterial artificial chromosome (GenBank accession no. AL035708). This bacterial artificial chromosome contains a gene (*At4g39850*) predicted to encode a protein resembling a subset of ABC-ATPase transporters. In yeast and humans, similar ATPases are required to import LCFAs into the peroxisome for catabolism (Shani and Valle, 1998; Dubois-Dalcq et al., 1999; Holland and Blight, 1999). We hypothesized that a defect in this protein would disrupt β-oxidation, resulting in an IBA-resistant mutant with developmental defects. We sequenced this gene using *pxa1* mutant DNA (see "Materials and Methods") and identified a G-to-A mutation at position 5,559 (where position 1 is the A of the initiator ATG; Fig. 5C).

To confirm that the nucleotide change in *pxa1* causes the mutant phenotypes, we complemented the mutant with a wild-type copy of *PXA1*. We inserted a full-length *PXA1* cDNA in the 35SpBARN plant transformation vector (LeClere and Bartel, 2001) behind the constitutive 35S cauliflower mosaic virus promoter. This construct (35S*PXA1*) was transformed into *pxa1* mutant plants, and transformants were selected using gluphosinate ammonium (Basta, Crescent Chemical Co., Hauppauge, NY) herbicide. Mutant plants transformed with this construct developed normally on medium without Suc (Fig. 5D), suggesting a restored ability to β-oxidize LCFAs. In addition, transformants became sensitive to root elongation inhibition on IBA (Fig. 5E) and regained the ability to make lateral roots both in response to IBA (Fig. 5F) and without induction (data not shown). This phenotypic rescue indicates that we have identified the mutation causing the defect in *pxa1*. Therefore, we named this gene *PXA1* (*p*ero*x*isomal *A*BC transporter *1*).

To examine the protein sequence and the intron/exon junctions of the gene, we obtained and sequenced an apparently full-length cDNA (see "Materials and Methods"), which revealed a predicted coding sequence of 1,338 amino acids (GenBank accession no. AF378120). Comparing the cDNA and genomic sequences revealed that *PXA1* is interrupted by 24 introns. The G-to-A change in the *pxa1* mutant is at the exon/intron junction following the 24th exon. It is interesting that sequencing the *PXA1* cDNA also revealed that the protein predicted by the sequencing project (T5J17.20, GenBank accession no. AL035708) was incorrectly spliced at 15 (of 48) intron/exon junctions, including three exons that were missing completely (data not shown). These discrepancies reinforce the continued importance of cDNA analysis in protein predictions.

The consensus 5′-splice site for wild-type exon/intron junctions is AG/gt, and previous work has established that the intronic GT bases are absolutely required for splicing (Brown et al., 1996). The *pxa1* mutation alters an essential G in the 5′-exon/intron splice site (Fig. 5C), converting the sequence AA/gt to AAAT. To determine how the splicing mutation affects the mutant protein, we made RNA from 5-d-old wild-type and mutant plants. We reverse transcribed the RNA to make the *PXA1* and *pxa1* cDNAs and amplified these templates using primers spanning the mutation (see "Materials and Methods"). We initially expected that a second AAGT immediately following the original splice site would reinitiate splicing, removing the intron but shifting the remainder of the protein out of frame. Sequencing the *pxa1* cDNA, however, revealed that the entire intron was present in the mutant cDNA, which would cause translation through the intron and the coding of a premature stop codon (Fig. 5C). In the resultant pxa1 protein, 19 amino acids (encoded by intron 24) replace the final 32 amino acids (encoded by exon 25) of PXA1.

DISCUSSION

pxa1 originally was identified as an IBA-response mutant (Zolman et al., 2000). It is resistant to the inhibitory effects of IBA on root elongation (Fig. 1A) but remains sensitive to IAA (Fig. 1B) and the synthetic auxin naphthalene-1-acetic acid (data not shown). The mutant also is resistant to the IBA analog 2,4-DB (Zolman et al., 2000), which is converted

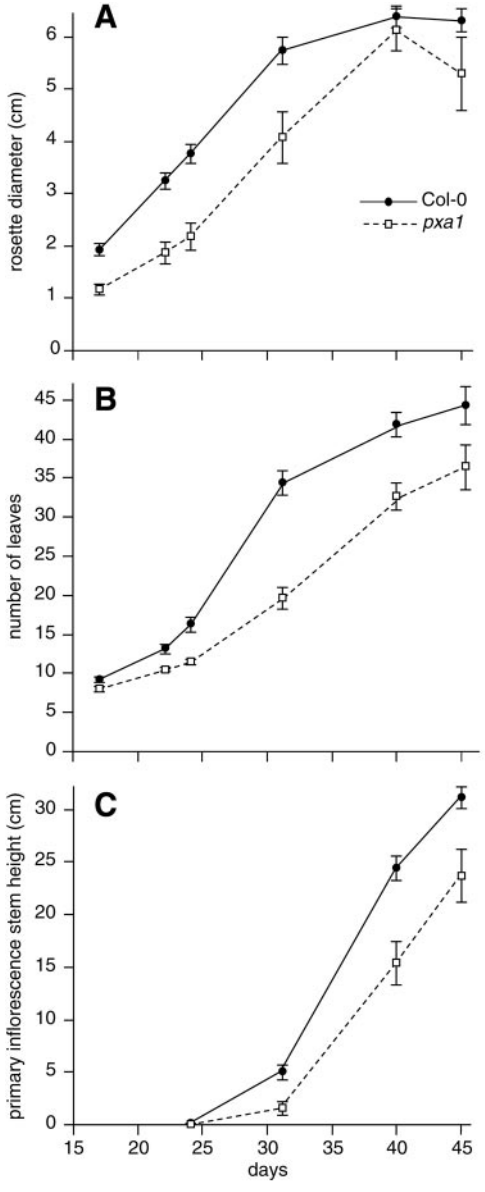

Figure 3. *pxa1* grows more slowly than wild type. Ten-day-old wild-type and *pxa1* mutant plants were transferred to soil and examined weekly. A, Rosette diameter at widest point. B, Number of rosette leaves. C, Height of the primary inflorescence stem. Error bars indicate SE values ($n > 13$).

to the synthetic auxin 2,4-D in a mechanism similar to the β-oxidation of IBA (Wain and Wightman, 1954; Hayashi et al., 1998). In addition, *pxa1* is defective in lateral root initiation, making fewer lateral roots than wild type in the absence of hormone and particularly in response to IBA induction (Fig. 2).

The *pxa1* mutant also has developmental defects. Although mutant seeds germinate normally, mutant plants do not develop beyond germination unless provided with exogenous Suc (Table I). This phenotype suggests severe peroxisomal defects, because peroxisomal β-oxidation mutants cannot catabolize stored fatty acids for energy before photosynthesis begins (Hayashi et al., 1998). Throughout development, *pxa1* mutant plants grow more slowly than wild-type plants, with smaller rosettes, fewer leaves, and shorter inflorescence stems (Fig. 3). *pxa1* also has a slightly shorter root than wild type (Fig. 1).

The IBA resistance of the *pxa1* mutant is dominant; heterozygous *PXA1/pxa1* plants elongate roots on IBA similarly to homozygous *pxa1/pxa1* plants (Fig. 4A). In contrast, the Suc dependence of mutant seedling establishment appears fully recessive (Fig. 4B). Also, the *ped1* mutant, which is defective in a thiolase acting in fatty acid β-oxidation (Hayashi et al., 1998), the *ped2* mutant, which is defective in the peroxisomal membrane protein PEX14 (Hayashi et al., 2000), and the *ped3* mutant are dominant for resistance to the IBA analog 2,4-DB but recessive for Suc dependence during germination (Hayashi et al., 1998). These results suggest that haplo-insufficiency, rather than a gain-of-function in the pxa1 protein, causes the dominance of *pxa1* IBA resistance. Because het-

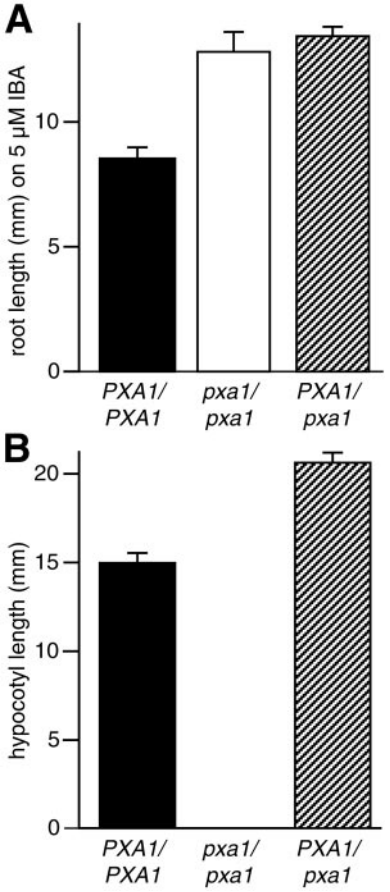

Figure 4. Genetic analysis of *pxa1* phenotypes. A, *pxa1* IBA-resistant root elongation is dominant. Root elongation on 5 μM IBA was measured as described in the legend to Figure 1. Error bars indicate SE values ($n > 13$). B, *pxa1* Suc dependence is recessive. Seedlings were grown on Suc-free medium for 1 d in the light and 5 d in the dark, and hypocotyl length was measured. Error bars indicate SE values ($n > 16$). For A and B, *PXA1/pxa1* seeds are F_1 progeny from a backcross of *pxa1* to wild-type Columbia (Col-0).

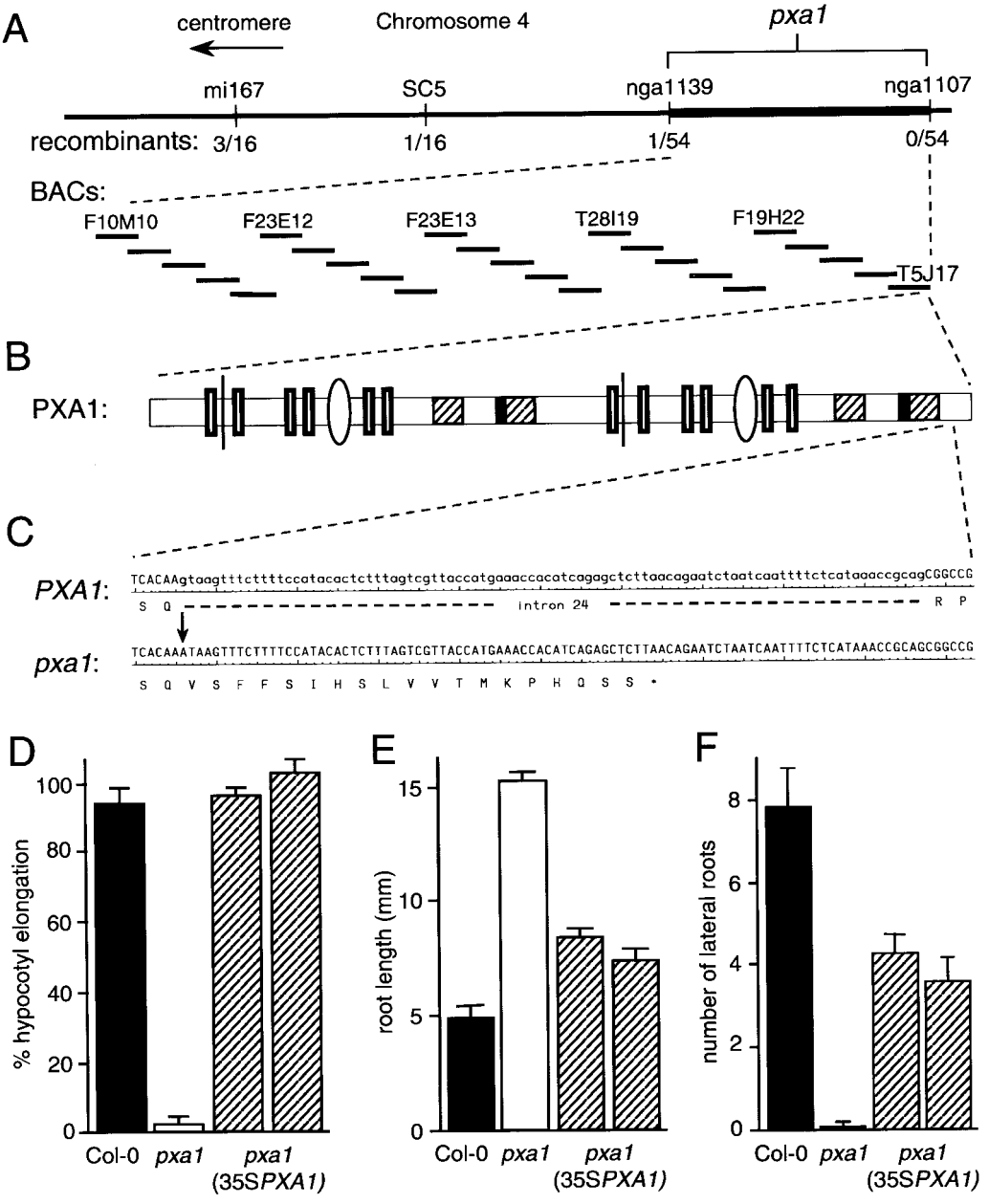

Figure 5. Positional cloning of *PXA1*. A, Recombination mapping of *pxa1*. Mapping with PCR-based markers mi167 (see "Materials and Methods"), SC5 (http://Arabidopsis.org/maps/CAPS_Chr4.html), nga1139, and nga1107 (Bell and Ecker, 1994) localized the defect between nga1139 and the telomere. Examination of the sequenced BACs (thick lines) in this region revealed the *PXA1* gene (At4g39850) on T5J17. B, PXA1 structure. PXA1 contains several conserved domains in its predicted protein structure, including 12 transmembrane (TM) domains (hollow boxes), two loop 1 conserved regions (thin lines), two EAA-like domains (ovals), Walker A and B ATP-binding domains (hatched rectangles), and two ABC signature motifs, or C sequences (black rectangles). C, *pxa1* has a G-to-A mutation at position 5559 (where 1 is the A of the initiator ATG) that destroys the 5′-splice site of the last intron. Reverse transcription-PCR analysis indicates that the last intron is not removed from the protein, and thus translation continues into the intron sequence. Nucleotides 7,349 through 7,452 are shown, corresponding to the end of the 24th exon and beginning of the 25th exon in wild type and the 24th exon in the mutant, which continues coding through the intron to a premature termination codon. D through F, 35S*PXA1* rescues the *pxa1* mutant phenotype. D, Seeds from wild-type (Col-0), *pxa1-1*, and two independent homozygous lines from *pxa1-1* plants transformed with the 35S*PXA1* construct were analyzed for hypocotyl elongation as described in the legend of Figure 4B. Error bars indicate SE values ($n > 12$). E, Root elongation inhibition by 10 μM IBA was measured for wild-type, *pxa1*, and transformed plants as described in the legend of Figure 1A. Error bars indicate SE values ($n > 13$). F, Lateral root initiation by wild-type, *pxa1*, and transformed plants was examined in response to 5 μM IBA after 8 d as described in the legend of Figure 2. Error bars indicate SE values ($n > 13$).

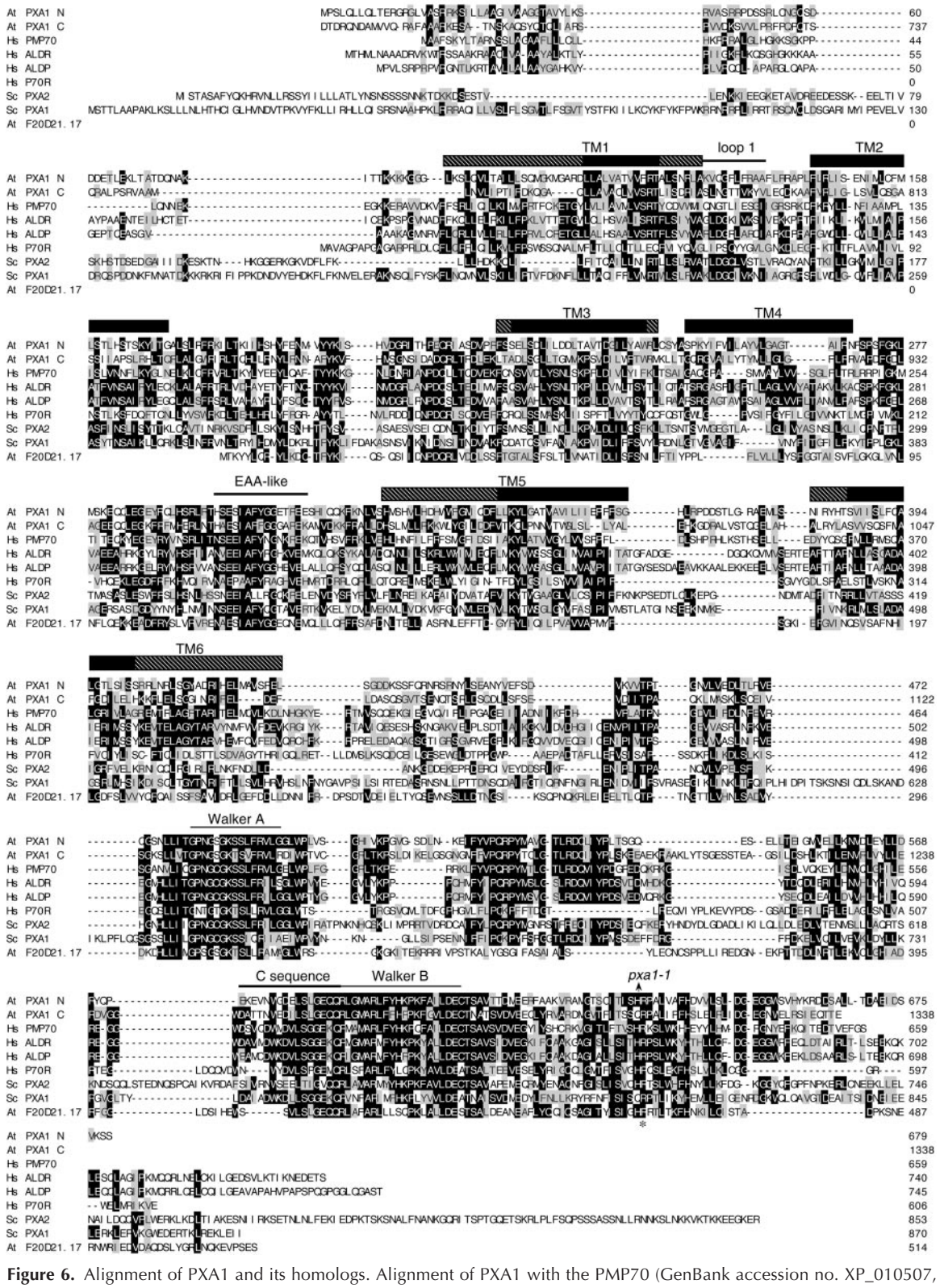

Figure 6. Alignment of PXA1 and its homologs. Alignment of PXA1 with the PMP70 (GenBank accession no. XP_010507, 45% identical to Arabidopsis PXA1), ALDR (NP_005155, 42% identical), ALDP (XP_010174, 42% identical), and P70R (NP_064731, 36% identical) proteins from human (Hs); the Pxa2p (NP_012733, 30% identical) and Pxa1p (NP_015178, 24% identical) proteins from yeast (Sc); and the F20D21.17 (AAD25615, 21% identical) protein from Arabidopsis (At). The PXA1 protein is divided into halves to show its homology with the hemitransporters; PXA1 N is the N terminus of the protein from amino acids 1 to 679; PXA C is the C terminus of the protein from amino acids 680 to 1,338. Sequences were aligned

(*Legend continues on next page.*)

erozygous plants apparently metabolize sufficient fatty acids for normal development, but do not β-oxidize enough IBA to inhibit root elongation, IBA resistance appears to provide a more sensitive assay for β-oxidation defects than does Suc dependence. The defect in *ped3* has not been reported, but *ped3* maps to the same interval as *pxa1* on the bottom of chromosome 4 and also is Suc dependent for seedling establishment (Hayashi et al., 1998), suggesting that it may also have a defect in *PXA1*.

The gene defective in *pxa1* encodes a protein homologous to members of the ABC-ATPase superfamily, which are ATP-driven pumps or channels transporting substrates ranging from small ions to large polypeptides across membranes (for review, see Holland and Blight, 1999; Davies and Coleman, 2000). More than 100 putative ABC transporters have been identified in Arabidopsis (Davies and Coleman, 2000; Sánchez-Fernández et al., 2001). As shown in Figure 6, the Arabidopsis PXA1 protein resembles two yeast peroxisomal ABC transporters (Pxa1p/Pat2p/Pal1p and Pxa2p/Pat1p; Shani et al., 1995; Hettema et al., 1996; Shani and Valle, 1996; Swartzman et al., 1996) and four human transporters (PMP70/PXMP1, P70R, ALDP, and ALDRP; Gärtner et al., 1992, 1998; Kamijo et al., 1992; Mosser et al., 1993; Lombard-Platet et al., 1996; Holzinger et al., 1999). Several of these proteins have been localized to the peroxisomal membrane (Kamijo et al., 1992; Contreras et al., 1994; Imanaka et al., 1996, 1999; Swartzman et al., 1996; Holland and Blight, 1999), and experiments using semi-intact yeast cell systems show that oleic acid enters the peroxisome as a CoA ester using PXA2 in an ATP-dependent manner (Verleur et al., 1997). Because Arabidopsis PXA1 closely resembles these proteins and the *pxa1* mutant has β-oxidation defects, we predict that this protein is an ABC transporter acting in peroxisomal import. To our knowledge, this is the first characterization of a potential peroxisomal fatty acid transporter in plants.

ABC transporters are composed of two homologous halves, which can be encoded either by one gene as a single polypeptide or by separate genes that each encode a hemitransporter (Dubois-Dalcq et al., 1999; Holland and Blight, 1999; Davies and Coleman, 2000). Each half has four to six TM domains, a cytoplasmic ATP-binding and hydrolysis region containing the highly conserved Walker A and B domains (GX_2GXGKS/T; Figs. 5B, hatched boxes, and 6), and a 19-amino acid C sequence (Figs. 5B, black boxes, and 6; Dubois-Dalcq et al., 1999; Holland and Blight, 1999; Davies and Coleman, 2000).

The yeast and human peroxisomal ABC transporters are composed of two hemitransporters (Dubois-Dalcq et al., 1999; Holland and Blight, 1999; Liu et al., 1999). In contrast, the Arabidopsis PXA1 protein appears to be a complete transporter with two homologous halves: amino acids 1 through 679 make up the first half of the transporter, whereas amino acids 680 through 1,338 make up the second half (Figs. 5B and 6). These peroxisomal ABC transporters contain two additional highly conserved motifs. The loop following the TM domain 1 (loop 1) is in the peroxisomal matrix and probably controls membrane insertion (Figs. 5B, black lines, and 6; Dubois-Dalcq et al., 1999). The EAA-like domain (Figs. 5B, ovals, and 6) is a second conserved region on the cytoplasmic face of the protein between TM domains 4 and 5 and is thought to control substrate specificity by binding fatty acids (Shani et al., 1995; Shani and Valle, 1996). The mechanism of fatty acid transport by these peroxisomal proteins has not been elucidated. Some evidence indicates that the human ABC protein P-glycoprotein acts as a flippase that transports phospholipids between leaflets of the plasma membrane (Romsicki and Sharom, 2001), although other mechanisms also have been suggested for protein P-glycoprotein and other ABC transporters (van Veen and Konings, 1997; van Veen, 2001).

Yeast *PXA1* and *PXA2* deletion mutants have morphologically intact peroxisomes but have reduced (approximately 20%–50%) LCFA β-oxidation and consequently cannot use oleic acid as a carbon source (Shani et al., 1995; Hettema et al., 1996; Shani and Valle, 1996). The β-oxidation of short-chain fatty acids is unaffected, indicating that β-oxidation enzymes and matrix protein import remain intact (Hettema et al., 1996). The phenotype of the yeast *pxa1/pxa2* double mutant is comparable with the single mutants and Pxa1p and Pxa2p interact in yeast two-hybrid assays and co-immunoprecipitation experiments, suggesting that the two proteins function together (Shani et al., 1995; Hettema et al., 1996; Shani and Valle, 1996).

Figure 6. (*Legend continued from preceding page.*)
with the MegAlign program (DNASTAR, Inc., Madison, WI) using the ClustalW method. Amino acid residues identical in at least three of the sequences are boxed in black and similar amino acids are boxed in gray. Hyphens indicate gaps introduced to maximize alignment. The arrow above the alignment marks the position of the *pxa1* splicing defect and conserved domains are indicated above the sequence. The 12 TM domains were predicted using homology to the human (Shani and Valle, 1998; Dubois-Dalcq et al., 1999) and yeast proteins (Shani and Valle, 1996; Swartzman et al., 1996) and by TM prediction programs TMAP (Perrson and Argos, 1994, 1996), SMART; (Schultz et al., 1998, 2000), and TMpred (Hofmann and Stoffel, 1993). TM domains are indicated by rectangles above the sequence; regions of high certainty are indicated by black boxes; regions of lower certainty are indicated by hatched boxes. An asterisk marks an Arg residue in ALDP that is a site of recurrent mutations in X-ALD patients (Dubois-Dalcq et al., 1999), which coincides with the first amino acid affected in the *pxa1* mutant.

In humans, disruption of the PXA1 homolog ALDP is lethal. Patients with X-ALD accumulate very LCFAs (VLCFAs) in serum and tissues, resulting in adrenal insufficiency and myelin destruction in the central nervous system (Hettema et al., 1996; Dubois-Dalcq et al., 1999; Gärtner, 2000). Fibroblasts from X-ALD patients have decreased LCFA β-oxidation, causing the fatty acid accumulation (Lazo et al., 1989; Braiterman et al., 1999). The reduced β-oxidation and consequent accumulation of LCFAs in yeast and humans with defects in these ABC-type transporters suggests that these proteins facilitate the peroxisomal import of LCFA CoA esters. Consistent with this hypothesis, β-oxidation of long-chain CoA esters requires Pxa2p in a semi-intact yeast system (Verleur et al., 1997).

However, other data suggest that VLCFA transport across the peroxisomal membranes is normal in X-ALD fibroblasts (Singh et al., 1992). In addition, X-ALD fibroblasts can β-oxidize long-chain CoA esters but not LCFAs (Hashmi et al., 1986; Lazo et al., 1988). The VLCFA synthetase responsible for activating the fatty acids to the CoA ester has reduced activity in X-ALD fibroblasts (Lazo et al., 1988, 1989; Wanders et al., 1988; Singh et al., 1992), leading to a second hypothesis that ALDP acts in the activation of VLCFAs or the stabilization of VLCFA synthetase (Smith et al., 2000). Further characterization of fatty acid transport and enzyme activity in the Arabidopsis *pxa1* mutant may allow determination of how PXA1 functions and its role in fatty acid β-oxidation in plants.

Examination of the virtually complete Arabidopsis genome sequence reveals only one PXA1 homolog (F20D21.17/At1g54350, Fig. 6). Unlike PXA1, this protein is a hemitransporter, containing one TM domain region followed by a single nucleotide-binding fold. The identity between the two Arabidopsis proteins (21%) is less than the identity between PXA1 and the yeast (24%–30%) and mammalian (36%–45%) proteins. Furthermore, the human and yeast proteins are more similar to Arabidopsis PXA1 than to F20D21.17, with the single exception of P70R. It is interesting that the predicted F20D21.17 protein apparently contains only four TM domains (Fig. 6), whereas each of the homologous hemitransporters has six TM domains and PXA1 has 12 predicted TM domains. Because humans and yeast have multiple proteins that dimerize to form the functional peroxisomal transporter, F20D21.17 may homodimerize and form an Arabidopsis transporter. It remains to be determined whether both the full transporter (PXA1) and the hemitransporter (F20D21.17) play similar roles in plant peroxisomal β-oxidation, or whether the proteins have different expression patterns or substrate specificities making their roles unique.

The use of IBA resistance coupled with Suc-dependent seedling development as a screen to identify Arabidopsis peroxisomal β-oxidation mutants, such as *pxa1*, is providing an unbiased approach to explore the specifics of both IBA and fatty acid metabolism in plants (Bartel et al., 2001). The identification of *PXA1* as the gene defective in an IBA-response mutant supports the hypothesis that peroxisomes convert IBA to IAA using a pathway resembling fatty acid β-oxidation. A model of this process, based on IBA- and 2,4-DB-resistant mutants, is shown in Figure 7. PXA1 likely transports fatty acyl-CoA esters into the peroxisome, where they are β-oxidized to acetyl-CoA, which is metabolized to succinate via the glyoxylate cycle (Gerhardt, 1992; Olsen, 1998). Because *pxa1* is resistant to IBA and the IBA analog 2,4-DB (Zolman et al., 2000), PXA1 probably also imports IBA-CoA and 2,4-DB-CoA into peroxisomes for oxidation to IAA-CoA and 2,4-D-CoA, respectively. These compounds presumably are hydrolyzed and exit the peroxisome to elicit specific phenotypic effects, including root elongation inhibition and lateral root initiation.

IBA is used widely to propagate plant cuttings because it efficiently induces lateral and adventitious

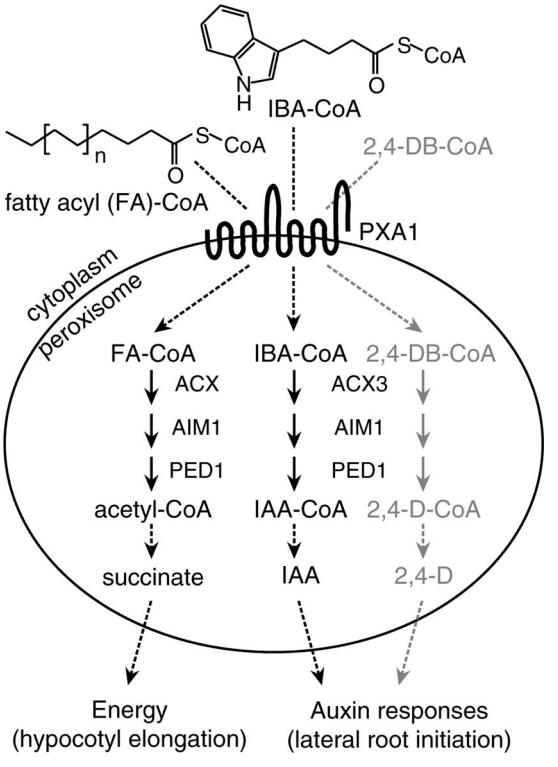

Figure 7. Proposed model for PXA1 function in Arabidopsis. Based on its homology to human and yeast proteins, PXA1 is predicted to be a transporter localized in the peroxisomal membrane. Because of the mutant phenotype, PXA1 is likely to import fatty acyl (FA)-CoA esters, IBA-CoA, and 2,4-DB-CoA into the peroxisome, where they are catabolized to succinate, IAA, and 2,4-D, respectively. 2,4-DB-resistant mutants defective in an acyl-CoA oxidase (*acx3*, Eastmond et al., 2000), a multifunctional protein (*aim1*, Richmond and Bleecker, 1999), and a thiolase (*ped1*, Hayashi et al., 1998) also are IBA resistant (Zolman et al., 2000; B. Zolman and B. Bartel, unpublished data), suggesting that these isozymes act directly in IBA and 2,4-DB β-oxidation. See text for details.

roots (Hartmann et al., 1990). One hypothesis explaining the high rooting ability of IBA is that IBA acts as a "slow-release" form of IAA, similar to certain auxin conjugates (Hangarter and Good, 1981). Because IBA is β-oxidized to IAA and because numerous mutants defective in β-oxidation do not form lateral roots in response to IBA (Zolman et al., 2000), it is likely that the enhanced lateral root production by wild-type plants in response to IBA is based on its slow conversion to IAA. Furthermore, the observation that *pxa1* makes fewer lateral roots on unsupplemented medium suggests that the β-oxidation of endogenous IBA to IAA may be important for the development of lateral roots in wild-type seedlings.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis accessions Col-0 and Ws were used. The *pxa1* mutant was previously described as B40, an ethyl methanesulfonate-induced IBA-response mutant in the Col-0 background (Zolman et al., 2000). Plants were grown in soil (Metromix 200, Scotts, Marysville, OH) at 22°C to 25°C under continuous illumination by Cool White fluorescent bulbs (Sylvania, Danvers, MA). Plants grown aseptically were plated on PNS (plant nutrient medium with 0.5% [w/v] Suc; Haughn and Somerville, 1986) solidified with 0.6% (w/v) agar, either alone or supplemented with hormones (from 0.1, 1, or 100 mM stocks in ethanol) or Basta (from a 50-mg mL^{-1} stock in 25% [v/v] ethanol). Plates were wrapped with gas-permeable surgical tape (LecTec Corp., Minnetonka, MN) and grown at 22°C under continuous light. Plates containing auxin were incubated under yellow filters to slow the breakdown of indolic compounds (Stasinopoulos and Hangarter, 1990).

Phenotypic Analyses

The *pxa1* mutant was backcrossed at least once prior to analyses, and all assays were conducted at least twice with similar results. Seeds were surface sterilized (Last and Fink, 1988) and plated on PNS with the indicated hormone concentration. In root elongation assays, seedlings were grown for 8 d and removed from the agar, and the length of the primary root was measured (Figs. 1, 4A, and 5E). In lateral root assays (Figs. 2 and 5F), seeds were grown on PNS for either 4 or 6 d, transferred to medium containing IBA, IAA, or no hormone, and grown for an additional 4 or 6 d. The number of lateral roots was counted under a dissecting microscope. In the seed germination assay (Table I), seeds were plated on either PNS or PN (plant nutrient medium without Suc) and grown in the light for 6 d or in the light for 1 d to induce germination, followed by 5 d in the dark. Germination, defined as radicle emergence from the seed coat, was scored using a dissecting microscope. Establishment was defined as seedling emergence and cotyledon expansion in the light or hypocotyl elongation in the dark. Seeds that germinated but did not establish were transferred to PNS and scored again after an additional 4 d of growth under white light. For hypocotyl elongation assays (Figs. 4B and 5D), seeds were plated on PN (without Suc) or PNS and incubated for 24 h under white light before being transferred to the dark. The length of the hypocotyl was measured after an additional 5 d.

In the adult growth studies, wild-type and mutant plants were grown on PNS for 10 d under white light before being transferred to soil. Rosette diameter was measured at the widest point of the plant without disturbing any leaves (Fig. 3A). For flowering time determination, plants were examined two to three times per week and the number of leaves was counted at the appearance of the first bud in the shoot apex.

Genetic Analysis and Mutant Complementation

The mutant was outcrossed to Ws for mapping, and DNA was isolated (Celenza et al., 1995) from IBA-resistant F_2 plants that had 100% Suc-dependent progeny. The mutation was mapped using published simple sequence length polymorphisms (Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993). For the marker mi167, PCR amplification with the primers 5′-CACTAGATCTTCAAGCGCTCGATG-3′ and 5′-GACATATCCATAGAGTAACTTCAC-3′ yield a 390-bp product with two *Rsa*I sites in Col-0 and no sites in Ws.

A candidate gene (*PXA1*) within the mapping interval was examined for defects in the mutant. Genomic DNA extracted from mutant plants was amplified using seven pairs of oligonucleotides (5′-CTTCAGGTGTTTTGGACACTTGTTGTCAAG-3′ and 5′-CATCCAGTATAAGATCGCTCAACTCTGAGG-3′, 5′-ATATCACACGTGGATGGTCGGATTACGC-3′ and 5′-CAGAAGATTAGACCCTTGCTCAACTCG-3′, 5′-GTTACTCCAACCGGAAATGTTTTGGTGG-3′ and 5′-CCATCCTCCTTCACCGTCTAATGACAGAAC-3′, 5′-GCACAAGTGCTGTCACAACTGATATGG-3′ and 5′-GAGCAATTTAGTAAGTCGTGACAAGGTG-3′, 5′-GGACCACTGTGAAGTATGTCTTGGAGCAAG-3′ and 5′-CAATTCCAAGTTCACTTCCGGTAGCTTTG-3′, 5′-CTTAATTGCCCTAGCTATAGCTGCTGG-3′ and 5′-CCATTCCCTGACCCAAGTTCTTTGATATCC-3′, and 5′-GAAATAGTTTCAGGGAAAAGCCTGCTCGTC-3′ and 5′-ATTCTCTTCCACTCCTTGCGATCGAGGAAG-3′) with a program of 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 3 min. The resulting overlapping fragments were approximately 1200 bp each and covered the gene from 90 bp upstream of the putative translation start site to 140 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 (Rice University Sequencing Facility, Houston) with the primers used for amplification.

An apparently full-length *PXA1* cDNA in pBluescript II SK(−) (H1A6T7) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus; Kieber et al., 1993). This cDNA was sequenced using vector-derived and internal primers (GenBank accession

no. AF378120). To remove an open reading frame upstream of the *PXA1* open reading frame, we linearized the plasmid with *Ase*I and removed the 3′ overhang with T4 DNA polymerase. The cDNA was excised by digestion with *Eco*RI and ligated into pBluescript II KS(+) cut with *Sma*I and *Eco*RI, forming pKS-*PXA1*c. The cDNA was excised by digestion with *Not*I and subcloned in the sense orientation behind the constitutive 35S cauliflower mosaic virus promoter in the 35SpBARN plant transformation vector (LeClere and Bartel, 2001). This plasmid (35S*PXA1*) was electroporated (Ausubel et al., 1999) into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1992), which was used to transform *pxa1* mutant plants using the floral dip method (Clough and Bent, 1998). Transformants were identified on PNS plates supplemented with 7.5 μg mL^{-1} Basta after 10 d under white light. Rescue assays were done using seeds from homozygous progeny of Basta-resistant transformants (Fig. 5, D–F).

RNA Analyses

Wild-type and *pxa1* mutant plants were grown for 5 d under white light on PNS plates covered with filter paper. Tissue was harvested by immersion in liquid nitrogen, and RNA was isolated as described before (Nagy et al., 1988). Reverse transcription of the RNA was done using the Retroscript reverse transcription-PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions. PCR amplification of the cDNA was performed using primers spanning the *pxa1* mutation, and the resulting products were purified and sequenced with the primers used for amplification (see above).

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