Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants

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In many higher plants, cellulose synthesis is inhibited by isoxaben and thiazolidinone herbicides such as 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidinone. Semidominant mutations at the *IXR1* and *IXR2* loci of *Arabidopsis* confer isoxaben and thiazolidinone resistance. Isolation of the *IXR1* gene by map-based cloning revealed that it encodes the *AtCESA3* isoform of cellulose synthase. The two known mutant alleles contain point mutations that replace glycine 998 with aspartic acid, and threonine 942 with isoleucine, respectively. The mutations occur in a highly conserved region of the enzyme near the carboxyl terminus that is well separated from the proposed active site. Although the *IXR1* gene is expressed in the same cells as the structurally related *RSW1* (*AtCESA1*) cellulose synthase gene, these two *CESA* genes are not functionally redundant.

Isoxaben (*N*-3[1-ethyl-1-methylpropyl]-5-isoxazolyl-2,6, dimethoxybenzamide, EL-107, Flexidor, Gallery) is a preemergence broad-leaf herbicide used primarily on small grains, turf, and ornamentals. This compound is extremely toxic, with an I_{50} for *Brassica napus* of 20 nM (1). Isoxaben inhibits the incorporation of glucose into the cellulose-rich acid-insoluble fraction of isolated cell walls and has been proposed to be a specific inhibitor of cellulose biosynthesis (2, 3). Treated cells of sensitive species fail to elongate normally and consequently grow isodiametrically (1). Analysis of the effects of the compound on the polysaccharide composition of cell walls and other aspects of plant physiology has led to the proposal that the herbicidal action of isoxaben can be explained entirely by its effect on cellulose biosynthesis (4). Other cellular processes, such as seed germination, mitosis, respiration, photosynthesis, and lipid and RNA synthesis, are unaffected by isoxaben (1, 5).

Mutations at two genetic loci in *Arabidopsis thaliana*, originally termed *ixrA* and *ixrB* but since renamed *ixr1* and *ixr2*, respectively, confer resistance to isoxaben (6, 7). Homozygous *ixr1–1* and *ixr1–2* mutant plants are 300 and 90 times, respectively, more resistant to isoxaben than wild-type plants (6). The *ixr* mutations appear to directly affect the herbicide target, because resistant cell lines show no alterations in uptake or detoxification of the herbicide (4).

Thiazolidinones such as 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidnone (TZ) are a new class of *N*-phenyl-lactam-carbamate herbicides (8). TZ has a similar syndrome of effects on plants to isoxaben. The *ixr1–1* mutant of *Arabidopsis* exhibits resistance to both isoxaben and TZ, indicating that isoxaben and TZ share a common mode of action (8). At 12 μM, TZ kills wild-type *Arabidopsis* but reduces growth of the *ixr1–1* mutant by only 50% (8).

Here, we describe the isolation and characterization of the *IXR1* gene and the identity of the amino acids changes associated with herbicide resistance in two *ixr1* alleles. Resistance is caused by changes in the amino acid sequence of the presumed catalytic subunit of a cellulose synthase gene designated *AtCESA3* (9, 10). Identification of the site of action of isoxaben and TZ enhances the potential utility of these compounds in mechanistic studies of cellulose synthase structure and function.

Materials and Methods

Nomenclature. The 10 cellulose synthase genes in *Arabidopsis* have been designated *CESA1* to *CESA10* (9, 10). Some of these genes also have clone or genetic locus designations as follows: *RSW1* = *CESA1* (11), *Ath-A* = *CESA2* (11), *Ath-B* = *IXR1* = *CESA3* (11), *PROCUSTE* = *IXR2* = *CESA6* (H. Höfte, personal communication), *IRX3* = *CESA7* (12). Because the results presented here are primarily related to the characterization of several mutants, we have used the genetic locus symbols throughout.

Genetic Materials. Seeds from homozygous isoxaben-resistant mutants *ixr1–1* (strain DH47; stock no. CS6201) and *ixr1–2* (strain DH48; stock no. CS6202) (6) were obtained from the Arabidopsis Biological Resource Center, Columbus, OH. Plants were grown in a commercial peat–vermiculite–perlite mix (Premier Horticulture, Red Hill, PA) in a glasshouse under natural light conditions at a temperature of ≈25°C (day) to ≈20°C (night).

Transgenic plants containing *RSW1* and *IXR1* promoters fused to the *Escherichia coli* β-glucuronidase (GUS) coding sequence were obtained as follows. The promoters were amplified from genomic DNA with Expand High Fidelity polymerase (Roche Molecular Biochemicals) by using primer pairs CTG-GTCGACGAGAAGAGATGGTAAAGAGAG and GGACT-GCAGCCATGGCGCAGCCACCGACACACAGAG for *RSW1* and GACGTCGACCTGGTGATACGAGGTGATGG and GACGTCGACCTGGTGATACGAGGTGATGG for *IXR1*. The 2,170-bp amplified fragment for the *IRX1* promoter was cloned into pRITA1 as a *Sal*I/*Pst*I fragment, and then this construct was cut with *Nco*I and a snapback ligation was done to remove the small section of linker between the 3′ end of the promoter and the ATG of GUS. The 1,111-bp promoter fragment of *RSW1* was cloned directly as a *Sal*I/*Nco*I fragment into pRITA1. The clones were sequenced and then subcloned as *Not*I promoter-GUS fragments into Ti plasmid pMLBART, introduced into *Agrobacterium tumefaciens*, and used to transform Col-0 *Arabidopsis* (13). Plasmids pRITA1 and pMLBART were obtained from Bart Janssen and Kim Richardson at the Horticultural and Food Research Institute of New Zealand in Auckland.

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Abbreviations: TZ, 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidinone; GUS, β-glucuronidase

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Scoring Isoxaben Resistance. Isoxaben (95%), supplied by Dow-Elanco (Indianapolis, IN), was solubilized in DMSO. Beginning 2 days after germination, seedlings were sprayed 4 times at 3-day intervals with 1 ml/6 cm^2 soil surface of 2.0 μM isoxaben in an aqueous solution of 0.025% DMSO. The growth of wild-type plants was completely inhibited by isoxaben. No effect of 0.025% DMSO on plant growth was noted. Homozygous mutants grew normally, although *ixr1–1* grew better than *ixr1–2*. All heterozygous plants had clearly reduced growth as compared with the homozygous mutants.

Genetic Mapping of the *ixr*1 Locus. Approximately 5,000 F_2 seeds from an *ixr1–2* × Landsberg *erecta* ecotype cross were planted at a density of ≈1 plant per 2.3 cm^2. The seedlings were treated with 2.0 μM isoxaben, leaving only F_2-plants homozygous for the *ixr1* locus. DNA was prepared from tissue samples of 1,056 plants by alkaline lysis, as described (14).

Four percent agarose gels were used to resolve SSLP markers for mapping. Conditions for PCR amplification of SSLP markers were: 50 mM KCl/10 mM Tris·HCl (pH 9.0 at 25°C)/0.1% Triton X-100/200 μM each of dATP, dGTP, dTTP, dCTP/5 pmols of each primer/2.0–2.5 mM $MgCl_2$/1.0 unit *Taq* polymerase (Promega)/10–50 ng of genomic DNA, final volume 20 μl. PCR conditions: 1 min 94°C; 40 cycles (20 sec 94°C, 20 sec 48–55°C, 40 sec 72°C), 3 min 72°C. The simple sequence length polymorphism and clearable amplified polymorphic sequence markers used for mapping were identified from the information provided by The Arabidopsis Information Resource (http://www.arabidopsis.org).

DNA Sequencing. Overlapping PCR fragments, 2,034, 2,064, and 2,395 bp in length and spanning the entire coding sequence of the *IXR1* gene on TAC-clone K2A11 (AB018111 bases 10620–15972), were amplified from genomic wild-type Columbia, *ixr1–1* and *ixr1–2* DNA with three oligonucleotide primer pairs (TTAGCCATCCCAAGATTCT, CTTCAAGGGGTCAACAGTA; TACCGAGCGTTTTTCCTAT, CCAGCACCTAAGTTTCACA; GTTCAGTTCCCACAAAGATT, TCATTCCGACCAAAAGTT). Genomic DNA for each genotype was prepared from a mixture of young growing leaves and inflorescence tissue by using a cetyltrimethylammonium bromide-detergent extraction method (14). The fragments from each genotype were PCR-amplified by using a mixture of *Taq* and proofreading *Pfu* polymerases (Promega) and sequenced with a set of 20 primers by cycle sequencing by using Big-Dye dideoxy-terminator reaction-mix (Perkin–Elmer Applied Biosystems) and resolved on an ABI310 sequenator. The fragments that carried the *ixr1–1* and *ixr1–2* point mutations were independently amplified and sequenced three times.

Cosmid Library Screen and Complementation. Cosmids carrying *IXR1* were isolated from a Landsberg *erecta* ecotype library constructed in the binary Ti cosmid vector pBIC20 (15).

Mutant *ixr1* plants were transformed by *A. tumefaciens* (GV3101) carrying the various cosmid clones according to Bent and Clough (13), and T_1 transformants were selected on MS plates containing kanamycin (50 μg ml^{-1}). To score for isoxaben resistance, surface-sterilized seeds of the transgenic T_1 plants were germinated on 0.8% agar-solidified medium containing Murashige and Skoog mineral salts (Sigma) and 600 nM isoxaben. The plates were incubated vertically at 25°C under continuous fluorescent illumination (≈50 μmols photons m^{-2}·s^{-1}) so that the roots grew on the surface of the agar. Isoxaben resistance was scored after 7 days of incubation.

RNA Gel Blot Analysis. Total RNA was isolated from various tissues of 6-week-old flowering plants grown on soil or from roots and rosettes of 3-week-old plants grown in a hydroponic system, by using TRIzol reagent (Life Technologies, Rockville, MD). Fifteen micrograms of total RNA was electrophoresed in 1.2% formaldehyde agarose gels, transferred to a nylon membrane, immobilized by UV irradiation, and probed with random-primed DNA fragments encoding stretches of variable region 1 of the *IXR1*, *RSW1*, *AtCESA2*, and *IRX*3 cellulose synthase genes. The probes, 188–274 bp in length, were amplified by PCR from wild-type Columbia seedling cDNA with the following primers: *IXR1* [AAATTTCAGAGCGGATGC, CAAGCTACATTCCCGAGTC], *AtCESA2* [CGTGGTGGATTGGATTCAG, GCTTTTCGCCTTGTCGTC], *RSW1* [GAGCTAACAAGGCGAGAC, ACATTACCAAGCCCATAAG], *IRX3* [GGAGATTCCCACCTGTTAT, TTCTGGCCCAAGATTTC].

Microscopy. Seeds were germinated and grown at 25°C for 5 days in continuous illumination (≈50 μmols photons m^{-2}·s^{-1}) on MS agar plus 1% sucrose. Whole seedlings were infiltrated under mild vacuum for 10 min with 50 μM ImaGene Green C_{12}FDGlcU (Molecular Probes) in 0.1% Silwet L-77 detergent (Lehle Seeds, Round Rock, TX) and incubated for 4–6 h at room temperature before examination. Tissue was counterstained with 0.1 μg ml^{-1} propidium iodide just before visualization. Cleavage of C_{12}FDGlcU by GUS generates 5-dodecanoylaminofluorescein, which was visualized by using a Bio-Rad 1024 confocal microscope (excitation 488 nm; emission 522/535 nm). Wild-type controls incubated with C_{12}FDGlcU showed no evidence of background staining (16).

Results

Positional Cloning of the *IXR1* Locus. The *IXR1* locus maps to the top arm of chromosome 5 within 3 cM of the visible marker *lutescens*. To obtain a high-resolution map position, 1,056 homozygous mutant plants were selected from the F_2 progeny of a cross of the *ixr1–2* mutant (Col background) to the Landsberg *erecta* line. DNA from each isoxaben-resistant F_2 plant was examined with the two codominant PCR-based SSLP-markers, CIW13, and CIW14. CIW13 is ≈310 kb distal to g3715, and CIW14 is ≈150 kb distal to m217 (Fig. 1*A*). Initial mapping resulted in the identification of 52 lines with a recombination event between CIW13 and *ixr1* and 39 lines with a recombination event between *ixr1* and CIW14. By using six additional SSLP or CAPS markers, the genomic interval containing the *IXR*1 locus was subsequently narrowed down to a fully sequenced ≈50-kb genomic fragment between markers CIW16 and CIW17 (Fig. 1*B*).

Inspection of the region of chromosome V between CIW16 and CIW17 revealed the presence of a gene for cellulose synthase previously designated as *AtCESA3* (9, 10). An additional SSLP marker, CIW18 (Fig. 1 *B* and *C*), located within this gene, did not yield any recombinant chromosomes, indicating that the *IXR1* locus was in very close proximity. A cDNA clone corresponding to this same cellulose synthase gene was previously described by Arioli *et al.* (11). Comparison of the sequence of the *AtCESA3* cDNA (GenBank accession no. AF027174) to the genomic sequence present on TAC-clone K2A11 (GenBank accession no. AB018111) indicated that the cDNA clone had 87 nucleotides at the 5′ end that are not present in the genomic sequence. This extra sequence corresponds to a 59-nt multiple cloning site (G *GACTC GCGCGC CTGCAG GTCGAC ACTAGT GGATCC* AAA *GAATTC* G *CGGCCG* C *GTCGAC*, restriction enzyme sites are shown in italics) that was introduced during cloning of the cDNA and an additional 28-nt fragment of DNA (TACGGCTGCGAGAAGACGACAGAAGGGG) that was also introduced at some stage during the cloning of the cDNA. A search of GenBank indicated that this 28-bp sequence is also found at the 5′ ends of other cDNA clones; thus, it is a common artifact in some libraries.

The first nucleotide of the *AtCESA3* mRNA that corresponds

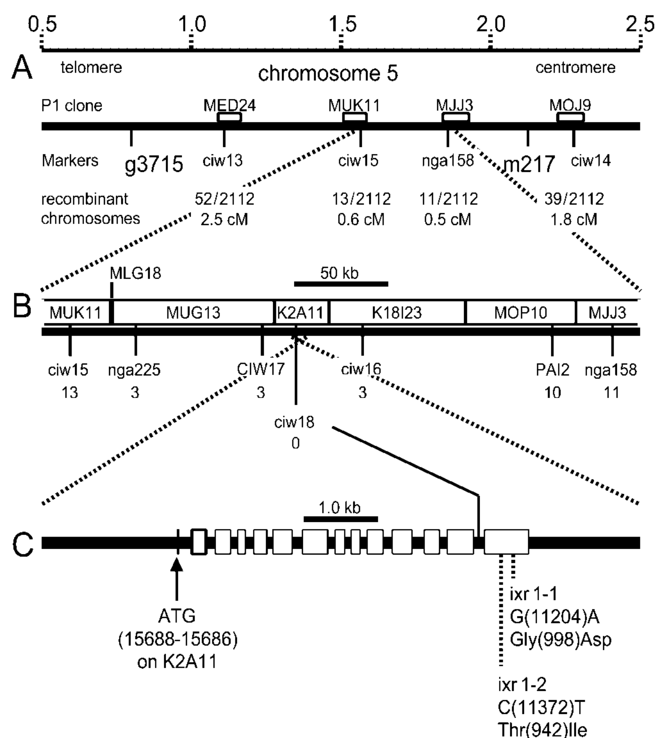

Fig. 1. Map-based cloning of the *IXR1* gene. (*A*) Representation of a region of *Arabidopsis* chromosome 5 showing a megabase-scale (*Upper*), the position of RFLP markers g3715 and m217, SSLP markers CIW13, 14, 15, and SSLP marker nga158. Chromosome 5 P1 clones (http://www.kazusa.or.jp/arabi/) containing the SSLP markers are represented by white boxes. The number of recombinant chromosomes (meiotic breakpoints) in a total of 2,112 examined chromosomes, found for each marker and their calculated genetic distance to the *IXR*1 locus [in centimorgans (cM)], is given. (*B*) The region containing the flanking SSLP-markers CIW15 and nga158, showing the position and the number of recombinants found for CAPS (CIW17, PAI2) and SSLP (CIW16, 18) markers and the nonoverlapping parts of P1 and TAC clones spanning the region. (*C*) An 8-kb segment of TAC clone K2A11 (nucleotides 17, 500–9, 500) showing the intron/exon structure of the *IXR1* gene (note crossover of the dotted lines). The start codon and the point mutations in the *ixr1–1* and *ixr1–2* alleles in the last exon and the predicted amino acid exchanges in the mutant gene products are indicated.

to the genomic clone is nucleotide 15966 of TAC-clone K2A11. The ORF of the gene begins at nucleotide 15688 (start codon) of K2A11 and ends at nucleotide 10999 (stop codon). The genomic clone contains an intron in the 5′ nontranslated leader sequence that corresponds to nucleotides 15736–15845 of K2A11 and another 13 introns that divide the coding sequence into 14 exons (Fig. 1*C*), as predicted by GRAIL (http://grail.sourceforge.net), GENSCAN 1.0 (http://genes.mit.edu/GENSCAN.html), and NETPLANTGENE (http://www.cbs.dtu.dk/services/netpgene).

In view of the evidence indicating that the mechanism of action of the isoxaben and thiazolidinone herbicides is to inhibit cellulose synthesis, this observation suggested that *AtCESA3* corresponds to the *IXR1* gene. To test this hypothesis, the gene was cloned and sequenced from Columbia wild type and from the *ixr1–1* and *ixr1–2* mutants. Sequencing revealed that the *AtCESA3* genes from the two *ixr1* mutants were identical to the wild type except for a G to A change at nucleotide 11204 in *ixr1–1* and a C to T change at nucleotide 11372 in *ixr1–2* on the coding strand of TAC-clone K2A11. The point mutation in *ixr1–1* leads to replacement of a conserved glycine at position 998 in the *AtCESA3* protein with an aspartic acid residue, and the point mutation in *ixr1–2* results in replacement of a conserved threonine residue at position 942 in the *AtCESA3* protein with an isoleucine residue (Fig. 1*C*). Thus, both of the *ixr1* mutations occurred in exon 14 of the *AtCESA3* cellulose synthase gene.

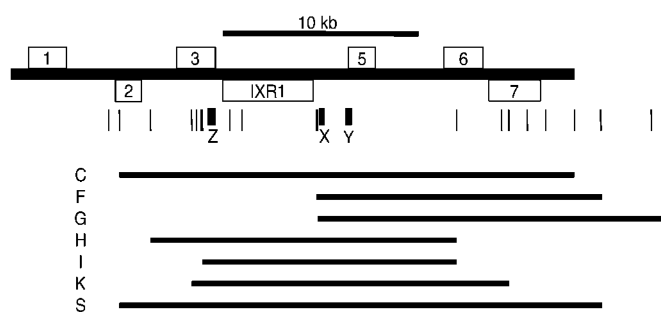

Fig. 2. Representation of the positions and sizes of various cosmid clones (C-S) tested for ability to complement an *ixr1* mutation. The genomic DNA contained in TAC clones K2A11 and K18I23 (Fig. 1*B*) is represented as bold black and gray lines, respectively. Predicted and known genes (white boxes) were annotated by Kaneko *et al.* (23). *Hin*DIII restriction sites (vertical thin lines) and the position of hybridization probes X, Y, and Z (small black boxes) used for cosmid library screening are shown.

Transformation of *ixr1* Mutants with Wild-Type *IXR1* Results in Isoxaben Sensitivity. To confirm that the mutations found in the *ixr1* mutants are responsible for the isoxaben-resistant phenotype, a series of overlapping clones was isolated from a genomic cosmid library of the Columbia wild type (15) and transformed into the *ixr1–1* and *ixr1-2* mutants. Cosmids C, H, I, K, and S (see Fig. 2) all contained the full *IXR1* coding sequence along with variable amounts of 5′ upstream sequence. T_2-progeny of *ixr1* mutants

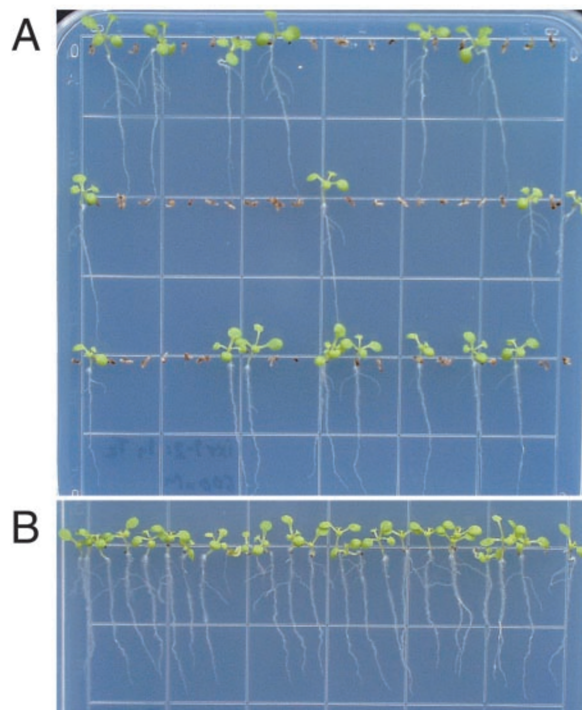

Fig. 3. Transformation of the *ixr1–2* mutant with a wild-type *IXR1* gene results in isoxaben sensitive progeny. (*A*) Segregating T_2 progeny of an *ixr1–2* mutant transformed with cosmid I (Fig. 2). Approximately 75% of the progeny, representing heterozygous and homozygous *ixr1–2* transformants, are herbicide-sensitive when grown on vertical MS plates containing 600 nM isoxaben. (*B*) Progeny of an *ixr1–2* mutant transformed with cosmid F were 100% isoxaben-resistant.

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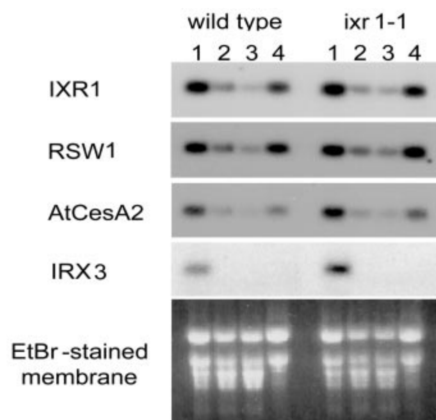

Fig. 4. Northern analysis of cellulose synthase genes (*IXR1*, *RSW1*, *AtCESA2*, and *IRX3*) in (1) stems, (2) rosette leaves, (3) cauline leaves, and (4) flowers of wild-type and *ixr1–1* mutant.

transformed with these cosmid clones segregated for isoxaben-resistance (Fig. 3*A*), whereas the T_2-progeny of *ixr1* mutants transformed with cosmid clones F and G, which lack a complete *IXR1* gene, were completely isoxaben-resistant (Fig. 3*B*). Because the only complete gene carried in common by the cosmids that produced isoxaben sensitivity was the *IXR1* gene, we conclude that this gene is responsible for isoxaben sensitivity.

The *IXR1* Gene Is Expressed in Both Roots and Shoots. Total RNA was extracted from stems, rosette, and cauline leaves and flowers (Fig. 4), and from shoots and roots of *Arabidopsis* plants that were treated with or without isoxaben (Fig. 5). Northern blots of these RNA samples were probed with PCR-amplified DNA fragments encoding variable region 1 (9) of the *IXR1* gene as well as the related *CesA* genes *RSW1*, *AtCESA2* (11), and *IRX3* (12). *IXR1*, *RSW1*, and *AtCESA2* were highly expressed in stems, flowers, roots, and shoots, whereas *IRX3* was expressed only in stems (Fig. 4). *IXR1* appeared to be expressed more highly in roots than shoots.

To examine the possibility that one or more of these *CESA* genes might be induced by a deficiency in cellulose, wild-type and *ixr1* plants were treated with isoxaben for 2 days, and a steady-state amount of mRNA was assessed by Northern blots (Fig. 5). The results of this experiment indicate that there was no detectable increase in mRNA for any of the *CESA* genes as a result of the isoxaben treatment.

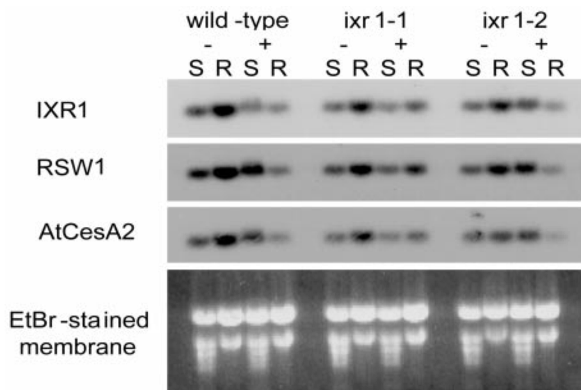

Fig. 5. Northern analysis of cellulose synthase genes (*IXR1*, *RSW1*, and *AtCESA2*) in shoots (S) and roots (R) of wild-type, *ixr1–1*, and *ixr1–2* mutants grown in hydroponic culture and treated with (+) or without (−) 0.5 μM isoxaben for 2 days. No signal was detectable for *IRX3*.

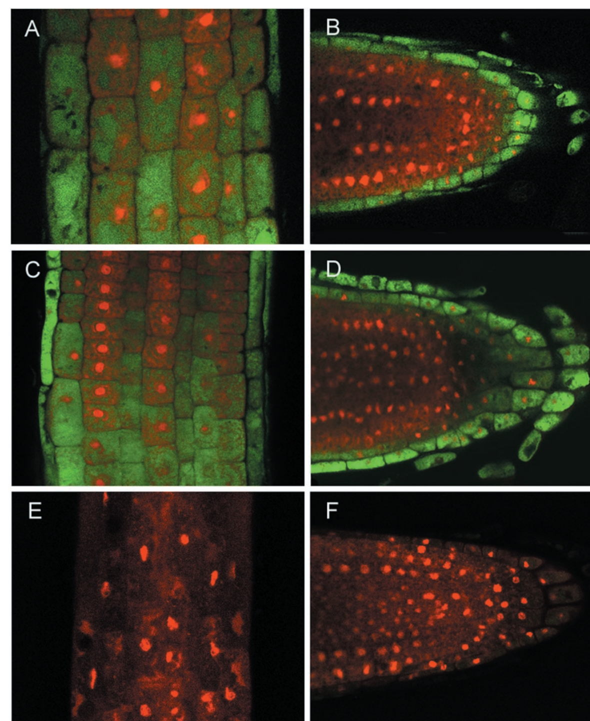

Fig. 6. Medial longitudinal sections of roots from transgenic plants containing the *RSW1*:*GUS* promoter fusion (*A*, *B*), *IXR1:GUS* promoter fusion (*C*, *D*) or nontransformed (*E*, *F*). *A*, *C*, and *E* show sections made just above the boundary of the root meristem and the elongation zone. Green fluorescence indicates GUS activity.

Because we noted that the mRNA expression patterns for *IXR1* and *RSW1* were almost identical in the Northern blot experiments, we further explored the possibility that these two genes might be expressed in the same cell types and at the same time during development. The expression pattern of the GUS reporter gene was examined in transgenic plants containing the *IXR1* or the *RSW1* promoter fused to GUS. Five-day-old seedlings stained for GUS activity with 5-bromo-4-chloro-3-indoyl-β-D-glucuronide showed intense blue staining in most cell types (see Fig. 8, which is published as supplemental data on the PNAS web site, www.pnas.org). Staining of the root meristem and the newly emerging true leaves was absent or substantially reduced by comparison with staining in other tissues.

To obtain high-resolution images, GUS activity was visualized by confocal microscopy by using ImaGene Green as a substrate and propidium iodide as a nonspecific fluorescent indicator for substrate penetration into the tissue (Fig. 6). Longitudinal medial sections of root tips showed that the root cap and the epidermal layer of the transgenics had high levels of GUS activity, but activity was not detected in the internal cells of the meristem region of the root (Fig. 6 *B* and *D*). However, all of the cells of the root above the meristematic region exhibited strong GUS activity (Fig. 6 *A* and *C*). Activity could not be detected in the wild type (Fig. 6 *E* and *F*). Thus, *IXR1* and *RSW1* have indistinguishable patterns of expression with respect to cell type and stage of root development.

Discussion

Results presented here show that resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis ixr1* mutants is because of amino acid changes in the *CESA3* cellulose synthase gene. The mutations corresponding to the *ixr1–1* and *ixr1-2* mutations cause amino acid substitutions in a 56-aa region near the carboxyl

terminus of the protein. In the *ixr1–1* mutation Gly-998 becomes Asp-998 and in the *ixr1–2* Thr-942 becomes Ile-942. In the absence of a structural model for the protein, it is not currently possible to understand why these mutations render the enzyme resistant to two structurally dissimilar inhibitors. The simplest assumption is that these compounds bind to a site on the enzyme that includes, or is affected by, the indicated amino acids. However, the amino acids are located at a site on the enzyme quite distant from the highly conserved aspartic acid residues that have been proposed to be a component of the active site (17). Although it is formally possible that the identified residues fold into the proposed active site, this seems unlikely because the residues are near the center of a predicted transmembrane helix (*ixr1-1*) and in a short extracellular loop that connects two proposed transmembrane helices (*ixr1–2*) (9). If the herbicides are acting directly on the active site of the enzyme, this observation could suggest the unlikely possibility that the conserved aspartic acid residues are not in the active site but have another role in enzyme action. Another possibility for the mode of action of the herbicides is that these regions are necessary for binding of regulatory molecules and that the *ixr1* mutations may alter these sites rather than directly affecting the catalytic site. According to this model, the herbicides may act by binding directly to the Ixr1 protein or to a regulatory subunit that binds near the site of the *ixr1-1* and *ixr1-2* mutations. Yet a third possibility is that these regions, being in or around proposed transmembrane helices, are critical for formation of a proposed pore (9) for glucan chain secretion, and that the hydrophobic herbicides bind to these regions and disrupt pore formation. Conceivably, mutations that prevent herbicide binding might allow proper pore formation in the presence of the herbicides.

Previous genetic studies of the mode of inheritance of the *ixr1* mutations had shown that the mutations conferred a semidominant resistance phenotype in which heterozygous (*ixr1/IXR1*) plants had a level of herbicide resistance that was intermediate between that of mutant and wild type (6). Consistent with previous studies, transgenic *ixr1* mutant plants that had been transformed with the wild-type *IXR1* gene were much less resistant to isoxaben than the mutant. Our interpretation of this phenotype is based on a model in which cellulose synthase forms a multisubunit complex in the plasma membrane (9, 17–19). According to this hypothesis, the ≈36 individual β-1,4-glucan chains that comprise cellulose microfibrils in higher plants are produced by a corresponding number of catalytic centers in a multisubunit complex in such a way that the individual molecules are coordinately synthesized and hydrogen bond to form the microfibrils as they emerge from the complex. In a heterozygous *ixr1IXR1* plant the putative cellulose synthase complexes would be composed of a mixture of resistant and sensitive subunits. Presumably in a complex that contained some sensitive subunits, those subunits would not be able to synthesize β-1,4-glucan chains in the presence of the herbicide. This could, in principle, lead to two outcomes: the resistant subunits might synthesize aberrant cellulose fibrils with fewer than the normal number of β-1,4-glucan chains. Alternatively, the presence of a certain number of sensitive subunits might cause the entire complex to stall, thereby preventing synthesis of a microfibril. Either possibility may cause weakening or disruption of the cell wall leading to cessation of growth.

The *IXR1* gene is a member of the *CesA* multigene family (9, 10) that contains at least 10 closely related members in *Arabidopsis* (http://cellwall.stanford.edu/). At least two of the other *CESA* genes (*RSW1, AtCESA2*) appear to be expressed at levels similar to *IXR1* on Northern blots of mRNA from roots, rosette, and cauline leaves, stems, and flowers. Thus, *IXR1* is just one of several *CesA* genes expressed in the major tissues. Indeed, histochemical analyses presented here show that *IXR1* and *RSW1* are expressed in the same cells. These two *CESA* genes are also

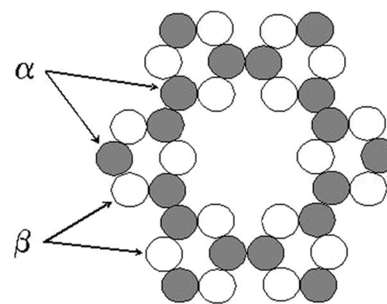

Fig. 7. A heteromeric model for the structure of cellulose synthase. In this model, the complex is composed of 36 CesA subunits that are distinguished by the number of subunit binding sites. The α subunits have binding sites for three CesA subunits (one α and two β), whereas the β subunits have only two binding sites.

by far the most highly represented by *Arabidopsis* expressed sequence tags (http://cellwall.stanford.edu/cesa/species/arabidopsis_atcesa/). In view of the fact that *rsw1* mutants are not viable at the nonpermissive temperature (11), it is apparent that *RSW1* and *IXR1* do not have redundant functions. Similarly, the observation that isoxaben and TZ inhibit the growth of wild-type plants implies that a functional Ixr1 protein is also required for cellulose synthesis, even though Rsw1 protein is present. Thus, we infer that at least two closely related cellulose synthase genes that are expressed in the same tissues at similar levels of steady-state mRNA are required for cellulose synthesis. The implication seems to be that *IXR1* and *RSW1* are not functionally redundant. This conclusion is similar to that of Taylor *et al.* (20), who found that both the *IRX1* and *IRX3* genes are required for synthesis of cellulose in secondary cell walls of xylem tissue. In addition, Taylor *et al.* (20) obtained direct biochemical evidence that the Irx1 and Irx3 proteins bound each other in detergent solubilized extracts, suggesting that they are normally present in the same complex.

Several possible explanations have been proposed for the requirement for expression of at least two nonidentical *CESA* genes in the same cells. One possibility is that two nonidentical subunits are required to catalyze β-1,4-glucan chain elongation (20, 21). However, an equally compelling hypothesis arises from consideration of the geometry of a self-assembling planar multisubunit complex such as cellulose synthase (21). Because there are estimated to be approximately 36 β-1,4-glucan chains in a cellulose microfibril (9, 19), it seems likely that the cellulose synthase complex has approximately that many CesA subunits arranged in a planar configuration in the membrane. One conceivable way of packing 36 structurally similar proteins into a regular structure is shown in Fig. 7. This arrangement resembles the six-subunit rosette structures observed by electron microscopy (17–19). At least two types of CesA subunits, designated α and β, would be required to allow such a structure to spontaneously assemble. Each α subunit must be able to interact with three other subunits (i.e., one α subunit and two β subunits). By contrast, each β subunit needs only to interact with two other subunits (i.e., two α subunits). More complex variants of this model are also possible, but less complex models seem unlikely, based on simple geometric considerations.

In addition to the *ixr1* mutations, Heim *et al.* (7) isolated mutations at another locus, now designated *ixr2*, that also conferred isoxaben resistance. The gene corresponding to this mutation has recently been identified as *CESA6* (H. Höfte, personal communication). Mutations at the *PROCUSTE* locus lead to loss of *CESA6* function and result in a phenotype that is similar to the *rsw1* mutants (22). This observation led Fagard *et al.* (22) to suggest that *PROCUSTE* and *RSW1* also have at least

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partially nonredundant functions. By contrast, that either an *ixr1* or an *ixr2* mutation can give rise to herbicide resistance indicates that these two genes are functionally redundant with regard to each other. This would be consistent with the observation that *ixr1 ixr2* double mutants do not show a higher level of isoxaben resistance than the single mutations (7). Because *ixr* mutations are sufficient to confer herbicide resistance, we infer that the Rsw1 enzyme and possibly other CesA proteins are resistant to isoxaben and TZ. None of the wild-type *CESA* genes, including *RSW1*, has either of the amino acids that confer herbicide resistance in the *ixr*1 mutants. However, all of the CesA proteins do exhibit other amino acid differences with Ixr1 in the carboxyl terminal region of the Ixr proteins, and it is possible that these differences may render them insensitive to the herbicide. Within the context of the model proposed here (Fig. 7), it is also possible that the herbicides act only on a function or a structural feature that is unique to either the α or β subunits.

We anticipate that, with further progress in understanding the structure and function of the cellulose synthase complex, it will eventually become possible to carry out detailed *in vitro* biochemical studies of catalysis by this enigmatic enzyme. Because it is now clear that isoxaben and TZ act either directly on cellulose synthase or on some other factor that interacts directly with the enzyme, these compounds may prove useful in future mechanistic studies of enzyme action.

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