# Resistance against Herbicide Isoxaben and Cellulose Deficiency Caused by Distinct Mutations in Same Cellulose Synthase Isoform CESA6[1]

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Isoxaben is a pre-emergence herbicide that inhibits cellulose biosynthesis in higher plants. Two loci identified by isoxaben-resistant mutants (*ixr1-1*, *ixr1-2*, and *ixr2-1*) in Arabidopsis have been reported previously. *IXR1* was recently shown to encode the cellulose synthase catalytic subunit CESA3 (W.-R. Scheible, R. Eshed, T. Richmond, D. Delmer, and C. Somerville [2001] Proc Natl Acad Sci USA **98:** 10079–10084). Here, we report on the cloning of *IXR2*, and show that it encodes another cellulose synthase isoform, CESA6. *ixr2-1* carries a mutation substituting an amino acid close to the C terminus of CESA6 that is highly conserved among CESA family members. Transformation of wild-type plants with the mutated gene and not with the wild-type gene conferred increased resistance against the herbicide. The simplest interpretation for the existence of these two isoxaben-resistant loci is that CESA3 and CESA6 have redundant functions. However, loss of function *procuste1* alleles of *CESA6* were previously shown to have a strong growth defect and reduced cellulose content in roots and dark-grown hypocotyls. This indicates that in these mutants, the presence of CESA3 does not compensate for the absence of CESA6 in roots and dark-grown hypocotyls, which argues against redundant functions for CESA3 and CESA6. Together, these observations are compatible with a model in which CESA6 and CESA3 are active as a protein complex.

Cellulose synthesis remains a poorly understood process (for review, see Delmer, 1999). This linear 1,4-$\beta$-linked glucan is synthesized in terrestrial plants by a hexameric protein complex referred to as the terminal complex or rosette embedded in the plasma membrane (Kimura et al., 1999). The composition of this complex is unknown. Each of the six particles in the complex is thought to contain several catalytic subunits with an unknown stoichiometry. The relationship between the rosette and the crystalline microfibril structure remains controversial. It is commonly held that one rosette produces one microfibril. This idea was based on calculations based upon the spacing of 1,4-$\beta$-glucan chains in algal celluloses and it assumed the presence of 36 glucan chains in a single microfibril (Herth, 1983). Each rosette particle would then contribute six glucan chains to the microfibril. More recent studies using solid-state nuclear magnetic resonance lead to the conclusion that microfibrils in the primary wall consist of 2-nm crystalline units or elementary fibrils, each of which would not contain more than 10 to 15 glucan chains (Ha et al., 1998). The authors propose a scenario in which each of the six particles in the rosette produce the glucan chains of an elementary fibril, which in turn associate into a hexagonal 8- to 10-nm microfibril observed in most primary cell walls.

Genes encoding the cellulose synthase catalytic subunits have been identified in bacteria and plants (Pear et al., 1996). The Arabidopsis genome encodes 10 isoforms of the cellulose synthase catalytic subunit, CESA (http://cellwall.stanford.edu). The strong cellulose-deficient phenotypes observed for mutants in *CESA1* (Arioli et al., 1998), *CESA7* (Taylor et al., 1999), *CESA8* (Taylor et al., 2000), *CESA4* (S. Turner, personal communication), or *CESA6* (Fagard et al., 2000) indicate specific nonredundant functions for each of these genes. It is interesting that the mutant phenotypes also suggest that CESA isoforms have specialized roles in primary or secondary wall synthesis. Mutants for *CESA4*, *7*, and *8* specifically show a cellulose defect in the secondary wall of the xylem (Taylor et al., 1999, 2000; N. Taylor and S. Turner, personal communication), whereas mutants for *CESA1* and *6* have defects in the primary wall (Arioli et al., 1998; Fagard et al., 2000). Furthermore, evidence is accumulating indicating the requirement for more than one isoform in the same cell (Fagard et al., 2000; Taylor et al., 2000), and pull-down experiments suggested that at least CESA7 and CESA8

physically interact and may operate as a heterodimer or multimer (Taylor et al., 2000). Genetic studies finally also indicate a crucial role for a membrane-bound endo-1,4-β-glucanase, KORRIGAN, in the synthesis of cellulose (Nicol et al., 1998; Lane et al., 2001; Sato et al., 2001).

In addition to mutants, chemical inhibitors can be powerful tools for the molecular dissection of biological processes. Several herbicides inhibiting cellulose synthesis have been described such as dichlorobenzonitrile (DCB), isoxaben, and CGA325′615 (Sabba and Vaughn, 1999; Peng et al., 2001). Isoxaben, *N*-[3(1-ethyl-1-methylpropyl)-5-isoxazolyl] is a pre-emergence, broad leaf herbicide used primarily on small grains, turf, and ornamentals (Huggenberger et al., 1982; Technical report on EL-107 [1987] Lilly Research Laboratories, Indianapolis). It is selectively phytotoxic to dicotyledonous plants, whereas most monocotyledonous species are tolerant. This herbicide is extremely active, with $IC_{50}$ values in the nanomolar range (Heim et al., 1989). Isoxaben specifically inhibits radioactive Glc incorporation into the acid insoluble cellulosic cell wall fraction (Heim et al., 1990a). Two isoxaben-resistance loci (*IXR1* and *IXR2*) have been described in Arabidopsis (Heim et al., 1989, 1990b).

*IXR1* was recently cloned and shown to encode a cellulose synthase catalytic subunit isoform CESA3 (Scheible et al., 2001). Here, we show that isoxaben resistance in *ixr2-1* is caused by a mutation in another cellulose synthase isoform, CESA6. This gene was previously identified by knockout mutations (*prc1*) causing a cellulose-deficient short hypocotyl phenotype (Fagard et al., 2000). These and other data provide new insights into the complexity of the cellulose synthesis machinery in plants.

## RESULTS

### Phenotype of Isoxaben-Treated Wild-Type and *ixr2-1* Seedlings

Isoxaben is a potent inhibitor of cellulose synthesis in dicots, including Arabidopsis seedlings (Heim et al., 1990b; unpublished data). Figures 1 and 2 show that isoxaben strongly inhibits hypocotyl and root elongation of dark-grown seedlings. In addition, cells expand radially, callose, lignin, and other phenolic compounds (Fig. 2) accumulate, and seedlings eventually die at concentrations above the $IC_{50}$ value. In the light, seedlings germinate in the presence of the herbicide, but hypocotyl growth and the development of the root and shoot meristem is blocked ($IC_{50}$ around 5 nM, data not shown), and seedlings become necrotic and die. The accumulation of callose and lignin is frequently associated with the inhibition of cellulose synthesis as shown for seedlings treated with DCB, another cellulose biosynthesis inhibitor (Fig. 2) or cellulose-deficient mutants (Lukowitz et



**Figure 1.** The effect of isoxaben on hypocotyl growth in wild type and *ixr2-1*. Wild-type Col0 and *ixr2-1* seedlings were grown for 4 d in the dark in the absence (−) or presence (+) of 5 nM isoxaben.

al., 2001; G. Refrégier, S. Pagant, and H. Höfte, unpublished data). Transverse sections through the hypocotyl of dark-grown seedlings showed the presence of gapped cell walls in isoxaben- or DCB-treated seedlings (Fig. 2). Similar phenotypic characteristics have been described previously for cellulose-deficient mutants (Arioli, et al., 1998; Nicol et al., 1998; Fagard et al., 2000; Lane et al., 2001).

Seedlings homozygous for *ixr2-1* showed an increased tolerance to the herbicide as shown by the lack of inhibition of hypocotyl growth of dark-grown seedlings by 5 nM isoxaben (Fig. 1). The same was observed for *ixr1-2* homozygotes (Scheible et al., 2001 and data not shown).

### *ixr2-1* Carries a Missense Mutation in *CESA6*

*IXR2* was previously shown to map at 0.5 cM from the visual marker *yi* on the bottom of chromosome 5 (Heim et al., 1990a). Using a cross between a C-24 accession containing a T-DNA insertion north of *yi* (Van Lijsebettens et al., 1996; Fagard et al., 2000) and *ixr2-1*, the *IXR2* locus was fine mapped (Fig. 3A). It is interesting that *IXR2* mapped to the same position as *CESA6*, a gene encoding a cellulose synthase isoform previously identified by loss of function mutations (*prc1*) causing a growth defect and cellulose deficiency specifically in roots and etiolated seedlings. Given the fact that low concentrations of isoxaben phenocopy the Prc1⁻ phenotype (Fig. 5; Fagard et al., 2000), the hypothesis that *IXR2* is a *prc1* allele was tested by sequencing the *CESA6* gene in *ixr2-1*. A single point mutation was detected toward the 3′ end of the coding sequence, causing the amino acid
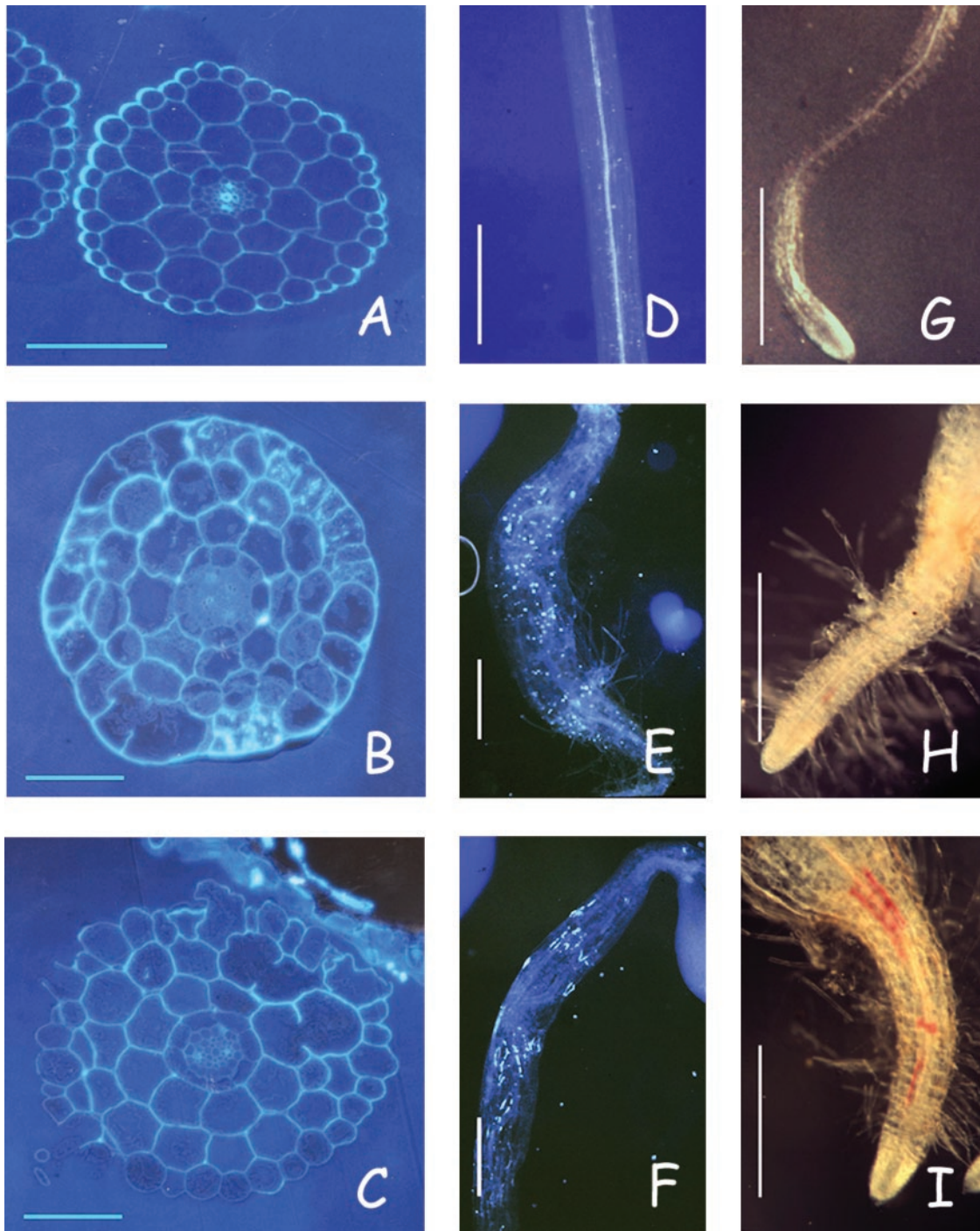
**Figure 2.** Similar effects of isoxaben and DCB are displayed on hypocotyl and root of wild-type seedlings. A–C, Transverse sections halfway through hypocotyls show gapped walls in seedlings treated with either herbicide. D--F, Hypocotyls stained with sirofluor present an accumulation of callose for both herbicides. G–I, Roots stained with phloroglucinol show an accumulation of lignin for both herbicides, with a stronger staining for DCB. A, D, and G, Control: 4-d-old dark-grown seedlings. B, E, and H, In the presence of 5 nM or (B) 7 nM isoxaben. C, F, and I, In the presence of 0.5 $\mu$M or (C) 1.5 $\mu$M DCB. Bars in A through C = 100 $\mu$m; D through I = 250 $\mu$m.

change $R_{1064}$W. This region of the predicted amino acid sequence is highly conserved between all 10 Arabidopsis *CESA* genes (Fig. 3B), and the charged residue $R_{1064}$ delimits the C-terminal end of the 8th predicted transmembrane anchor of CESA6.

**Wild-Type Plants Transgenic for *IXR2-1* Show Reduced Sensitivity to Isoxaben**

To confirm that the $R_{1064}$W change is responsible for the isoxaben-resistant phenotype, transgenic Ara-
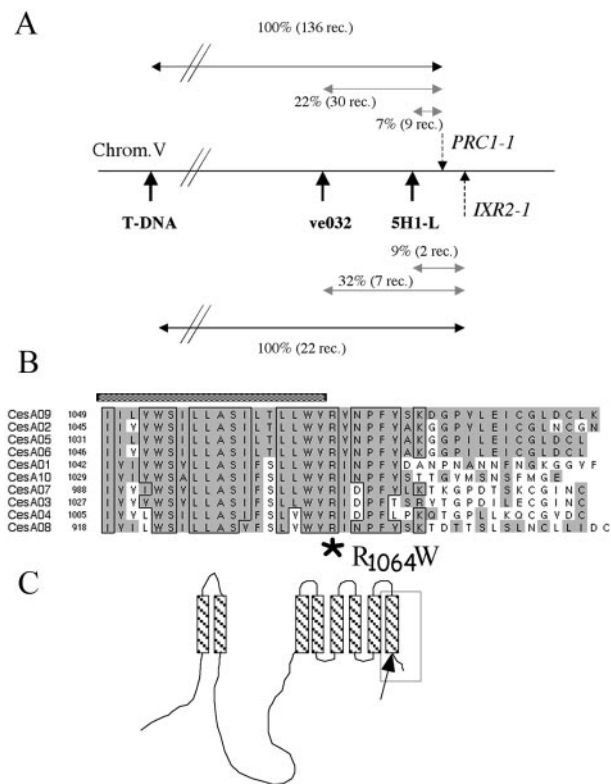
**Figure 3.** The *ixr2-1* mutation causes a $R_{1064}W$ change in a residue conserved for all CESA isoforms in Arabidopsis. A, Fine mapping of *IXR2*. Twenty-two recombinants were isolated between a T-DNA insertion in a C-24 background, and *ixr2-1* in Col0 and recombination breakpoints were mapped using the PCR markers ve032 and 5H1-L. *PRC1* was mapped using the same markers on 136 recombinants between *prc1-1* in Col0 and the same T-DNA insertion in C-24 (Fagard et al., 2000). The comparison of the maps showed a very close proximity between *IXR2* and *PRC1*. B, Multiple alignment of the C-terminal amino acid sequences of the 10 Arabidopsis CESAs. The numbers indicate the positions of the first amino acid presented in the alignment. Hatched bar indicates the last predicted transmembrane domain. Shaded residues represent a high consensus value of 85%, specified in the program. Boxed residues are identical for at least seven isoforms. The position of the $R_{1064}W$ mutation in the *IXR2-1* allele of CESA6 is indicated by the star below the alignment. C, Hypothetical diagram of the membrane topology of CESA proteins. Area in rectangle refers to the part of the sequence shown in B. Predicted transmembrane domains are hatched. The arrow indicates the position of the *ixr2-1* mutation.

bidopsis plants were constructed carrying a 12.74-kb genomic clone containing *CESA6* with or without the $R_{1064}W$ mutation. This fragment carries a functional *PRC1* gene as shown by its ability to complement the *prc1* phenotype (data not shown). $T_2$ progenies of four of four transformants carrying the wild-type *CESA6* fragment did not show an increased resistance against 5 nM isoxaben (e.g. The length of dark-grown Columbia [Col0] hypocotyls was 11.8 ± 1.6 mm and 2.1 ± 0.3 mm, respectively, in the absence and presence of 5 nM isoxaben. For a $T_2$ family of Col0 transformed with the wild-type *CESA6* fragment, hypocotyls measured 12.9 ± 0.9 mm and 2.3 ±

0.3 mm, respectively, in the absence and presence of 5 nM isoxaben. The three other $T_2$ families behaved in the same way). In contrast, $T_2$ progenies of five of five independent transformants carrying the $R_{1064}W$ mutation segregated for an increased isoxaben-resistance phenotype (data not shown). A homozygous $T_3$ line (line 201.3) containing *CESA6-$R_{1064}$W* was selected and analyzed more in detail. Measuring the inhibition of hypocotyl growth of dark-grown seedlings is a sensitive way to quantify isoxaben activity. Wild-type seedlings showed a steep dose-response curve with an $IC_{50}$ of around 1.5 nM (Fig. 4). This result was confirmed by the inhibition of the incorporation of $^{14}$C-Glc into the acid-insoluble cellulosic fraction ($IC_{50}$ around 1 nM, data not shown). The line homozygous for *ixr2-1* showed an increased resistance against isoxaben with $IC_{50}$ values 15 times higher than those for the wild-type control (Fig. 4). In the absence of isoxaben, hypocotyls of the line carrying *CESA6-$R_{1064}$W* (line 201.3) were, for unknown reasons, slightly shorter than those of wild-type plants. The isoxaben dose-response curve for 201.3 (Fig. 4) revealed a resistance intermediate between that of the wild-type and *ixr2* homozygotes ($IC_{50}$ of 6 nM). Together, these results demonstrate that the $R_{1064}W$ point mutation in *CESA6* is sufficient to confer increased isoxaben resistance.

## Other Functionally Redundant Isoforms of CESA6 Also May Be Isoxaben Targets

All six *prc1* alleles sequenced so far contain premature stop codons and are complete loss-of-function alleles of *CESA6* (Fagard et al., 2000). The results reported here show that CESA6 is a target for isoxaben. As a result, the Prc1⁻ phenotype is expected to be comparable with that of the wild-type treated with isoxaben. This is only in part true. Dark-grown hypocotyls of *prc1-1* were as long as those of the wild
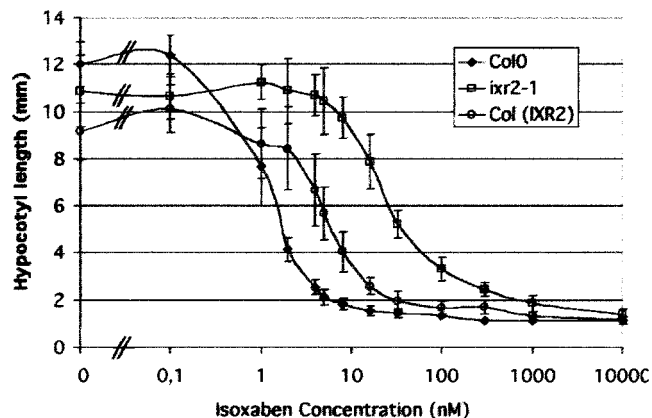


**Figure 4.** Transgenic Col0 homozygous for an insertion carrying *CESA6-$R_{1064}$W* shows increased resistance to isoxaben. Isoxaben dose-response curve for hypocotyl length of 4-d-old dark-grown seedlings. Curves for wild-type Col0, *ixr2-1* homozygotes, and Col0 plants homozygote for *CESA6-$R_{1064}$W* [Col(IXR2)] are shown.
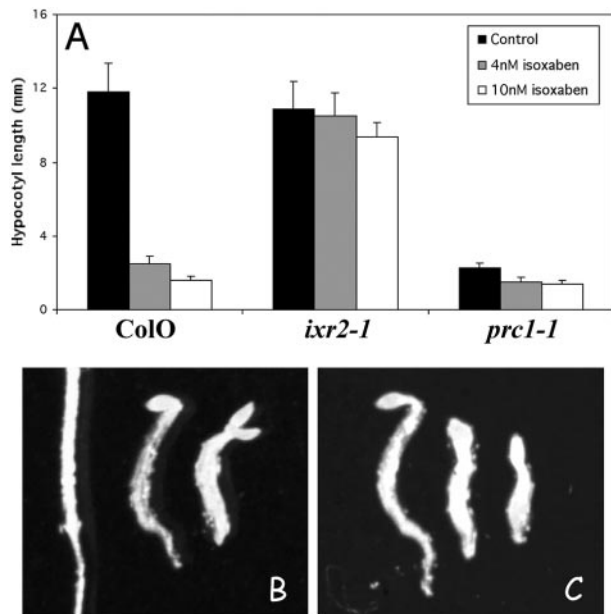
**Figure 5.** Evidence for additional isoxaben targets. A, Dark-grown hypocotyl length of 4-d-old seedlings of Col0, *ixr2-1* homozygotes, and *prc1-1* homozygotes in the absence or presence of 5 or 10 nM isoxaben. B, Seedling phenotype of Col0 grown without (left) and with (middle) 5 nM or (right) 10 nM isoxaben. C, Same conditions as in B, but for *prc1-1* seedlings. Note the stronger phenotype for wild-type seedlings grown in the presence of 10 nM isoxaben than that of *prc1-1* homozygotes.

type grown on 4 nM isoxaben (Fig. 5). However, higher concentrations of isoxaben further inhibited hypocotyl growth. Isoxaben also had a strong effect on light-grown seedlings (data not shown), whereas aerial parts of *prc1-1* grown in the light did not show a detectable phenotype (Fagard et al., 2000). Paradoxically, despite the nonessential role for CESA6 in these conditions, *ixr2-1* seedlings were also resistant against isoxaben when grown in the light. These observations indicate that isoxaben recognizes other targets besides CESA6, which are partially or totally redundant with CESA6. This was confirmed by the observation that despite the absence of a growth defect in the light, hypocotyl growth of *prc1* was hypersensitive to isoxaben. Hypocotyls of 7-d-old wild-type and *prc1-1* seedlings grown in the absence of isoxaben showed comparable lengths (0.67 ± 0.03 cm and 0.63 ± 0.04 cm, respectively, $n > 20$). In contrast, the hypocotyls of *prc1-1* seedlings grown for 7 d on 3 nM isoxaben were significantly shorter than those of the wild-type controls (0.32 ± 0.02 cm versus 0.62 ± 0.03 cm, respectively, $n > 20$).

## DISCUSSION

### *IXR2* encodes CESA6

The following evidence shows that resistance against isoxaben in the *ixr2-1* mutant is caused by a mutation in CESA6. First, fine mapping showed a

colocalization of *IXR2-1* with *PRC1*, which encodes CESA6. Second, CESA6 in *ixr2-1* carried an amino acid change, $R_{1064}W$. Third, transformation of a genomic fragment carrying the *CESA6* gene with this mutation, but not with the wild-type control, conferred increased isoxaben-resistance to wild-type plants.

The observed reduced resistance level of homozygotes for the mutant transgene compared with *ixr2-1* homozygotes is not surprising. The semidominant nature of the mutation has been reported previously, with *ixr2-1* heterozygotes showing a 10-fold higher $IC_{50}$ value than homozygotes (Heim et al., 1990a). The same observation was reported for *ixr1* mutants. In addition, *ixr1* homozygotes transformed with the wild-type gene showed a lower resistance to isoxaben than nontransformed *ixr1* controls (Scheible et al., 2001). This observation is consistent with the idea that multiple copies of CESA6 and/or CESA3 constitute the rosette complex. The different catalytic subunits within a rosette would coordinately synthesize the different glucan chains that constitute the microfibril. It is not clear how the activity of the subunits is coordinated, but it can be expected that the presence of even a few isoxaben-sensitive subunits within a, for the rest resistant, complex may lead to the poisoning of the entire complex in the presence of the herbicide.

### Redundancy within the CESA Gene Family

It is important to know the function of all 10 CESA isoforms in Arabidopsis (Fig. 6). Most isoforms appear to have an evolutionary conserved, specialized function, as shown by the presence of orthologs in unrelated plant species such as cotton (Fig. 6). Mutants *rsw1* (Arioli et al., 1998) and *prc1* (Fagard et al., 2000) have a strong seedling phenotype, indicating that the encoded isoforms (CESA1 and CESA6, respectively) are required for cellulose synthesis in the primary wall. Mutants for CESA7 (*irx3*), CESA8 (*irx1*), and CESA4 (*irx5*; Taylor et al., 1999, 2000; N. Taylor and S. Turner, personal communication) are characterized by their collapsed xylem phenotype, and the isoforms appear to be specialized for cellulose synthesis during secondary wall formation. The very similar dark-grown seedling phenotype for *rsw1* and *prc1* (Fagard et al., 2000) suggests that at least in the hypocotyl, the two encoded isoforms are required in the same cell types and have nonredundant functions. The same was observed for *irx1*, *3*, and *5* in xylem cells (Taylor et al., 1999, 2000; Taylor, 2001). Expression in the same cell types was further suggested for *CESA1* and *CESA3* using promoter-β-glucuronidase fusion constructs (Scheible et al., 2001). The requirement for several isoforms in the same cell type may indicate the coexistence of several specialized cellulose synthase complexes or the presence of one or more isoforms in the same complex.
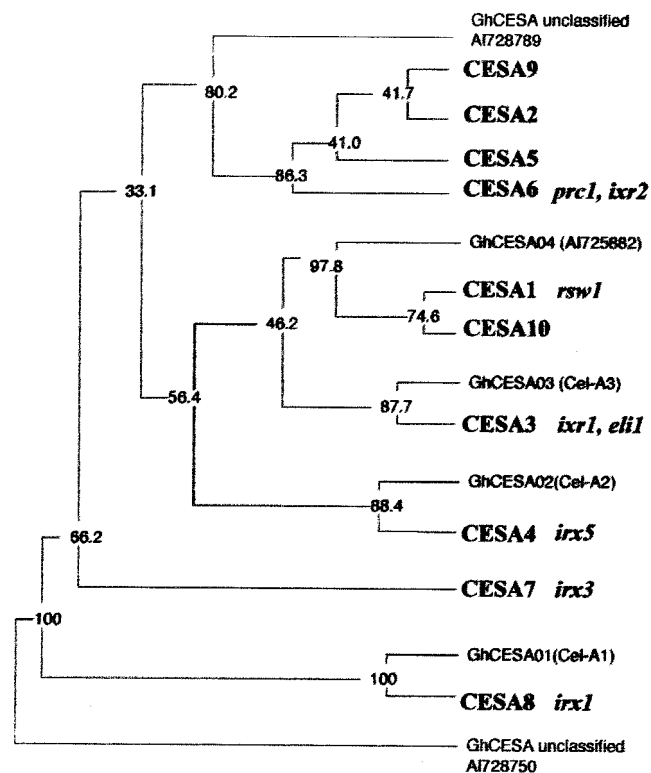
**Figure 6.** Members of the CESA family are functionally specialized as shown by loss-of-function and isoxaben-resistant alleles. A phylogenetic tree based on a hypervariable region among CESA protein sequences is shown (Fagard et al., 2000). Sequences from Arabidopsis (in bold) were aligned with those from cotton (*Gossypium hirsutum*), and a parsimonious consensus tree was constructed. Numbers are bootstrapped values (*n* = 100). Corresponding mutant alleles are indicated in italics and mutant phenotypes are discussed in the text. Clustering of one or more specific Arabidopsis sequences with cotton homologs suggests a functional specialization of the corresponding isoforms. CESA6 clusters with three other Arabidopsis isoforms, suggesting that they have arisen by more recent gene duplications.

The latter idea was supported by pull-down experiments showing that CESA7 and CESA8 physically interact in vitro.

The cloning of *ixr1* and *ixr2* provides additional functional information for the encoded isoforms. The existence of two resistance loci on first sight would suggest redundant roles for the corresponding proteins. However, the strong phenotype observed for dark-grown hypocotyls of *prc1* suggests that CESA3 is absent in this organ or that CESA3 is present but unable to compensate for the absence of CESA6 in the mutant. Although CESA3 protein has not yet been detected directly, two lines of evidence strongly suggest that CESA3 is present in dark-grown hypocotyls. First, *CESA3* transcripts are constitutively expressed (Scheible et al., 2001), including in dark-grown and light-grown seedlings (A. Van Tuinen, T. Desprez, and H. Höfte, unpublished data), and second, *ixr1* hypocotyl growth in the dark was also resistant against isoxaben. Given the fact that *ixr1* mutations do not alter the expression pattern of *CESA3* tran-

scripts (Scheible et al., 2001), it is reasonable to conclude that in dark-grown hypocotyls, the function of CESA3 is not redundant with that of CESA6. The absence of redundancy is further suggested by the strong phenotype observed for dark-grown seedlings carrying recessive loss of function mutations in *CESA3* (M. Bevan, personal communication), This leads to the interesting possibility that at least in dark-grown hypocotyls, CESA3 and CESA6 are active as a complex. This is similar to the complex reported for CESA7 and CESA8 in cells synthesizing secondary walls. In this scenario, isoxaben may bind directly, or via another protein, at the interface between the two monomers or may recognize a conformation requiring the presence of both wild-type isoforms.

A closer examination of the Prc1⁻ phenotype, however, shows that in the absence of CESA6, other targets for isoxaben exist in dark-grown hypocotyls. Complete knockout mutants for CESA6 showed a milder phenotype than wild-type plants treated with 10 nM isoxaben, a concentration that did not significantly affect growth in *ixr2-1*. This residual isoxaben-sensitive CESA activity may correspond to CESA3, which in that case would only partially replace CESA6 in the *prc1* mutant, but it may also correspond to other CESA isoforms. More strikingly, aerial parts of *prc1* mutants grown in the light did not show a growth phenotype, but were nevertheless hypersensitive to isoxaben action. Therefore, other CESA isoforms must have redundant roles with CESA6 in these conditions. In theory, this may be CESA3, but this would imply that the specificity of CESA3 is altered in light because CESA3 was unable to completely replace CESA6 in dark-grown *prc1* seedlings. In an alternate manner, isoforms CESA2, 5, and 9, which are highly similar to CESA6, may be redundant with CESA6 and constitute other isoxaben targets. As shown by expressed sequence tag data, *CESA2* and *CESA5* mRNAs are also expressed in dark- and light-grown seedlings, but at lower levels than *CESA6* (http://cellwall.stanford.edu). *CESA9* has a very low expression level as shown by the single expressed sequence tag that was found in a library from rosette leaves. The exact function of these three isoforms remains to be determined and will require the isolation of the corresponding mutants.

The genetic data indicate that CESA1 is also essential for cellulose synthesis, including in dark-grown hypocotyls (Fagard et al., 2000). From this, it can be inferred that CESA1 is still active in the presence of isoxaben in an *ixr2* or *ixr1* background, which would suggest that CESA1 is not an isoxaben target. However, it is not formally excluded that isoxaben recognizes a conformation that requires the association of not only CESA3 and CESA6, but also of CESA1, and that mutations in any of these proteins may destabilize the complex. These two possibilities can be now

distinguished by expressing CESA1 versions carrying their equivalent of the *ixr2-1*, *ixr1-1*, or *ixr1-2* mutations in transgenic plants and assessing the resistance against isoxaben.

The $R_{1064}W$ mutation in *ixr2-1* removes a positive charge at the end of the last predicted membrane spanning domain. This may cause a dramatic conformational change associated with a change in the membrane topology of the protein, which may prevent the binding of isoxaben or isoxaben-associated protein(s). *ixr1-1* and *ixr1-2* also carry mutations close to the C terminus of CESA3 (Scheible et al., 2001), suggesting again that this region is targeted by the herbicide. It is unlikely, however, that the amino acids mutated in the *ixr1* and *ixr2* alleles fold together in a single isoxaben-binding site. According to the predicted topology, $R_{1064}W$ in IXR2-1, and $G_{998}D$ in IXR1-1 would be on the opposite side of the membrane compared with $T_{942}I$ in IXR1-2. The prediction of the topology is not correct or one has to assume that the mutations all induce a conformational change of the protein complex, leading to the loss of isoxaben binding. In the absence of a comprehensive three-dimensional structure for the cellulose synthase complex, it is not clear how isoxaben interferes with the synthesis of cellulose. In *ixr2* and *ixr1* alleles, the mutated amino acid is far downstream from the active site residues, which makes it unlikely that the compound directly interferes with the catalytic site. In an alternate manner, the extrusion of the glucan chain supposedly requires a membrane channel that may be formed by the transmembrane domains. Isoxaben could block such a channel. Isoxaben binding could interfere with the interaction between CESA subunits and in this way destabilize the hexameric rosette or each particle that constitutes the rosette. A destabilizing effect on the rosettes was also suggested for the herbicide CGA325'615 (Peng et al., 2001).

In conclusion, the identification of the isoxaben target CESA6 provides further insights in the complexity of the cellulose synthesis machinery and the role of the 10 cellulose synthase isoforms. Indirect evidence for complex formation between CESA3 and CESA6 is provided and partial redundancy between CESA6 and other CESA isoforms, possibly CESA2, CESA5, or both. With the targets characterized, isoxaben becomes an excellent tool for the study of cellulose synthesis in Arabidopsis, and also in other plant species.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

*prc1-1*(Desnos et al., 1996), *ixr1-2* (*DH48*), *ixr2-1* (*DH1*; kindly provided by the Arabidopsis Biological Resource Center), and wild-type Arabidopsis plants were of the Col0 ecotype. A KanR5 line used for *ixr2-1* map-based cloning that bears a T-DNA insertion close to marker LFY3 (Van

Lijsebettens et al., 1996) at the bottom of chromosome 5 (Lister and Dean, 1993) was of C24 ecotype. Seedlings were grown in dark conditions as described in Fagard et al. (2000).

### Measurement of Hypocotyl Length

Growth of seedlings was arrested by addition of an aqueous solution of 0.4% (w/v) formaldehyde. Seedlings were spread on agar plates and an image was captured using a digital camera. The lengths of hypocotyls were measured using image analysis software (Optimas 5.2; IMASYS, Surennes, France) as described in Gendreau et al. (1997).

### Cross Sections and Calcofluor Staining

For light microscopy, seedlings were fixed in 4% (w/v) paraformaldehyde and 0.2% (w/v) glutaraldehyde in phosphate-buffered saline buffer and were embedded in historesin (Technovit 7100; Kulzer, Wehrheim, Germany) following the manufacturer's instructions. Sections 3 $\mu$m thick were cut using a microtome (Jung RM2055; Leica, Wetzlar, Germany). The cross sections were stained with a 0.005% (w/v) aqueous solution of Calcofluor (Fluorescent Brightener 28; Sigma, St. Louis) for 2 min and they were visualized under UV light using a microscope (microphot FXA; Nikon, Tokyo).

### Callose and Lignin Staining and Microscopic Observations

Plants were grown in vitro for 4 d in dark conditions on a medium containing 1% (w/v) Suc and 5 nM isoxaben or 0.5 $\mu$M DCB. Seedlings were directly spread on slides and were gently squashed under the coverslip before adding the staining components between the slide and the coverslip. Callose staining was done with Aniline Blue Fluorochrome or sirofluor (Biosupplies Australia, Parkville, Victoria, Australia) at a concentration of 0.1 mg mL$^{-1}$. Slides were kept in the dark for 30 to 120 min before observation under UV light.

Phloroglucinol in hydrochloric solution (Prolabo, Fontenay, France) was used for lignin staining and was observed under white light with Nomarski optics.

### Mapping of the *ixr2* Mutation

The *ixr2* mutant (Col0 ecotype; Heim et al., 1990a) was crossed to a T-DNA line (C24 ecotype) bearing an insertion close to RI marker LFY3. The F$_1$ hybrid was allowed to self-pollinate and the progeny was collected. To identify seedlings homozygous for the *ixr2* mutation, the F$_2$ progeny was plated directly on 6 nM isoxaben-containing growth medium and cultured in the dark for 4 d. In these conditions, it is possible to distinguish homozygous *ixr2/ixr2*, heterozygous *ixr2/IXR2*, and homozygous wild-type (IXR2/IXR2) seedlings (data not shown). Homozygous mutant seedlings (*ixr2/ixr2*) were then transferred to fresh

medium containing 50 mg $L^{-1}$ kanamycin. After 10 d, 22 kanamycin-resistant seedlings were transferred to greenhouse and were considered recombinant between the *ixr2-1* mutation and the T-DNA insertion. Genomic DNA was extracted from leaves and flower buds as described previously (Bouchez and Camilleri, 1998). These recombinant seedlings were further analyzed using the markers ve032 and 5H1-L generated during the map-based cloning of the *CESA6* gene (Fagard et al., 2000).

## DNA Construction and Plant Transformation

Standard molecular cloning techniques were performed as described by Sambrook et al. (1989). A 6.4-kb *Eco*RI-*Hin*dIII DNA fragment of the P1 clone MVP7 (accession no. AB025637) was cloned into the site *Eco*RI-*Hin*dIII of the binary vector pDE 1001 containing a kanamycin resistance marker (Denecke et al., 1992) to obtain the pDE-EHMVP7 clone. A 6.3-kb *Hin*dIII DNA fragment of the P1 clone MVP7 was introduced into the *Hin*dIII site of pDE-EHMVP7 to obtain the pDE-CESA6 clone.

A PCR reaction was performed using the oligonucleotides TGGTTATGGAGGTGGGTTGA, forward, and ATTTTCAATTTAGAAGACCGCAT, reverse, that surrounds the *ixr2-1* mutation. The resulting 2.6-kb DNA fragment was cleaved with *Sal*I-*Xba*I enzymes, and the digested fragment was cloned into the *Sal*I-*Xba*I sites of pDE-CESA6, giving rise to the pDE-CESA6-$R_{1064}$W clone. The binary T-DNA constructs were mobilized into *Agrobacterium tumefaciens* C58 Pmp90. The pDE-CESA6 and pDE-CESA6-$R_{1064}$W constructs were introduced into Col0 seedlings using the infiltration protocol described by Bechtold and Pelletier (1998). The $T_1$ transformants were selected on a kanamycin-containing medium (50 mg $L^{-1}$). The $T_2$ transformants were obtained after selfing $T_1$ kanamycin-resistant seedlings. Four Col0(CESA6) $T_2$ transformants were analyzed on a kanamycin-containing medium, and the line 206 carrying an unique transgene was selected for the analyses.

Five Col0(CESA6-$R_{1064}$W) = Col(IXR2) $T_2$ transformants were tested for their isoxaben resistance. The $T_2$ transformants were plated on $10^{-9}$ M isoxaben-containing medium in dark conditions so that the wild-type seedlings were classified as sensitive. We observed a segregating population representing homozygous and heterozygous *ixr2* states. The $F_3$ progeny of the most resistant line (line 201.3) was obtained and it was 100% kanamycin-resistant, confirming the homozygous state of the unique transgene.

## DNA Sequencing

Genomic DNA was extracted from adult seedlings grown in normal greenhouse conditions using standard protocol. PCR fragments covering the entire coding sequence were generated and sequenced by Genome Express (Meylan, France).

The *ixr2* mutation was detected in the PCR fragment amplified with the specific primers TGGTTATGGAGGT-GGGTTGA, forward, and ACGTGGCACAATATGGCTGA, reverse.

## Alignments and Phylogenetic Tree

The alignment presented in the Figure 3 was obtained with the Multalign program (http://www.toulouse.inra.fr/multalin.html). For multiple alignment, selected sequences (from the last transmembrane segment through the end of each AtCESA) were combined in SeqVu. A high consensus value of 85% was chosen in the program. For the dendrogram (Fig. 6), sequences from the HVR2 domains corresponding to amino acids 690 and 743 in CESA6 were aligned with the program ClustalW (http://www.infobiogen.fr/services/analyseq/cgi-bin/clustalw_in.pl). An additional sequence, which is more divergent, was inserted as an outlier (AI727450). This alignment was analyzed by PROTPARS to construct a consensus tree from 100 bootstrapped data sets.

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