

Impairment of Cellulose Synthases Required for *Arabidopsis* Secondary Cell Wall Formation Enhances Disease Resistance ^W

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Cellulose is synthesized by cellulose synthases (CESAs) contained in plasma membrane-localized complexes. In *Arabidopsis thaliana*, three types of CESA subunits (CESA4/IRREGULAR XYLEM5 [IRX5], CESA7/IRX3, and CESA8/IRX1) are required for secondary cell wall formation. We report that mutations in these proteins conferred enhanced resistance to the soil-borne bacterium *Ralstonia solanacearum* and the necrotrophic fungus *Plectosphaerella cucumerina*. By contrast, susceptibility to these pathogens was not altered in cell wall mutants of primary wall CESA subunits (CESA1, CESA3/ISOXABEN RESISTANT1 [IXR1], and CESA6/IXR2) or *POWDERY MILDEW-RESISTANT5* (*PMR5*) and *PMR6* genes. Double mutants indicated that *irx*-mediated resistance was independent of salicylic acid, ethylene, and jasmonate signaling. Comparative transcriptomic analyses identified a set of common *irx* upregulated genes, including a number of abscisic acid (ABA)-responsive, defense-related genes encoding antibiotic peptides and enzymes involved in the synthesis and activation of antimicrobial secondary metabolites. These data as well as the increased susceptibility of ABA mutants (*abi1-1*, *abi2-1*, and *aba1-6*) to *R. solanacearum* support a direct role of ABA in resistance to this pathogen. Our results also indicate that alteration of secondary cell wall integrity by inhibiting cellulose synthesis leads to specific activation of novel defense pathways that contribute to the generation of an antimicrobial-enriched environment hostile to pathogens.

INTRODUCTION

The presence of a cell wall confers many of the gross morphological characteristics of plants and is one of the features that distinguish them from animals. The cell wall is a complex com-

posite of cellulose, high molecular weight polysaccharides, proteins, and aromatic substances that undergoes dynamic changes. In addition to providing structural support and a passive barrier against invading pathogens and pests, the cell wall controls cell expansion and is involved in the exchange of water and substances throughout plant development (Carpita and McCann, 2000). It also constitutes a reservoir of antimicrobial compounds and is a source of signaling molecules (Carpita and McCann, 2000).

Cellulose is a linear polymer of β -(1-4)-linked glucose, which is synthesized at the plasma membrane by a large membrane-bound complex whose only identified components are several structurally similar cellulose synthase (CESA) subunits (Somerville et al., 2004). *Arabidopsis thaliana* contains 10 CESA genes, six of which encode proteins with known functions (Somerville et al., 2004). The CESA1 (RADIAL SWELLING1 [RSW1]), CESA3 (ISOXABEN RESISTANT1 [IXR1]), and CESA6 (PRC1/IXR2) subunits are largely responsible for cellulose production during the formation of the primary cell wall in most tissues (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Somerville et al., 2004), whereas cellulose synthesis for the secondary cell wall, which takes place after the arrest of cell expansion, requires CESA4

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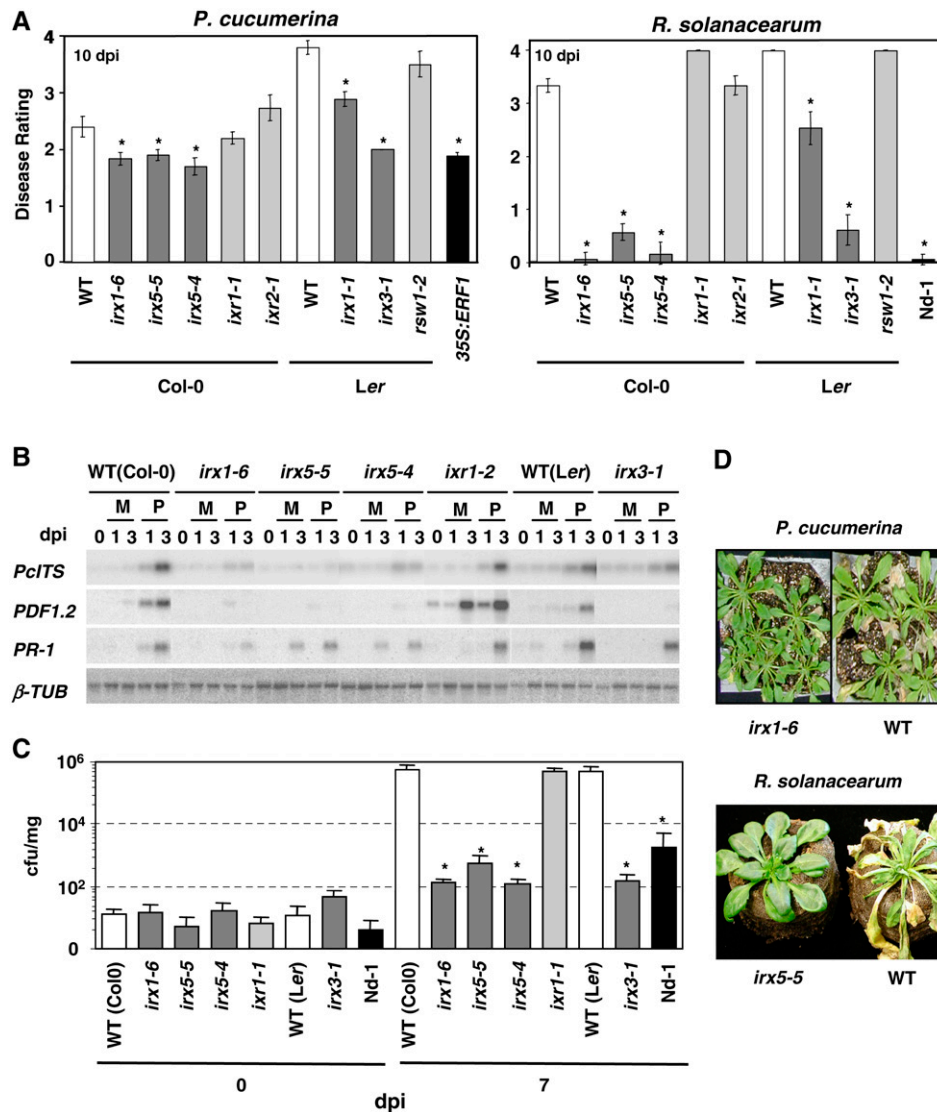


Figure 1. Alteration of Secondary Cell Walls by Loss of Function of *IRX/CESA* Genes Reduced *Arabidopsis* Susceptibility to Pathogens.

(A) Average disease rating (\pm SD) of wild-type plants (white bars) and mutants in secondary cell walls (*ixr1-6/em1*, *ixr5-5/nws2*, *ixr5-4*, *ixr1-1*, and *ixr3-1* [dark gray bars]) or primary cell walls (*ixr1-2*, *ixr2-1*, and *rsw2-1* [light gray bars]) at 10 d after inoculation (dpi) with *P. cucumerina* or *R. solanacearum*. The disease rating varies between 0 (no symptoms) and 4 (dead plants). The alleles used were in either the Col-0 or Ler genetic background. The transgenic 35S:ERF1 plants (Col-0) and the Nd-1 ecotype that showed enhanced or full resistance to *P. cucumerina* and *R. solanacearum*, respectively, were included as controls (black bars) (Berrocal-Lobo et al., 2002a; Deslandes et al., 2003). Asterisks indicate values significantly different ($P < 0.01$, *t* test) from those of wild-type plants.

(B) RNA gel blot analysis of the expression of the *P. cucumerina* Interspace Transcribed Sequence (*PcITS*) and of the defense genes *PR1* and *PDF1.2* in wild-type (Col-0 and Ler), *ixr*, and *ixr* plants. Total RNA (2.5 μ g/lane) was extracted from plants collected at the indicated times after inoculation with the fungus (P) or mock-inoculation (M). β -Tubulin (β -*TUB*) was included as a loading control.

(C) *R. solanacearum* GMI1000 growth (log cfu [colony-forming units]/mg fresh tissue) in wild-type (Col-0 and Ler), *ixr*, and *ixr* plants at 0 and 7 d after inoculation. The resistant Nd-1 ecotype (Deslandes et al., 2003) was included for comparison. Error bars represent SD. At least 10 plants per genotype were tested. Asterisks indicate values significantly different ($P < 0.01$, *t* test) from those of wild-type plants.

(D) Disease symptoms of *ixr1-6*, *ixr5-5*, and wild-type plants inoculated with *P. cucumerina* (12 d after inoculation) or *R. solanacearum* GMI1000 (10 d after inoculation).

(IRX5), CESA7 (IRX3), and CESA8 (IRX1) and a diverse set of recently identified proteins (Taylor et al., 2003; Somerville et al., 2004; Brown et al., 2005; Persson et al., 2005). Mutations in any of these six *CESA* genes lead to a reduced level of cellulose synthesis and consequently to modifications in the composition and structure of either the primary or the secondary cell wall (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Taylor et al., 2003; Somerville et al., 2004; Brown et al., 2005; Persson et al., 2005).

Initial evidence for a connection between modifications of the cell wall structure and stress responses came from the finding that alteration of the primary cell wall of the *Arabidopsis ixr1/cev1* (for constitutive expression of *VSP1*) mutant, caused by partial loss of *CESA3* function, leads to the constitutive activation of the jasmonate (JA) and ethylene (ET) signaling pathways and to enhanced resistance to some pathogens (Ellis et al., 2002). Also,

disruption of the *Arabidopsis* secondary cell wall by inactivation of the *CESA8/IRX1* gene in the *leaf wilting2 (lew2)/ixr1* mutant causes an increase of the endogenous abscisic acid (ABA) levels and an enhanced tolerance to drought and osmotic stress (Chen et al., 2005). Cell wall polysaccharide composition is also a determinant of disease development (Vogel et al., 2002, 2004; Somerville et al., 2004). For example, a search for *Arabidopsis* mutants with enhanced resistance to virulent strains of powdery mildew (*Erysiphe cichoracearum*) led to the identification of *powdery mildew-resistant5 (pmr5)* and *pmr6* mutants, which exhibit an altered cell wall composition (Vogel et al., 2002, 2004).

Our knowledge of the plant molecular mechanisms controlling infections caused by the necrotrophic fungus *Plectosphaerella cucumerina* and the vascular bacterial pathogen *Ralstonia solanacearum* is limited. *Arabidopsis* resistance to the fungus depends on the ET, JA, and salicylic acid (SA) signaling pathways, whereas tolerance to the bacterium is just ET-dependent (Berrocal-Lobo et al., 2002a; Hirsch et al., 2002). *Arabidopsis* resistance to various strains of *R. solanacearum* is monogenic and depends on *RRS1-R*, a resistance gene present in the Niederzenz (Nd-1)-resistant ecotype but truncated in the susceptible Columbia (Col-0) ecotype (Deslandes et al., 2003). By contrast, *Arabidopsis* resistance to *P. cucumerina* is multigenic (Llorente et al., 2005). We have shown previously that the receptor-like kinase *ERECTA* was required for *Arabidopsis* resistance to both *P. cucumerina* and strain 14-25 of *R. solanacearum* (Godiard et al., 2004; Llorente et al., 2005).

To identify additional *Arabidopsis* genes that control the development of the infections caused by these pathogens, we searched for mutants unable to develop disease symptoms upon infection. Here, we show that mutations (*ixr*) in any of the three *Arabidopsis* *CESAs* required for secondary cell wall formation (*IRX1/CESA8*, *IRX3/CESA7*, and *IRX5/CESA4*) conferred enhanced resistance to these pathogens as well as to *Botrytis cinerea* and powdery mildew fungi. Genetic and transcriptomic analyses indicated that *ixr/cesa*-mediated resistance was independent of SA, ET, and JA signaling and allowed the identification in *ixr* plants of a group of constitutively upregulated genes, which included ABA-responsive, defense-related genes. These results suggest that modifications of plant cell wall integrity could activate specific defensive pathways, which could lead to plant enhanced resistance to different type of pathogens.

RESULTS

Isolation and Characterization of *ern1/ixr1* and *nws2/ixr5* Mutants

To identify *Arabidopsis* genes that affect the development of the diseases caused by the necrotroph *P. cucumerina* and the soil-borne pathogen *R. solanacearum* GMI1000, we inoculated an ethyl methanesulfonate-mutagenized population with the fungus and T-DNA-tagged mutants with the bacterium, and we searched for mutants unable to develop disease symptoms. In these two independent screens, we isolated the *ern1* (*enhanced resistance to necrotrophs1*) and *nws2* (*no wilt symptoms2*) mutants, which showed reduced disease symptoms or remained symptomless upon infection with the fungus and the bacterium,

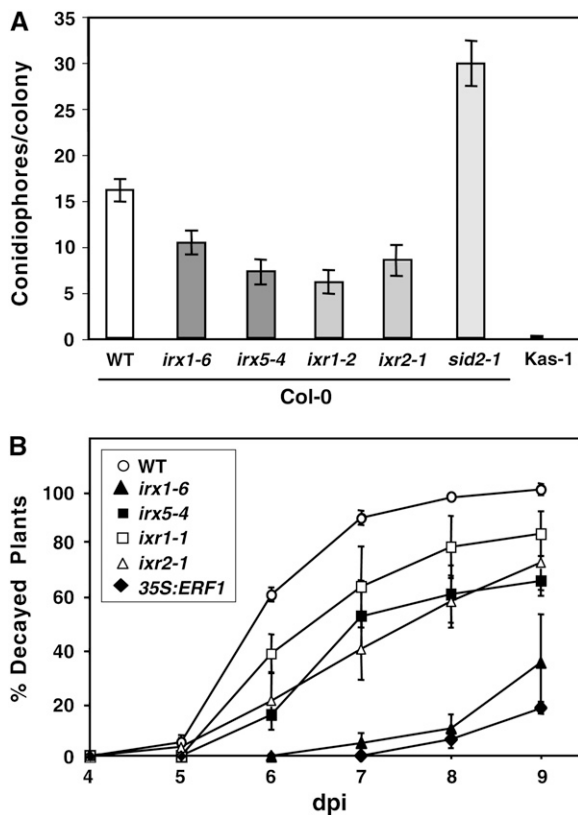


Figure 2. Susceptibility of *ixr* and *ixr* Mutants to *E. cichoracearum* USC1 and *B. cinerea*.

(A) Quantification of *E. cichoracearum* growth (conidiophores per colony at 6 d after inoculation) in wild-type plants (Col-0) and *ixr* and *ixr* mutants. Mean values \pm SD based on 15 colonies are represented. The resistant Kashmir (Kas-1) ecotype and the susceptible *sid2-1* mutant were included for comparison (Vogel et al., 2002).

(B) Percentage of wild-type (Col-0), *ixr*, and *ixr* decayed plants at several days after inoculation (dpi) with 5×10^4 spores/mL *B. cinerea*. Data values represent averages \pm SD of three independent experiments. Partially resistant, transgenic *35S:ERF1* plants were included for comparison (Berrocal-Lobo et al., 2002a).

Table 1. Response of *irx* Mutants to Various Pathogens

Pathogen	Wild Type	<i>irx5-5</i>	<i>irx1-6</i>
<i>Botrytis cinerea</i>	S	WR	WR
<i>Erysiphe cichoracearum</i> USC1	S	WR	WR
<i>Hyaloperonospora parasitica</i> Noco2	S	S	S
<i>H. parasitica</i> Emwa1	HR	HR	HR
<i>Pseudomonas syringae</i> DC3000	S	S	S
<i>P. syringae</i> DC3000 (<i>AvrRPM1</i>)	HR	HR	HR
<i>Xanthomonas campestris</i> pv <i>campestris</i> 8004	S	S	ND
<i>X. campestris</i> pv <i>campestris</i> 147	HR	HR	ND

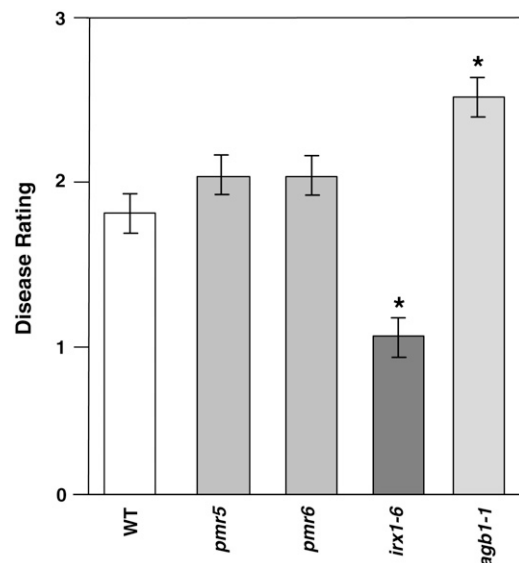
HR, hypersensitive response; ND, not determined; S, susceptible; WR, weak enhanced resistance.

respectively (Figures 1A and 1D). The *ern1* and *nws2* mutations were identified by map-based cloning and inverse PCR of T-DNA flanking sequences (see Supplemental Figure 1 online), and they were found to be impaired in the CESA8/IRX1 and CESA4/IRX5 proteins, respectively, required for secondary cell wall synthesis (Taylor et al., 2003; Brown et al., 2005; Chen et al., 2005; Persson et al., 2005). The *ern1* mutant was identical to the *lew2* mutant (Chen et al., 2005), in which a point mutation leads to the production of a truncated CESA8/IRX1 protein lacking the catalytic domain. The *ern1/lew2* mutant was renamed *irx1-6*, because it was allelic to the previously described *irx1-1* to *irx1-5* mutants (Taylor et al., 2003; Brown et al., 2005). In the *nws2* mutant, the T-DNA was inserted in the second exon of the *CESA4/IRX5* gene, and no transcript could be detected (see Supplemental Figure 1 online; data not shown). The *nws2* mutant was renamed *irx5-5*, because it was allelic to the loss-of-function mutants *irx5-1* to *irx5-4* (Taylor et al., 2003; Brown et al., 2005).

To further demonstrate the significance of the impairment of secondary cell wall CESAs in the reduced susceptibility to both pathogens, we examined the disease responses of additional alleles of *irx1* (*irx1-1*) and *irx5* (*irx5-4*) as well as that of *irx3-1*, a null mutant of CESA7/IRX3, the third subunit required for the synthesis of secondary cell wall cellulose (Taylor et al., 2003; Somerville et al., 2004; Brown et al., 2005). Consistently, all of these mutants showed reduced susceptibility to *P. cucumerina* comparable to that of transgenic *35S:ERF1* plants, in which the ET and JA defensive pathways are constitutively activated by the overexpression of the *ERF1* transcriptional factor, an integrator of both pathways (Figure 1A) (Berrocal-Lobo et al., 2002a). The resistance of these *irx* mutants to *R. solanacearum* was similar to that of the resistant Nd-1 ecotype (Figure 1A) (Deslandes et al., 2003). Interestingly, mutants of the three CESAs required for primary cell wall synthesis (*ixr1-1/cev1*, *ixr2-1*, and *rws1-1*) (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Ellis et al., 2002) were as susceptible to these pathogens as wild-type plants (Figure 1A). To verify whether the disease symptoms observed in these mutants correlated with a concomitant diminution of pathogen multiplication in planta, internal growth curves of *R. solanacearum* were plotted and determination of *P. cucumerina* biomass was performed in the inoculated plants. These analyses revealed that the reduced disease symptoms observed in the *irx* secondary cell wall mutants correlated with

a concomitant decrease in the proliferation of these pathogens in planta (Figures 1B and 1C). By contrast, in the *ixr* primary cell wall mutants, the two pathogens grew to similar levels than in wild-type plants (Figures 1B and 1C). These results proved that reduction of cellulose content in secondary cell walls specifically activates *Arabidopsis* resistance to *P. cucumerina* and *R. solanacearum*.

Besides *P. cucumerina* and *R. solanacearum*, we also tested the responses of the *irx1-6* and *irx5-5* mutant alleles to a broad range of pathogens, including oomycetes, fungi, and bacteria. These mutants were as susceptible as wild-type plants to most of these pathogens, except for the necrotrophic fungus *B. cinerea* and a virulent isolate of powdery mildew (Figure 2, Table 1). However, the reduced susceptibility to powdery mildew and *B. cinerea* may not be specific to secondary cell wall mutants, because the primary cell wall mutants *ixr1/cev1* and *ixr2* were also more resistant to these pathogens (Figure 2) (Ellis et al., 2002). To further determine the specificity of the disease resistance activated by the impairment of the CESA required for secondary cell wall formation, we analyzed the susceptibility to *P. cucumerina* and *R. solanacearum* of additional cell wall mutants, such as *pmr5* and *pmr6*, that showed, like the *irx* mutants, powdery mildew resistance (Vogel et al., 2002, 2004). Interestingly, the susceptibility of these cell wall mutants to *P. cucumerina*, *R. solanacearum*, and *B. cinerea* was similar to that of wild-type plants (Figure 3; data not shown). These results illustrate the specificity of the resistance of *irx/cesa* mutants toward *R. solanacearum* and *P. cucumerina*

**Figure 3.** Susceptibility of *pmr5* and *pmr6* Cell Wall Mutants to *P. cucumerina*.

Average disease rating (±SD) of wild-type plants and mutants at 10 d after inoculation with *P. cucumerina*. The disease rating varies between 0 (no symptoms) and 4 (dead plants). At least 10 plants per genotype were tested. The *agb1-1* mutant, which shows enhanced susceptibility to *P. cucumerina*, was included as a control (Llorente et al., 2005). Asterisks indicate values significantly different ($P < 0.01$, *t* test) from those of wild-type plants.

and also show that alterations of different components of primary or secondary cell walls lead to enhanced resistance to different subsets of pathogens.

irx-Mediated Resistance Is SA-, ET-, and JA-Independent

Distinct mechanisms control infection caused by the necrotroph *P. cucumerina* and the vascular pathogen *R. solanacearum*, two pathogens whose mode of colonization of host plants differ widely. Indeed, resistance to the fungus depends on the ET, JA, and SA signaling pathways, as revealed by the enhanced susceptibility to the fungus of *NahG* plants and *sid2* mutants, which are impaired in SA signaling, of the *ein2-5* mutant, which is blocked in the ET pathway, and of the *coi1-1* and *jar1-1* mutants, which are defective in JA signaling (Figures 4A and 4B) (Berrocal-Lobo et al., 2002a; Stein et al., 2006). By contrast, *Arabidopsis* tolerance to the bacterium is just ET- and JA-dependent, as disease development was reduced in *ein2-5* and *coi1-1* mutants compared with wild-type plants (Figure 4A) (Hirsch et al., 2002). To determine the relevance of these defense signaling pathways

(Glazebrook, 2001) in *irx*-mediated resistance, we disabled each of them in the *irx1-6* mutant by generating *irx1-6 NahG* lines and the *irx1-6 sid2-1*, *irx1-6 coi1-1*, *irx1-6 jar1-1*, and *irx1-6 ein2-5* double mutants. However, the inactivation of these defense pathways did not affect the response to *R. solanacearum* and *P. cucumerina*, demonstrating that they did not contribute to the observed reduced susceptibility of *irx* plants (Figures 4A and 4B; data not shown). Similarly, inactivation of the defense regulator protein NPR1 (Cao et al., 1994; Glazebrook, 2001) in the double *irx1-6 npr1-1* mutant did not affect *irx*-mediated resistance (data not shown).

In agreement with the infection data, the SA-responsive gene *PR1* and the ET/JA-associated gene *PDF1.2* were not constitutively expressed in uninfected *irx* plants (Figures 1B and 4C). Upon infection with the pathogens, the steady state levels of these genes were weakly increased in *irx* plants (Figures 1B and 4C). In the case of the *R. solanacearum*-inoculated plants, *PR1* and *PDF1.2* expression levels were similar to those found in the resistant Nd-1 plants (data not shown). Moreover, after fungal infection, the expression of *PDF1.2* and *PR1* in *irx1-6 NahG*,

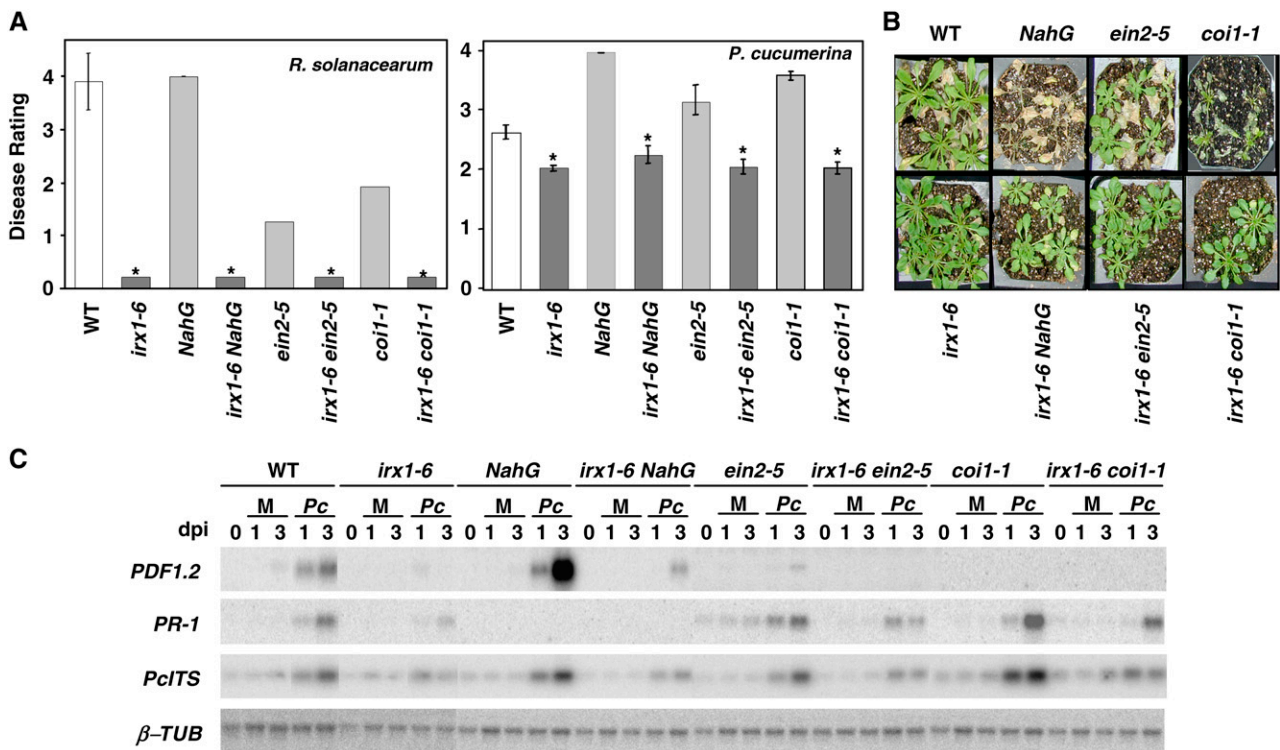


Figure 4. *irx*-Mediated Resistance Is SA-, ET-, and JA-Independent.

(A) Analysis of the susceptibility to *R. solanacearum* and *P. cucumerina* of *irx1-6* double mutants with defects in the SA (*irx1-6 NahG*), ET (*irx1-6 ein2-5*), or JA (*irx1-6 coi1-1*) signal transduction pathway. Average disease ratings (\pm SD) of the indicated genotypes at 10 d after inoculation are represented. The disease rating varies between 0 (no symptoms) and 4 (dead plants). At least 10 plants per genotype were tested. Asterisks indicate values significantly different ($P < 0.01$, *t* test) from those of wild-type plants.

(B) Disease symptoms of *P. cucumerina*-inoculated plants at 12 d after inoculation.

(C) Expression analysis of SA and ET/JA marker genes in *irx1-6* double mutants. RNA gel blot of *PR1* and *PDF1.2* defense genes in wild-type plants, single mutants, *NahG* transgenic plants, and double mutants. Total RNA (2.5 μ g/lane) was extracted from plants collected at the indicated days after inoculation (dpi) with *P. cucumerina* (Pc) or treated with water (mock [M]). Blots were hybridized with the *PR1*, *PDF1.2*, and *Pc ITS* probes. β -Tubulin (β -TUB) hybridization was included as a loading control. Three independent experiments were performed and gave similar results.

irx1-6 coi1-1, and *irx1-6 ein2-5* plants was similar or lower than that observed in the *NahG*, *coi1-1*, and *ein2-5* mutants (Figure 4C). These data demonstrated that these signaling pathways did not contribute significantly to the reduced susceptibility of the *irx* mutants to pathogens.

ABA-Responsive, Defense-Related Genes Are Expressed Constitutively in the *irx1-6* and *irx5-5* Mutants

To further investigate the mechanisms controlling *irx*-mediated resistance, comparative gene expression analysis of noninoculated wild-type, *irx5-5*, and *irx1-6* plants was performed. Of the 22,000 genes tested, 301 genes were constitutively upregulated and 265 genes were constitutively downregulated in both *irx1-6* and *irx5-5* mutants compared with wild-type plants (see Supplemental Tables 1 and 2 online). Most of these differentially expressed genes seemed to be specific to the secondary cell wall *irx* mutants, as quantitative RT-PCR analysis revealed that their expression in primary cell wall *irx* mutants was similar to that in wild-type plants (see Supplemental Figure 2 online; data not shown). A significant number of genes encoding cell wall–related proteins were differentially regulated in the *irx* mutants (see Supplemental Tables 1 and 2 online), as expected from the substantial changes in secondary cell wall structure and composition caused by the *irx* mutations (Taylor et al., 2003; Somerville et al., 2004; Brown et al., 2005; Persson et al., 2005).

Among the constitutively *irx* upregulated genes, a reduced number (6 to 7%) of ET- or SA-dependent defense-related genes was identified (Figure 5), which was in agreement with the genetic and RNA gel blot expression data (Figure 4). A noticeably greater percentage (37%) of constitutively upregulated genes were found to be JA-regulated (Figure 5). However, these genes may not be essential for *irx*-mediated resistance, as the phenotype of the *irx1-6* mutant was not affected by the JA-insensitive

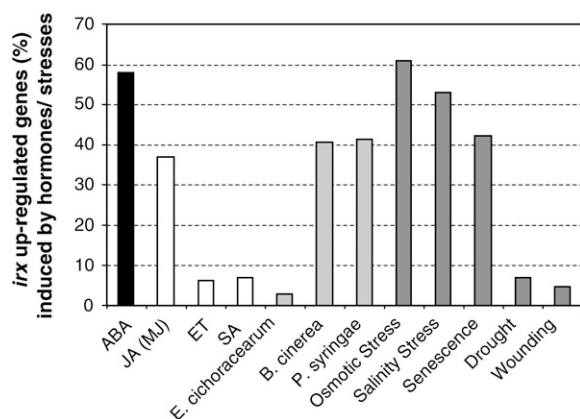


Figure 5. Expression of the *irx* Upregulated Genes in Response to Defense Signaling Compounds and to Abiotic and Biotic Stresses.

Expression of the 301 genes upregulated in the *irx5-5* and *irx1-6* genes was analyzed using the GeneInvestigator Meta-Analyzer tools (www.geneinvestigator.ethz.ch/at/). The corresponding percentages were determined by selecting upregulated genes showing fold (normalized) values > 2.

coi1 and *jar1* mutations (Figures 4A and 4B; data not shown). Interestingly, the majority (58%) of the genes strongly activated in the *irx* mutants were ABA-regulated (Figure 5; see Supplemental Table 1 online). Among those, we identified genes encoding key components of the ABA biosynthetic (e.g., NCED3) and signaling (e.g., ABI1, ABI2, and ABI4) pathways (Shinozaki et al., 2003) as well as defense-related proteins (e.g., Lipid Transfer Proteins [LTPs] and ATR1 [García-Olmedo et al., 1998; Grubb and Abel, 2006]). The constitutive activation of ABA signaling in the *irx* mutants was in agreement with the recent demonstration that disruption of the *CESA8/IRX1* gene in the *lew2-1/irx1-6* allele resulted in an increased accumulation of ABA in planta and an enhanced tolerance to drought and osmotic stresses (Chen et al., 2005). Consistently, a significant number of the *irx* upregulated genes also responded to these abiotic stresses (Figure 5).

Previous studies indicate that ABA functions as a negative modulator of the ET/JA-dependent defense response and as a positive regulator of JA-dependent defensive genes (Anderson et al., 2004; Lorenzo et al., 2004). In agreement with these data, some ET-associated genes were downregulated in the *irx* mutants compared with wild-type plants (see Supplemental Table 2 online).

Many constitutively upregulated genes in the *irx* mutants were defense-related genes, which responded to infection by different pathogens (e.g., *B. cinerea* and *Pseudomonas syringae*) (Figure 5). Among these pathogen-responsive genes, some encoded antimicrobial peptides belonging to four distinct families (e.g., LTPs, thionins [THs], snakin/GASA, and pEARLY), PR proteins, enzymes involved in the synthesis and activation of antimicrobial secondary metabolites (e.g., CYP79B2 and CYP79B3), as well as transcriptional regulators (e.g., ATR1) of this pathway (Table 2).

ABA Signaling Function in *irx*-Mediated Resistance

We further investigated the role of ABA signaling in the resistance to *P. cucumerina* and *R. solanacearum* by testing *abi1-1* and *abi2-1*, two ABA-insensitive mutants impaired in genes upregulated in *irx1-6/irx5-5* plants (see Supplemental Figure 2 online), as well as *aba1-6*, a mutant defective in ABA biosynthesis (Niyogi et al., 1998). These mutants displayed a higher susceptibility toward the bacterial pathogen (Figure 6A), which further corroborates the involvement of ABA signaling in the control of wilt disease development caused by *R. solanacearum*. By contrast, the disease symptoms caused by the necrotrophic fungus were reduced in the *abi1-1*, *abi2-1*, and *aba1-6* mutants compared with those observed in wild-type plants (Figure 6B). The reduced susceptibility to *P. cucumerina* of *abi1-1*, *abi2-1*, and *aba1-6* mutants was in agreement with the previously described reduced susceptibility of *Arabidopsis* ABA-insensitive and ABA-deficient mutants to the vascular pathogen *Fusarium oxysporum* and of ABA-deficient tomato (*Solanum lycopersicum*) plants and *Arabidopsis* ABA-insensitive mutants to the necrotroph *B. cinerea* (Audenaert et al., 2002; Anderson et al., 2004; AbuQamar et al., 2006).

To corroborate the function of ABA signaling in *irx*-mediated resistance, we disabled ABA biosynthesis and ABA signaling in the *irx1-1* and *irx3-1* mutants by generating *irx1-1 abi1-1* and *irx3-1 aba3-2* double mutants. A small number of dwarf plants

Table 2. Defensive Genes Constitutively Upregulated in the *irx5-5* and *irx1-6* Mutants

Gene Description	Locus	Normalized <i>irx5-5/irx1-6</i> ^a	References
Thaumatococin, pathogenesis-related protein	At1g20030	9.4	Hu and Reddy (1997)
Subtilase protein family	At1g20160	4.0	Jorda et al. (1999)
Myrosinase binding protein	At1g52000	3.9	Kliebenstein et al. (2005)
Myrosinase binding protein (MBP2)	At1g52030	2.9	Capella et al. (2001)
Myrosinase-associated protein	At1g54020	11.9	Kliebenstein et al. (2005)
Terpene synthase/cyclase protein	At1g61120	4.8	Kappers et al. (2005)
pEARLY peptide	At1g62510	16.1	Weyman et al. (2005)
Flavin monooxygenase (FMO)	At1g62540	4.8	Bartsch et al. (2006)
Flavin monooxygenase (FMO)	At1g62570	6.6	Bartsch et al. (2006)
Antimicrobial peptide thionin (THI2.1)	At1g72260	5.5	Molina et al. (1993a); Eppe et al. (1997)
Cytochrome P450 CYP79B3	At2g22330	3.5	Kliebenstein et al. (2005); Grubb and Abel (2006)
Flavin adenine dinucleotide binding domain-containing protein	At2g34810	6.0	Bartsch et al. (2006)
Protease inhibitor/antimicrobial	At2g37870	14.9	García-Olmedo et al. (1998)
Lipid transfer protein (LTP), putative			
Jacalin lectin family protein	At3g16450	4.2	Kliebenstein et al. (2005)
Antimicrobial peptide LTP	At3g53980	19.2	García-Olmedo et al. (1998)
Strictosidine synthase	At3g57010	7.5	Facchini and St-Pierre (2005)
C-S lyase (CORI3)	At4g23600	2.9	Grubb and Abel (2006)
Antimicrobial peptide LTP	At4g33550	7.8	García-Olmedo et al. (1998)
ELI3-2 defensive protein	At4g37990	5.5	Williamson et al. (1995)
Cytochrome P450 CYP79B2	At4g39950	2.3	Kliebenstein et al. (2005); Grubb and Abel (2006)
Anthranilate synthase, α -subunit (ASA1)	At5g05730	3.2	Niyogi and Fink (1992)
Gibberellin-regulated protein 4 (GASA4)	At5g15230	2.4	Berrocal-Lobo et al. (2002b)
Antimicrobial peptide LTP4	At5g59310	11.1	Segura et al. (1993)
Antimicrobial peptide LTP3	At5g59320	13.2	Segura et al. (1993)
Transcriptional factor ATR1 (MYB34)	At5g60890	4.3	Celenza et al. (2005)

^aNormalized, average fold ratios (*irx5-5/irx1-6* mutants versus wild-type plants). Genes were selected using values of $P < 0.01$ and raw data in the mutants >70 (see Methods).

with pronounced developmental abnormalities, corresponding to the double homozygous mutants, were identified under high-humidity growth conditions (see Supplemental Figure 3 online; data not shown). However, these *irx1-1 abi1-1* and *irx3-1 aba3-2* double mutants died 2 to 3 weeks after sowing, making it impossible to test their resistance to pathogens. These results suggested that ABA signaling played a significant role in the regulation of the *irx* developmental phenotype.

Function of Secondary Metabolites in *irx*-Mediated Resistance

A significant number of defensive genes were constitutively upregulated in the *irx/cesa* mutants, including some encoding antimicrobial peptides (e.g., LTPs and THs) (Table 2; see Supplemental Figure 2 online) and enzymes involved in the synthesis and activation of secondary metabolites. Secondary metabolites, such as glucosinolates and camalexin, act as antimicrobials (Grubb and Abel, 2006) that affect the interaction between *Arabidopsis* and various pathogens, including the necrotrophic fungus *B. cinerea* (Kliebenstein et al., 2005). The synthesis of these metabolites is regulated by several enzymes (e.g., CYP79B2 and CYP79B3) and transcriptional factors (e.g., ATR1) whose corresponding genes were constitutively upregulated in the *irx* mutants (Table 2). To further determine the relevance of the

secondary metabolites in *irx*-mediated resistance, we analyzed the susceptibility to *P. cucumerina* of the defective *cyp79b2* and *cyp79b3* mutants and found that they were slightly, although significantly, more susceptible to the fungus than wild-type plants (Figure 7). The susceptibility of *cyp79b2* and *cyp79b3* was lower than that of the highly susceptible *agb1-1* mutant, which is altered in the β -subunit of the heterotrimeric G protein (Figure 7) (Llorente et al., 2005). Based on these results, the simplest hypothesis to explain *irx*-mediated resistance is that the secondary metabolites accumulated in the *irx* mutants may create a hostile and antimicrobial-enriched environment for the pathogen, hindering its multiplication.

DISCUSSION

The role of the cell wall in resistance and/or disease development has been highlighted by several studies (Schulze-Lefert, 2004; Somerville et al., 2004). Apart from the previously mentioned *ixr1/cev1*, *pmr5*, and *pmr6* mutants, which do not support the growth of two powdery mildew species (Ellis et al., 2002; Vogel et al., 2002, 2004), inactivation of a cell surface arabinogalactan protein, RAT1, and of a putative glycosyltransferase, RAT4, confers enhanced resistance to *Agrobacterium tumefaciens* infection (Zhu et al., 2003). In this study, we demonstrated that the *irx* mutants, impaired in the CESA proteins required for the synthesis

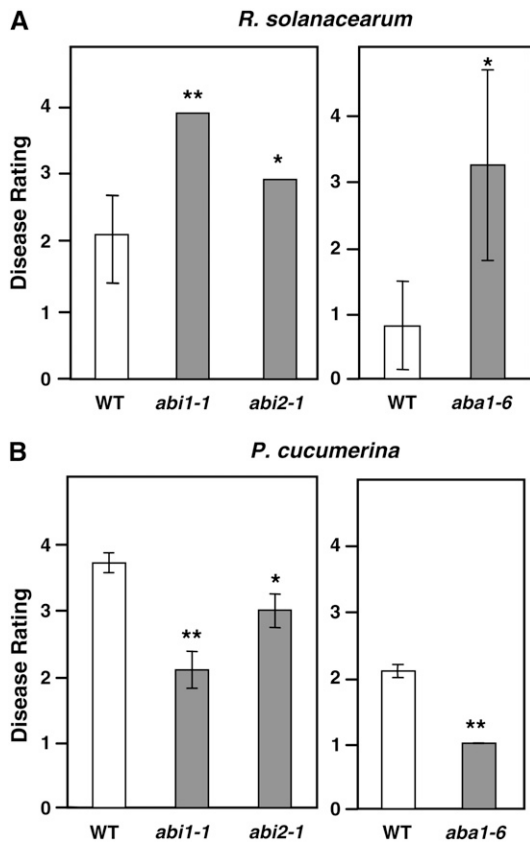


Figure 6. ABA Signaling Affects *Arabidopsis* Resistance to *R. solanacearum* and *P. cucumerina*.

The resistance of *abi1-1*, *abi2-1*, and *aba1-6* mutants to *R. solanacearum* (**A**) and *P. cucumerina* (**B**) was evaluated. Average disease ratings (\pm SD) of wild-type plants (Ler and Col-0), *abi1-1* and *abi2-1* mutants (Ler background), and the *aba1-6* mutant (Col-0 background) after inoculation with *R. solanacearum* GMI1000 (5 days after inoculation) or *P. cucumerina* (12 days after inoculation) are shown. The disease rating varies between 0 (no symptoms) and 4 (dead plants). At least 10 plants per genotype were tested. Asterisks indicate values significantly different (** $P < 0.01$, * $P < 0.05$, *t* test) from those of wild-type plants.

of secondary cell wall cellulose, show an enhanced resistance to fungal (*P. cucumerina*, *B. cinerea*, and powdery mildew) and bacterial (*R. solanacearum*) pathogens. The reduced susceptibility of *irx/cesa* mutants to these pathogens was SA-, ET-, and JA-independent, like that of *pmr5* and *pmr6* mutants (Vogel et al., 2002, 2004). By contrast, the enhanced resistance to powdery mildew of the primary cell wall mutant *cev1/ixr1*, which is impaired in the CESA3 protein, depended on a concomitant activation of the JA and ET pathways (Ellis et al., 2002). Interestingly, the susceptibility to *P. cucumerina* and *R. solanacearum* of CESA primary cell wall mutants, including *cev1/ixr1*, and of *pmr5* and *pmr6* mutants was similar to that of wild-type plants (Figures 1 and 3). These results illustrate the high level of specificity and complexity of the resistance associated with the impairment of primary and secondary cell wall integrity caused by the *irx*, *ixr*, and *pmr* mutations.

Most of the cell wall mutants present phenotypic alterations, which reveal the importance of the corresponding genes for plant development (Somerville et al., 2004). Developmental alterations have also been described for the majority of *Arabidopsis* mutants exhibiting a constitutive activation of disease resistance (Glazebrook, 2001). In the *pmr6* and *pmr5* mutants, a pectin component appears to be altered, and both mutants are reduced in stature compared with wild-type plants (Vogel et al., 2002, 2004). Similarly, mutations in the *CESA/IRX* genes cause significant modifications of the structure and noncellulosic carbohydrate composition of the cell wall, which lead to widespread morphological alterations, including the collapse of xylem vessels, that might affect pathogen attachment and spread within the plants (Taylor et al., 2003; Brown et al., 2005). This possibility is rather unlikely, because (1) microscopic examination showed that pathogen attachment was not impaired in *irx* mutants (data not shown); (2) bacteria inoculated by dipping wounded roots in a microbial suspension could be detected within minutes in the aerial parts of *irx* mutants (Figure 1D); (3) the induction of bacterial *hrp* genes, which is required for disease development (Aldon et al., 2000), was unaffected in the *irx* mutants (data not shown); and (4) the resistance to *P. cucumerina* of the *gpx1* mutant, which

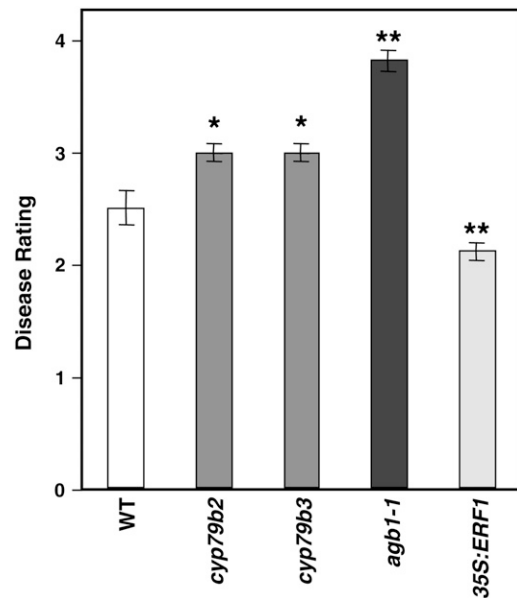


Figure 7. Secondary Metabolites Are Required for *Arabidopsis* Resistance to the Necrotrophic Fungus *P. cucumerina*.

Average disease ratings (\pm SD) of wild-type plants and of the *cyp79b2* and *cyp79b3* mutants, which are impaired in secondary metabolite biosynthesis, at 10 d after inoculation with *P. cucumerina* are shown. The disease rating varies between 0 (no symptoms) and 4 (dead plants). At least 10 plants per genotype were tested. The *agb1-1* mutant and the transgenic *35S:ERF1*, which show enhanced susceptibility and resistance to *P. cucumerina*, respectively, were included for comparison (Berrocal-Lobo et al., 2002a; Llorente et al., 2005). Asterisks indicate values significantly different (** $P < 0.01$, * $P < 0.05$, *t* test) from those of wild-type plants.

presents severe alterations in xylem vessels (Turner and Hall, 2000), did not differ from that of wild-type plants (data not shown).

Disruption of the *CESA8/IRX1* gene in the *irx1-6/ern1/lew2-1* allele leads to increased endogenous ABA levels in planta and to enhanced tolerance to drought and osmotic stresses (Chen et al., 2005) (Figure 8). Consistent with these data, transcriptomic analyses revealed that a majority (58%) of the constitutively upregulated genes in the *irx* mutants were ABA-responsive (Figure 5). Among the proteins encoded by these ABA-regulated genes, we identified enzymes and regulators of the ABA biosynthetic and signaling pathways (e.g., NECD3, ABI1, ABI2, and ABI4) (Shinozaki et al., 2003) as well as antimicrobial, defense-related peptides (e.g., LTPs) (Molina et al., 1993b) and transcriptional regulators of secondary metabolite biosynthesis (e.g., ATR1) (Grubb and Abel, 2006). ABA plays a significant role in the control of plant development and the adaptive response to osmotic stresses (Shinozaki et al., 2003). Also, ABA modulates disease resistance by interfering with biotic stress signaling. Thus, ABA can act as a negative regulator of the ET/JA-dependent defense response and as a positive modulator of JA-dependent defensive genes (Anderson et al., 2004; Lorenzo

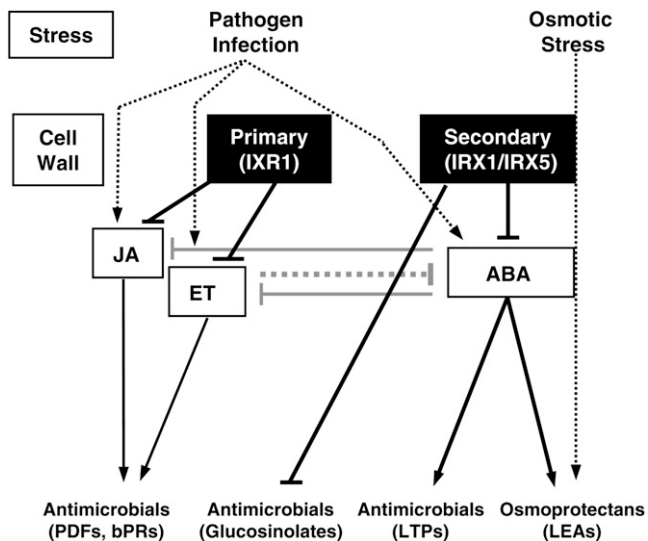


Figure 8. Scheme of the Interconnections between Alteration of Cell Wall Integrity and Hormone Signaling Pathways in the Regulation of *Arabidopsis* Resistance to Pathogens and Osmotic Stress.

Pathogen infection and abiotic stresses lead to the activation of signaling pathways (ET, JA, or ABA) that regulate the expression of different groups of antimicrobials and osmoprotectant compounds. Antagonistic interactions between the ET/JA and ABA signaling pathways have been described (Anderson et al., 2004; Lorenzo et al., 2004). Alteration of primary cell walls caused by mutations (*ixr1*) in the IXR1 protein leads to the constitutive activation of the ET/JA pathway, which controls the expression of antimicrobial proteins, such as defensins (PDFs) and basic PR proteins (bPRs) (Ellis et al., 2002). Modifications of the secondary cell walls caused by the impairment of IRX1 and IRX5 proteins, as occurs in the *irx/cesa* mutants, triggers ABA signaling and may lead to the accumulation of antimicrobial peptides (e.g., LTPs), secondary metabolites (e.g., glucosinolates), and osmoprotectants (e.g., late embryogenesis-associated proteins [LEAs]). Arrows indicate activation, and broken arrows indicate negative regulation.

et al., 2004). In agreement with these data, some ET-associated genes were constitutively downregulated in the *irx* mutants (see Supplemental Table 2 online), and upon infection, the induction of ET/JA-regulated genes, such as *PDF1.2*, was weaker in the *irx* mutants than in wild-type plants (Figures 1B and 4C).

The involvement of ABA in primed callose production at the site of pathogen penetration is one of the few examples of a positive function of this hormone in defense and may be the basis for the described β -aminobutyric acid-induced resistance (Ton and Mauch-Mani, 2004; Mauch-Mani and Mauch, 2005; Ton et al., 2005). However, callose deposition at the site of *P. cucumerina* infection was similar in *irx1-6* mutant and wild-type plants, which excludes priming for callose production as a mediator of *irx*-reduced susceptibility (data not shown). We now provide strong evidence for a direct involvement of ABA signaling in the control of *Arabidopsis* resistance to *R. solanacearum*. This ABA function was supported by different observations: (1) the ABA-insensitive mutants *abi1-1* and *abi2-1*, and the ABA-deficient mutant *aba1-6*, were more susceptible to the bacterium than were wild-type plants (Figure 6); (2) the constitutive expression in the *irx* mutants of some ABA signaling regulators, including *ABI1-1* and *ABI2-1*, was associated with an increased resistance to the bacterium (Figure 1, Table 2); and (3) the *ein2-1* mutant, a phenotypic suppressor of *abi1-1*, was shown to be more tolerant to virulent strains of the bacterium (Baudoin et al., 2000; Hirsch et al., 2002) (Figure 4A).

ABA was recently implicated as a regulator of defense responses to necrotrophic pathogens. Thus, ABA-deficient mutants exhibited reduced susceptibility to the necrotroph *B. cinerea* and the vascular pathogen *F. oxysporum* (Audenaert et al., 2002; Anderson et al., 2004) and ABA-sensitive mutants showed increased susceptibility to *B. cinerea* (AbuQamar et al., 2006). In agreement with these results, we previously demonstrated that the ABA-hypersensitive mutant *agb1-1* (Pandey et al., 2006) was more susceptible than wild-type plants to these fungi and *P. cucumerina* (Llorente et al., 2005). We now demonstrate that the constitutive activation of ABA signaling in the *irx* mutants, or the impairment of the ABA pathway, as it occurs in the *abi* and *aba* mutants, led to an enhanced resistance to *P. cucumerina* via the activation of separate pathways, which seem to regulate distinct sets of defense genes (Figure 8). In the *irx* mutants, ABA-regulated genes encoding antimicrobial peptides (e.g., LTPs and THs) (Molina et al., 1993b) or regulators of secondary metabolite biosynthesis (e.g., ATR1) (Grubb and Abel, 2006) were constitutively expressed. The accumulation of these antimicrobial peptides/metabolites in these mutants may explain their enhanced resistance to pathogens (Figure 8). Additionally, we have observed that in the *abi* and *aba* mutants some ET/JA-regulated, defense-related genes (e.g., *PDF1.2*) were overexpressed (data not shown), in agreement with the proposed function of ABA as a negative modulator of the ET/JA-dependent defense response (Anderson et al., 2004; Lorenzo et al., 2004). This observation may explain the reduced susceptibility of these *abi* and *aba* mutants to *P. cucumerina* (Figure 8). Indeed, we have demonstrated previously that the constitutive activation of the ET and JA pathways by overexpression of the *ERF1* transcriptional factor was sufficient to confer enhanced resistance to necrotrophs (Figures 1A and 7) (Berrocal-Lobo et al., 2002a).

The previous observations implicating ABA as a regulator of defense responses, those reported here, and the recent demonstration that stomata have an important role in defense against the bacterium *P. syringae* (Melotto et al., 2006) suggest that the ABA pathway, in addition to mediating the adaptive response to osmotic stress, may play a significant role in the fine-tuned regulation of the plant immune system. Examples of positive and negative crosstalk between biotic and abiotic stresses are emerging (Park et al., 2001; Mengiste et al., 2003). Interestingly, the enhanced resistance to biotic and abiotic stresses mediated by *irx/cesa* mutations was JA- and ET-independent.

Based on the transcriptomic analysis, the simplest hypothesis to explain *irx*-mediated resistance is that the antimicrobial compounds that might accumulate in these mutants (e.g., peptides, PR proteins, and secondary metabolites) create a hostile environment for pathogens, whose progression within the plant is hindered. Secondary metabolites have been suggested to constitute one of the determinants of nonhost and basal resistance to several pathogens, including *P. cucumerina* (Lipka et al., 2005; Stein et al., 2006), and to affect the interaction between *Arabidopsis* and the necrotrophic fungus *B. cinerea* (Kliebenstein et al., 2005). Also, the defensive functions of some plant antimicrobial peptides encoded by genes upregulated in the *irx* mutants are well established (Table 2) (García-Olmedo et al., 1998). Indeed, THs and LTPs, including those encoded by the *irx*-upregulated *LTP3* and *LTP4* genes (Table 2), possess in vitro antibiotic activity, and their constitutive overexpression in *Arabidopsis* increases plant tolerance to various pathogens (Molina et al., 1993a, 1993b; Segura et al., 1993; Epple et al., 1997; Molina and García-Olmedo, 1997; Titarenko et al., 1997). Our hypothesis is supported by the demonstration of an enhanced susceptibility to *P. cucumerina* of the *cyp79b2* and *cyp79b3* mutants, which are impaired in the accumulation of antimicrobial secondary metabolites (Figure 8). Moreover, we purified and tested the in vitro antibiotic activity of *Arabidopsis* LTPs encoded by genes upregulated in the *irx* mutants and found that they were active against *P. cucumerina* and *R. solanacearum* GMI1000 (data not shown). The activity of these LTPs was, as described previously for other pathogens, essentially equivalent to that of other plant LTPs (e.g., EC₅₀ values for *P. cucumerina* of $3.2 \pm 0.7 \mu\text{M}$ for barley [*Hordeum vulgare*] LTPs [LTP3 + LTP4] and $6.2 \pm 0.9 \mu\text{M}$ for *Arabidopsis* LTPs [Molina et al., 1993b; Segura et al., 1993]).

In yeast, there is a mechanism controlling cell wall integrity that is regulated by the WSC genes (for cell wall integrity and stress response), which encode integral membrane proteins acting as surface sensors responding to environmental stress (Philip and Levin, 2001; Merchan et al., 2004). Our data suggest that a similar sensing system to that of yeast may exist in plants, because modifications of the integrity of the primary or secondary cell wall activate different signaling pathways, at least partially ABA-dependent in the *irx* mutants, ET/JA-dependent in the *ixr* mutants, and still uncharacterized in the *pmr5* and *pmr6* mutants (Figure 8). Although the precise functions of most genes exhibiting altered expression in the *irx* mutants remain to be determined, some may encode putative sensors/receptors of cell wall integrity (see Supplemental Table 1 online). The characterization of plant sensors involved in the detection of pathogen-induced perturbations of cell wall integrity, and the identification of their

corresponding ligands and downstream effectors, are future challenges in the plant resistance field.

METHODS

Plant Lines and Growth Conditions

Arabidopsis thaliana accessions used in this study were Col-0, Landsberg *erecta* (Ler), Col-5 (a glabrous [*gl*] derivative of Col-0), Nd-1, and Kas-1. The primary and secondary cell wall mutants *ixr1-1*, *ixr2-1*, *rsw2-1*, *irx1-1*, *irx3-1*, and *irx5-4* analyzed here have been described previously (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Ellis et al., 2002; Taylor et al., 2003; Brown et al., 2005; Persson et al., 2005). The ABA mutants *aba1-6* (Col-0 background), *abi1-1*, *abi2-1*, and *aba3-2* (Ler background) analyzed in this study have been characterized previously (Niyogi et al., 1998; Gosti et al., 1999; Xiong et al., 2001). Plants were grown in growth chambers at 20 to 22°C with a 10-h photoperiod and a light intensity of $\sim 150 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, as described previously (Berrocal-Lobo et al., 2002a; Deslandes et al., 2003). For infection experiments with *Erysiphe cichoracearum* (*Golovinomyces cichoracearum*), plants were grown in growth chambers at 22°C with a 14-h photoperiod of $\sim 125 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in the 400- to 700-nm range.

Ralstonia solanacearum GMI1000 is a wild-type strain isolated from tomato (*Solanum lycopersicum*). *R. solanacearum* was grown at 28°C in B broth medium (Boucher et al., 1985). The ascomycete fungus *Plectosphaerella cucumerina* and the necrotroph *Botrytis cinerea* were grown at 28°C on potato (*Solanum tuberosum*) dextrose agar and their spores harvested as reported (Berrocal-Lobo et al., 2002a; Llorente et al., 2005). *Pseudomonas syringae* pv *tomato* DC3000 and *P. syringae* pv *tomato* DC3000 (*AvrRPM1*) were grown on nutrient broth at 28°C as described previously (Berrocal-Lobo et al., 2002a). Powdery mildew (*E. cichoracearum* UCSC1) was cultured and applied to *Arabidopsis* as described (Vogel and Somerville, 2000). *Hyaloperonospora parasitica* (*Peronospora parasitica*) isolates Noco-2 and Emwa1 were maintained on the genetically susceptible *Arabidopsis* accessions Col-0 and Ler, respectively, as described (Dangl et al., 1992). *Xanthomonas campestris* pv *campestris* bacteria were grown as described previously (Lummerzheim et al., 2004).

Pathogenicity Assays

The pathogens used in this study were *P. cucumerina*, *P. syringae* pv *tomato* DC3000 (*AvrRPM1*), and *P. syringae* pv *tomato* DC3000 (Berrocal-Lobo et al., 2002a), *B. cinerea* (Llorente et al., 2005), *R. solanacearum* GMI1000 (Deslandes et al., 1998), the isolates Noco2 and Emwa1 of *H. parasitica* (Dangl et al., 1992), *E. cichoracearum* UCSC1 (Vogel and Somerville, 2000), and the strains 8004 and 147 of *X. campestris* pv *campestris* (Lummerzheim et al., 2004).

Plant infection with *R. solanacearum* was done as reported by inoculating roots of 4-week-old plants with the bacterial suspension (Deslandes et al., 2003). Bacterial in planta growth curves were measured as described (Deslandes et al., 1998). Infection with *P. cucumerina* was performed by spraying 4-week-old plants with a spore suspension (2×10^6 spores/mL) of the fungus. After inoculation, plants were kept under the same growth conditions and the average disease rating (\pm SD) was measured at different times (days after inoculation) as reported (Llorente et al., 2005). Disease rating was as follows: 0, no symptoms, 1, 1 to 3 leaves showing some necrosis; 2, 4 to 8 leaves with some necrosis; 3, 8 to 12 leaves showing necrosis; 4, all leaves showing profuse necrosis or decayed/dead plant. At least 10 plants per genotype were inoculated in each of two to three experiments, and the disease rating means and SD were estimated at different times.

Plant inoculation with *B. cinerea* and disease resistance analysis were done as described (Llorente et al., 2005). *Arabidopsis* plants were dip-inoculated with *P. syringae* pv *tomato* DC3000 (*AvRPM1*) or *P. syringae* pv *tomato* DC3000, and in planta bacterial growth was determined at different times (Berrocal-Lobo et al., 2002a). Disease resistance analysis of *H. parasitica* was performed by spraying 2-week-old plants with a conidiophore suspension (4×10^4 spores/mL) of the isolates Noco2 or Emwa1, and the degree of infection was quantified as described (Llorente et al., 2005). For *E. cichoracearum* infections, 3-week-old *Arabidopsis* plants were inoculated as described above, phenotypes were monitored visually at 4, 5, 6, and 7 d after inoculation, and the number of conidiophores per colony was determined at the end of the experiment (Vogel and Somerville, 2000). For *X. campestris* infection, plants were kept at high humidity for 12 h before inoculation, and then the abaxial side of the leaves was injected with a bacterial suspension of 2×10^7 to 10^8 colony-forming units/mL using a blunt syringe (Lummerzheim et al., 2004). At least 10 plants per genotype were inoculated in each of two to three experiments performed.

Statistical analysis of the pathogenicity experiments was performed using a two-tailed Student's *t* test.

In Vitro Inhibition Tests

Arabidopsis LTPs and barley (*Hordeum vulgare*) LTPs (a mixture of LTP3 and LTP4) used for the in vitro inhibition assays were purified as described previously (Molina et al., 1993a; Segura et al., 1993). Pathogen inhibition was done on microtiter plates as reported (Molina et al., 1993b) by mixing 33 μ L of the corresponding peptide solution with 66 μ L of medium containing the fungal spores or bacteria (10^4 colony-forming units or spores/mL) to give the desired peptide concentrations. The experiment was repeated at least two times.

Microscopic Analysis

Four-week-old *irx1-6* and wild-type plants were mock-inoculated or challenged, as indicated above, with a spore suspension of *P. cucumerina*. Callose staining at different times (0, 1, 3, and 4 d after inoculation) was performed as reported previously (Llorente et al., 2005). At least 10 plants per genotype were analyzed, and the experiment was repeated three times.

Identification and Cloning of the *nws2/irx5-5* Gene

Thirty-five thousand T-DNA-tagged Col-5 plants were inoculated with the virulent GM11000 bacterium, and plants that did not develop wilt symptoms were selected and allowed to set seed. Cosegregation analysis in the F2 population resulting from the *nws2* backcross indicated that the resistant phenotype was tightly linked to the T-DNA insertion. The cloning of the *nws2/irx5-5* gene was performed by inverse PCR as described (http://www.darmouth.edu/tjack/cloning_flanking_DNA_from_.html). Primers used to amplify the right border were as follows: 5'-TCGGGCCTAACTTTTGGTG-3' (adjacent right border) and 5'-AGTGCCAAGCTTC-CATGC-3' (*Hind*III site in the polylinker). Genomic DNA (200 ng) was digested with various enzymes (*Xba*I, *Sal*I, *Bam*HI, and *Pst*I). For each digestion, 20 ng was used in a ligation reaction. PCR was performed using the following conditions: 94°C for 20 s, 54°C for 30 s, and 68°C for 10 min (35 cycles). An 8-kb fragment was obtained using, as a template, the self-ligated *Xba*I fragments.

Isolation and Map-Based Cloning of the *ern1/irx1-6* Mutant

Approximately 80,000 10-d-old plants of an ethyl methanesulfonate M2 mutagenized population of *Arabidopsis* Col-0 were sprayed with 2×10^6 spores/mL *P. cucumerina* fungus for the identification of *ern* mutants,

which showed reduced disease symptoms compared with Col-0 wild-type plants. This screen yielded the recessive *ern1-1* mutant. The *ERN1* gene was mapped using an *ern1-1* (Col-0) \times *Ler* F2 population of 430 individuals using standard PCR-based marker techniques (Lukowitz et al., 2000). New markers within the fine-mapping interval on chromosome IV were generated using the CERION database of Col and *Ler* polymorphisms (<http://www.arabidopsis.org/browse/Cereon/index.jsp>). The derived oligonucleotide sequences used for mapping were as follows: F15J5A, 5'-TCTAAGCGGGTCGGGTCGATTC-3' and 5'-AATCAGACCGGACCGGACCGG-3'; F28J12, 5'-CAAATCCATACATCTCTCCCTCTC-3' and 5'-GATGGAGTAGATAGAGAAAGGGAG-3'; F28A21A, 5'-AGGCATCTTTGCTAAGGGC-3' and 5'-AATTCACGTGGGACTTGGG-3'; F28A21B, 5'-AAAAGTTACGAGTCAGTTACATC-3' and 5'-GTTGGTGATGAATGATG-ATG-3'; F28A21C, 5'-CTTTGAGGTTAGCAAAAACCTCAC-3' and 5'-CAT-TGAACCTTTTTACATGGCAG-3'; and T16H5, 5'-ATTCTAGCTTTTGA-GAGGTCAG-3' and 5'-CCCAATCCAAATCAAATTC-3'.

Generation and Selection of Double Mutants

The mutant alleles used for the construction of double mutants with *irx1-6* were *coi1-1* (Feys et al., 1994), *npr1-1* (Cao et al., 1994), *ein2-5* (Roman et al., 1995), *NahG* (Lawton et al., 1995), *jar1-1* (Staswick et al., 1992), *sid2-1* (Wildermuth et al., 2001), *abi1-1* (Gosti et al., 1999), and *aba3-2* (Xiong et al., 2001). All of the mutant lines were in the Col-0 background except *coi1-1*, which was in the Col-6 background, and *abi1-1* and *aba3-2*, which were in the *Ler* background. We generated the double mutants by standard genetic crosses, following the mutations in the mutant alleles (*npr1-1*, *sid2-1*, *coi1-1*, *jar1-1*, *aba3-2*, and *abi1-1*) or the transgene (*NahG*) by PCR-based methods, or by selection on Murashige and Skoog plates containing 10 μ M 1-aminocyclopropane-1-carboxylic acid, the ET precursor (*ein2-5* [Roman et al., 1995]), 50 μ M JA (*coi1-1* and *jar1-1* [Feys et al., 1994]), or 1 μ M ABA (*abi1-1* [Gosti et al., 1999]). The *NahG*, *coi1-1*, *sid2-1*, *npr1-1*, and *jar1-1* oligonucleotides used for PCR-based detection have been described (Stein et al., 2006). Genotyping of the *irx1-6* mutation in all of the double mutants was confirmed by PCR amplification and sequencing of the mutation (oligonucleotides 5'-GGTTAATAGGAG-ACCATTAG-3' and 5'-TCCATCCAAATCTCAATCCC-3'). As described previously (Brown et al., 2005; Chen et al., 2005), the homozygous *irx1-6* and *irx5-5* plants were sterile; therefore, for the disease resistance and expression analysis, heterozygous populations were sown on soil and the corresponding *irx1-6* and *irx5-5* single or double mutants that showed a clear distinguishable phenotype were selected for the analyses. For the study of *irx1-6 coi1-1* plants, seeds of heterozygous *irx1-6 coi1-1* and *coi1-1* plants were sown on plates containing 50 μ M JA for *coi1-1* selection (Feys et al., 1994). Plants were then transferred to soil for the analysis, and the *irx1-6 coi1-1* double mutants were selected according to their phenotype. For the selection of *irx3-1 aba3-2* plants, a cleaved amplified polymorphism was used to identify the *irx3-1* mutation (5'-CGG-GAATCTTGGAAATTGAGATT-3' and 5'-GATCAGCAGTGTTGCCATTG-3' and *Mse*I digestion), and the *aba3-2* mutation was confirmed by DNA sequencing (5'-CTCAAGTCGCTTACACCTTCTG-3' and 5'-GGGATAA-CTCGAGATACTTTG-3'). Genotyping of the *irx1-1 abi1-1* double mutant was confirmed by PCR amplification of *abi1-1* (PCR amplification with oligonucleotides 5'-GCCTTTGTATGGTTTTACTTC-3' and 5'-CAGCCACG-TATCACCATCG-3' followed by *Nco*I digestion) and sequencing of *irx1-1* (oligonucleotides 5'-GGTTAATAGGAGACCATTAG-3' and 5'-TCC-ATCCAAATCTCAATCCC-3').

Expression Analysis

Total RNA isolation from plants challenged or not with *R. solanacearum* and RNA gel blotting were performed according to the protocol of Hirsch et al. (2002). RNA extraction of *P. cucumerina*-infected or mock-inoculated

plants and RNA gel blot analysis of these RNAs were done as described (Llorente et al., 2005). The hybridization probes (*PDF1.2*, *PR1*, *Pc ITS1*, and β -*TUB*) used in this study have been described (Llorente et al. 2005).

For quantitative RT-PCR analyses, 5 μ g of total RNA was treated with DNase and then reverse-transcribed for 90 min at 42°C in a 20- μ L reaction volume using the SuperScript II First-Strand Synthesis System kit (Invitrogen) and oligo(dT). Oligonucleotides used for cDNA amplification were designed with Primer Express (version 2.0; Applied Biosystems): At1g18710, 5'-GAGCTGCAGATTAAGGTGGCTT-3' and 5'-CCGAGAACAGCATGAAGTTGG-3'; At2g39800, 5'-ATGCAGTCTGTGTGCACTCC-3' and 5'-ACCATGAGTACTGTCCAAGGC-3'; At2g46680, 5'-CGATGATGTCAGCAGAGCCAT-3' and 5'-CAGTGACTTGATCTGCTCGTCG-3'; At3g1440, 5'-ACATCTTTACGCGGATAACCG-3' and 5'-TTCCATGTC-TTCTCGTCGTA-3'; At4g26080, 5'-TGTTTTCCCGTCTCACATCTTC-3' and 5'-CCGTTTATGGTCAACGGAT-3'; At4g39950, 5'-GCCGACCCACTTTGCTTTAAA-3' and 5'-GCACAACCTCTTTCCCGGTA-3'; At5g57050, 5'-TCAGTGCGGCGAGTAAAAGAA-3' and 5'-TCCGTTCCGAGCCAATC-3'; and At3g18780, 5'-GACCAGCTCTTCCATCGAGAA-3' and 5'-GCAGCTTCCATTCCACAA-3'.

Real-time quantitative PCR amplification and detection was performed in the 7300 real-time PCR system (Applied Biosystems). Reactions were done in a final volume of 20 μ L and contained 10 μ L of 2 \times SYBR Green Master Mix (Applied Biosystems), 0.5 μ M of each primer, and 8 ng of cDNA. PCR conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. At the end of each experiment, a dissociation stage (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) was performed to ensure that only single products were formed. *Actin2* (*At3g18780*) expression was used to normalize the transcript level in each sample. Data analysis was performed using the sequence detector software (version 1.2.2; Applied Biosystems).

Microarray Experiments and Analysis

Leaves from uninfected 4-week-old homozygous *irx5-5* and *irx1-6* plants and their corresponding heterozygous counterparts, which were fully susceptible to both *R. solanacearum* and *P. cucumerina* and showed a wild-type phenotype, were collected for microarray analysis. Three biological replicates from *irx5-5* and wild-type plants and two replicates from *irx1-6* and wild-type plants were harvested for microarray analysis. Total RNA was extracted using the NucleoSpin RNAII kit (Macherey-Nagel). Probes were synthesized from the RNA samples and hybridized to the Affymetrix GeneChip (ATH1, 22 K) according to the procedures provided by the manufacturer. The array images were analyzed with the Affymetrix Microarray Suite 5.0 with the target intensity set to 500. The expression levels of the genes were analyzed with GeneSpring 7.2 software (Silicon Genetics), and the chip-to-chip signal variation was minimized by normalizing signal intensities to the averaged intensity values of wild-type plants using the expression levels of the top 50th percentile of probe sets. Differentially expressed genes in both the *irx5-5* (three replicates) and *irx1-6* (two replicates) mutants relative to wild-type samples (five replicates) were identified using one-way analysis of variance and a Benjamini and Hochberg multiple testing correction (GeneSpring 7.2), as described previously (Stein et al., 2006). Genes were considered differentially expressed at $P \leq 0.01$. Upregulated and downregulated genes were selected using normalized values (fold) > 2 or < 0.5 relative to wild-type plants, respectively.

Accession Numbers

The data obtained by the microarray hybridizations have been submitted to NASCArrays (reference number /NASCARRAYS-368/). Arabidopsis Genome Initiative locus identifiers for the genes impaired in the mutants

characterized in this article are as follows: CESA8/IRX1/ERN1, At4g18780; CESA4/IRX5/NWS2, At5g44030.

Supplemental Data

The following materials are available in the online version of this article

Supplemental Figure 1. Cloning of the *ERN1/IRX1* and *NWS2/IRX5* Genes.

Supplemental Figure 2. Quantitative RT-PCR Analysis of Genes Differentially Upregulated or Downregulated in Secondary Cell Wall *irx* Mutants.

Supplemental Figure 3. Developmental Phenotype of the *irx3-1 aba3-2* Double Mutant.

Supplemental Table 1. Genes Constitutively Upregulated in the *irx5-5* and *irx1-6* Mutants.

Supplemental Table 2. Genes Constitutively Downregulated in the *irx5-5* and *irx1-6* Mutants.

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