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Repression of the Auxin Response Pathway Increases *Arabidopsis* Susceptibility to Necrotrophic Fungi

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ABSTRACT In plants, resistance to necrotrophic pathogens depends on the interplay between different hormone systems, such as those regulated by salicylic acid (SA), jasmonic acid (JA), ethylene, and abscisic acid. Repression of auxin signaling by the SA pathway was recently shown to contribute to antibacterial resistance. Here, we demonstrate that *Arabidopsis* auxin signaling mutants *axr1*, *axr2*, and *axr6* that have defects in the auxin-stimulated SCF (Skp1–Cullin–F-box) ubiquitination pathway exhibit increased susceptibility to the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea*. Also, stabilization of the auxin transcriptional repressor AXR3 that is normally targeted for removal by the SCF-ubiquitin/proteasome machinery occurs upon *P. cucumerina* infection. Pharmacological inhibition of auxin transport or proteasome function each compromise necrotroph resistance of wild-type plants to a similar extent as in non-treated auxin response mutants. These results suggest that auxin signaling is important for resistance to the necrotrophic fungi *P. cucumerina* and *B. cinerea*. *SGT1b* (one of two *Arabidopsis SGT1* genes encoding HSP90/HSC70 co-chaperones) promotes the functions of SCF E3-ubiquitin ligase complexes in auxin and JA responses and resistance conditioned by certain Resistance (*R*) genes to biotrophic pathogens. We find that *sgt1b* mutants are as resistant to *P. cucumerina* as wild-type plants. Conversely, auxin/SCF signaling mutants are uncompromised in *RPP4*-triggered resistance to the obligate biotrophic oomycete, *Hyaloperonospora parasitica*. Thus, the predominant action of *SGT1b* in *R* gene-conditioned resistance to oomycetes appears to be at a site other than assisting SCF E3-ubiquitin ligases. However, genetic additivity of *sgt1b axr1* double mutants in susceptibility to *H. parasitica* suggests that SCF-mediated ubiquitination contributes to limiting biotrophic pathogen colonization once plant–pathogen compatibility is established.

Key words: plant defense; innate immunity; necrotrophic fungi; auxin signaling; proteasome.

INTRODUCTION

Plants protect themselves from pathogen infection through a combination of constitutive and induced defenses (Holt et al., 2003; Chisholm et al., 2006). The effectiveness of induced resistance relies in large part on perception of pathogen-derived molecules by plant receptors (Chisholm et al., 2006; Nürnberger and Kemmerling, 2006). Plant–pathogen recognition triggers the biosynthesis of phytohormones such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) (Glazebrook, 2005; Ferry et al., 2004). The balance and interplay of these hormone systems has a pivotal role in the expression of resistance to particular pathogens and pests (Glazebrook, 2005; Ferry et al., 2004). For example, genetic evidence in *Arabidopsis* shows that extensive cross-talk between ET, JA, and SA signaling pathways determines resistance to different types of pathogens. Most apparent are cooperative and antagonistic interactions between SA and JA/ET signaling that affect local and systemic resistance responses (Berrocal-Lobo et al., 2002; Thomma et al., 1998, 1999; Laurie-Berry et al., 2006; Truman et al., 2007). Also, many plant pathogens are themselves able to produce phytohormones during infection that can interfere with host developmental processes and defense responses (Robert-Seilaniantz et al., 2007).

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Plant resistance to necrotrophic fungi such as *Plectosphaerella cucumerina* and *Botrytis cinerea* is genetically complex (Llorente et al., 2005) in contrast to monogenic gene-for-gene resistance that often conditions resistance to biotrophic fungi, oomycetes (e.g. *Hyaloperonospora parasitica*) or bacteria (e.g. *Pseudomonas syringae*; Holt et al., 2003). Multiple hormone pathways were found to contribute to *Arabidopsis* resistance to necrotrophic fungi, since the *ein2-5*, *coi1-1*, *sid2-1* mutants impaired, respectively, in ET, JA, and SA signaling, and *NahG* transgenic lines that are blocked in SA accumulation, were more susceptible than wild-type (WT) plants to *P. cucumerina* and *B. cinerea* (Thomma et al., 1998, 1999; Berrocal-Lobo et al., 2002; Ferrari et al., 2003). In contrast to the known contributions of SA, ET, and JA to plant disease resistance, the roles of other hormones such as abscisic acid (ABA), auxin and brassinosteroids in plant defense are less well defined (Robert-Seilaniantz et al., 2007). ABA appears to have multiple sites of action in plant–pathogen interactions (Mauch-Mani and Mauch, 2005). An ABA-mediated pathway promoted susceptibility in *Arabidopsis* infection with virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000; de Torres et al., 2007), but increased resistance to the soil-borne pathogens *Ralstonia solanacearum* and *Pythium irregulare* (Adie et al., 2007; Hernández-Blanco et al., 2007). ABA was also found to negatively regulate plant resistance to certain necrotrophic fungi, since ABA-deficient (e.g. *aba2*) and ABA-signaling mutants (e.g. *abi1*) were more resistant to these pathogens than WT plants (Audenaert et al., 2002; AbuQamar et al., 2006; Hernández-Blanco et al., 2007). Brassinosteroids can also affect the induction of plant defences. For example, treatment of rice or tobacco with brassinolide triggered enhanced resistance to different biotrophic fungi (Nakashita et al., 2003).

A negative effect of auxin signaling on plant resistance to biotrophic pathogens was recently described (Navarro et al., 2006; Wang et al., 2007). Auxin regulates many processes during plant development through direct interaction with TIR1-like F-box receptor proteins (Quint and Gray, 2006). Auxin binding to SCF^{TIR1} leads to enhanced removal of members of the AUX/IAA family of transcriptional factor (TF) repressors by the SCF (Skp1–Cullin–F-box) E3-ubiquitin ligase proteasome pathway (Gray et al., 2001; Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). The degradation of AUX/IAA proteins allows activation of Auxin Response Factors (ARFs) and the expression of auxin-responsive genes (Hagen and Guilfoyle, 2002). There is an increasing body of evidence that some plant pathogens either produce auxin themselves or increase plant auxin biosynthesis upon infection to manipulate host developmental processes (Manulis et al., 1998; Glickmann et al., 1998; Maor et al., 2004; Vandeputte et al., 2005). Repression of auxin-responsive genes also occurs upon plant treatments with the bacterial elicitor flg22 or the SA functional analog benzothiadiazole (BTH) (Navarro et al., 2006; Wang et al., 2007). Flg22 triggered the up-regulation of a canonical microRNA (miR393) that targets auxin receptors, thereby contributing to the down-regulation of auxin signaling (Navarro et al., 2006). Increasing the auxin response through overexpression of the TIR1 auxin receptor rendered plants more susceptible to *Pst*DC3000 and, conversely, attenuation of auxin signaling through miR393 overexpression increased resistance to bacteria (Navarro et al., 2006). These results show that repression of auxin signaling is part of a bacterial-induced plant immune response. Notably, SA treatment caused a stabilization of AUX/IAA repressor proteins and inhibition of the auxin response, suggesting that SA contributes to a general repression of the auxin pathway (Wang et al., 2007). Consistent with this view, the *axr2-1* mutant that is impaired in auxin responses restricted growth of virulent *P. syringae* pv. *maculicola* 4326 compared to WT plants (Wang et al., 2007).

Multiple auxin-resistant mutants have been isolated in *Arabidopsis* that define SCF ubiquitin-mediated protein degradation as a central component of auxin signaling (Gray et al., 1999, 2001). Ubiquitination of a target protein is operated by a multienzyme system consisting of ubiquitin-activating (E1), -conjugating (E2) and -ligating (E3) enzymes. Polyubiquitinated proteins are normally escorted to the 26S proteasome to be degraded (Devoto et al., 2003). Another *Arabidopsis* mutant, *sgt1b*, is defective in one of two highly related, functional *SGT1* genes (*SGT1a* and *SGT1b*) (Azevedo et al., 2006). SGT1 proteins structurally resemble and behave as HSP90/HSC70 co-chaperones (Shirasu and Schulze-Lefert, 2003; Azevedo et al., 2006; Noël et al., 2007). Plant SGT1 also co-immunoprecipitated with the SCF structural subunit SKP1 and the COP9 signalosome (CSN) that regulates SCF ubiquitin-proteasome degradation (Azevedo et al., 2002; Liu et al., 2002). Consistent with SGT1 assisting SCF ubiquitin E3-ligase activities, *Arabidopsis SGT1b* was found to contribute to the auxin response controlled by SCF^{TIR1} and the JA response mediated by SCF^{COI1} (Gray et al., 2001, 2003). *SGT1b* is also important for cell death-associated resistance responses to biotrophic oomycetes and hemi-biotrophic bacteria (Austin et al., 2002; Azevedo et al., 2006; Holt et al., 2005). However, it is unclear whether the role of *SGT1b* in resistance to these pathogens reflects an activity in SCF-mediated ubiquitination or a different co-chaperone function (Muskett and Parker, 2003; Noël et al., 2007).

Here, we explore the role of auxin in *Arabidopsis* resistance to necrotrophic fungi. We show that repression of auxin signaling either through mutations in the auxin pathway or by pharmacological interference with the auxin response impairs resistance to the necrotrophic fungi *P. cucumerina* and *B. cinerea*. We further provide evidence that ubiquitin-mediated proteolysis by the proteasome contributes to the restriction of the fungal diseases caused by *P. cucumerina*. The differential effects of *sgt1b* and auxin signaling mutants on resistance to the necrotroph *P. cucumerina* and the biotroph *H. parasitica* point to SCF-mediated ubiquitination being important for resistance to necrotrophic fungi, but contributing less to gene-for-gene resistance to biotrophic oomycetes.

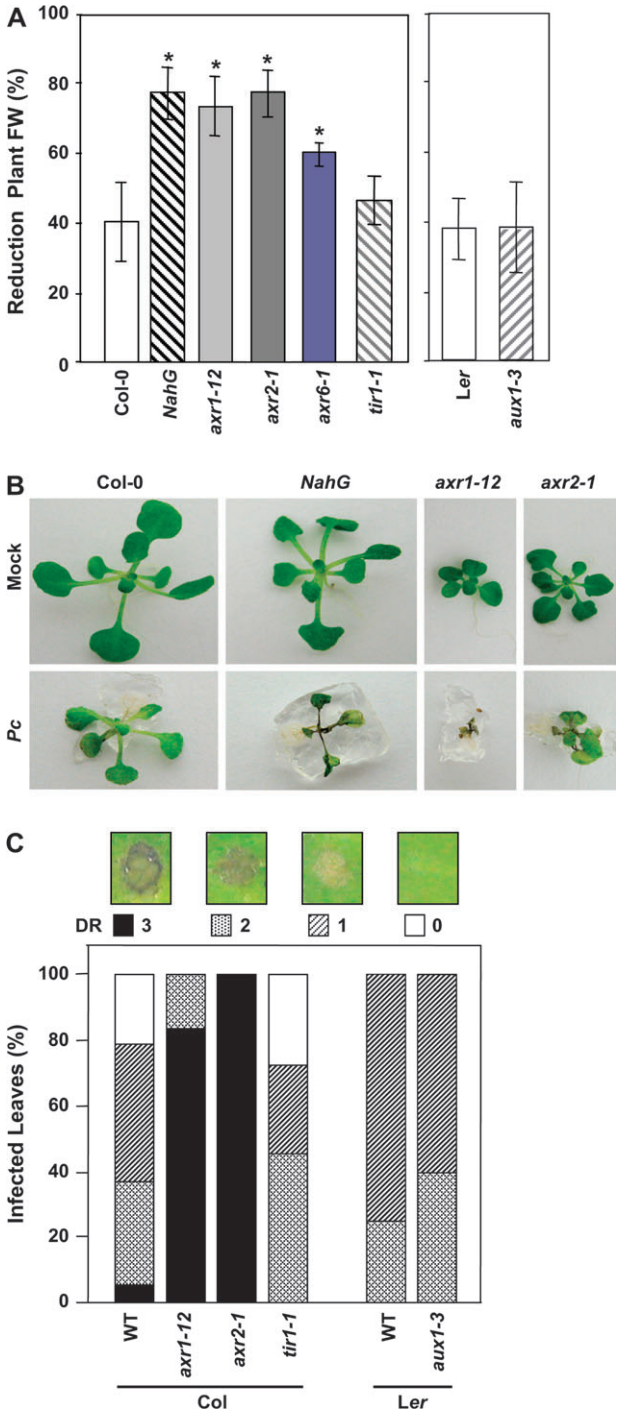

Figure 1. Enhanced Susceptibility of Auxin Signaling Mutants to the Necrotrophic Fungus *Plectosphaerella cucumerina*.

(A) Reduction in plant fresh weight (FW) caused by *P. cucumerina*-infection in WT (Col-0 and L*er*) and *NahG* plants, or *axr1-12*, *axr2-1*, *axr6-1*, *tir1-1* (in Col-0 background), and *aux1-3* (in *Ler*) mutants was measured 10 d post inoculation (dpi) with 4×10^5 spores ml^{-1}. Asterisks indicate data significantly different from WT ($P > 0.99$; *t*-test). Data values represent one of three independent experiments with similar results.

(B) Disease symptoms caused by *P. cucumerina* infection of WT (Col-0) and *NahG* plants, *axr1-12* and *axr2-1* mutants at 12 dpi.

(C) Graphical representation of disease symptoms caused by *P. cucumerina* 7 dpi of leaves from 4-week-old plants of the indicated genotypes with a 5-μl suspension of 2×10^6 spores ml^{-1}. Disease Rating (DR) is represented as percent of leaves showing no symptoms (0), chlorosis (1), necrosis (2), or severe tissue maceration (3). Data values represent one of three independent experiments that gave similar results.

RESULTS

Resistance to Necrotrophic Fungi Is Reduced in Auxin Signaling Mutants

In order to test whether auxin signaling competence affects the response of *Arabidopsis* to necrotrophic fungi, we infected wild-type (WT) Columbia (Col-0) and Landsberg-*erecta* (L*er*) plants and different auxin response mutants in these two accessions with the necrotrophic ascomycete fungus *P. cucumerina*. The mutants selected for the analysis are impaired in distinct components of the auxin signaling pathway: (1) the recessive mutant *tir1-1* is defective in the F-box TIR1 protein that is one of four auxin-binding proteins (ABPs) expressed in *Arabidopsis* (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005); (2) the dominant *axr2-1* mutant displays an enhanced stabilization of the auxin repressor transcriptional factor AXR2/IAA17 (Nagpal et al., 2000; Ramos et al., 2001); (3) the recessive *aux1-3* mutant is blocked in AUX1, one of the multiple *Arabidopsis* auxin importers (Marchant et al., 1999; Woodward and Bartel, 2005); and (4) the mutants *axr1-12* and *axr6-1* are defective in AXR1/RUB1 and AXR6/CUL1 proteins, two components of different SCF E3-ubiquitin ligase complexes (Leyser et al., 1993; Hellmann et al., 2003; del Pozo et al., 2002). Ten-day-old seedlings of WT, *P. cucumerina*-susceptible Col-*NahG* transgenic plants (Berrocal-Lobo et al., 2002) and the auxin response mutants axr*1-12*, *axr2-1*, *axr6-1*, *tir1-1*, and *aux1-3* grown on MS medium were sprayed with water (mock-treatment) or with a spore suspension of *P. cucumerina* (4×10^5 spores ml^{-1}). Susceptibility to *P. cucumerina* was estimated as the percent reduction of plant fresh weight (FW) at 12 d post inoculation (dpi), as described previously (Berrocal-Lobo and Molina, 2004; Llorente et al., 2005). This method to determine susceptibility to necrotrophs is not distorted by the different starting sizes of the genotypes analyzed (Llorente et al., 2005). As shown in Figure 1A, the reduction in plant FW caused by fungal infection was higher in the *axr1-12*, *axr2-1*, and *axr6-1* mutants than in WT plants. In *axr1-12* and *axr2-1*, the loss of plant FW was as extreme as in the highly susceptible *NahG* line (Berrocal-Lobo et al., 2002). By contrast, the level of susceptibility of *tir1-1* and *aux1-3* mutants to *P. cucumerina* did not differ from corresponding WT seedlings (Figure 1A). The progression of fungal infection in the *axr1-12*, *axr2-1*, and *axr6-1* mutants, as in the *NahG* line, correlated with the spread of necrosis in infected leaves that eventually consumed seedlings (Figure 1B and data not shown). In WT seedlings and

the *tir1-1* and *aux1-3* mutants, disease symptoms were less pronounced, although a reduction in plant FW and wilting of leaves were detected (Figure 1B and data not shown). Auxin signaling affects many developmental processes and, consequently, auxin response mutants have alterations in growth and development (Woodward and Bartel, 2005). To exclude the possibility that the observed susceptibility to *P. cucumerina* of the auxin response mutants analyzed could be related to the age of seedlings or the growing conditions, we also tested the resistance of adult (4-week-old) plants grown on soil. Individual leaves of WT plants (Col-0 and L*er*) and *axr1-12*, *axr2-1*, *tir1-1*, and *aux1-3* mutants were drop-inoculated with a 5-μl suspension of *P. cucumerina* spores (2×10^6 spores ml^{-1}) or water (control). After inoculation, the progression of infection was followed for 10 d, and a disease rating (DR; from 0, no symptoms, to 3, severe tissue maceration) was recorded daily (1–12 dpi). As shown in Figure 1C, the DR scores at a representative time point of 7 dpi were higher in the *axr1-12* and *axr2-1* mutants than in WT Col-0, whereas the DR scores of *tir1-1* and *aux1-3* were similar to those observed in corresponding WT Col-0 and L*er* plants, respectively. These results show that the enhanced susceptibility of *axr1-12* and *axr2-1* mutants to *P. cucumerina* is independent of plant developmental stage or growing conditions.

We tested whether the increased susceptibility of auxin signaling mutants extended to a different necrotrophic fungus, *Botrytis cinerea.* Ten-day-old seedlings of wild-type (Col-0 or L*er*), *axr1-12*, *axr2-1*, *tir1-1*, and *aux1-3* grown on MS medium were spray-inoculated with water (mock-treatment) or with a spore suspension of *B. cinerea* (5×10^4 spores ml^{-1}). Disease susceptibility was measured as the percent plant decay at different dpi, as described previously (Llorente et al., 2005). The level of plant decay caused by *B. cinerea* infection progressed more rapidly in *axr1-12* and *axr2-1* mutants than in WT Col plants (Figure 2). By contrast, progression of the infection in *tir1-1* and *aux1-3* mutants did not differ significantly from that of WT plants (Figure 2). Thus, the auxin signaling mutants *axr1-12* and *axr2-1* exhibit enhanced susceptibility to two necrotrophic fungi. These results point to a potential role of the auxin response pathway in regulation of *Arabidopsis* resistance to these necrotrophic pathogens.

Auxin affects many signaling processes (Woodward and Bartel, 2005). Thus, some auxin response mutants have been shown to be impaired either in JA (e.g. *axr1* and *axr6*), or JA/ET (e.g. *axr2*) signaling pathways (Woodward and Bartel, 2005). Moreover, SA was found to inhibit the auxin response pathway (Wang et al., 2007). We therefore investigated whether the enhanced susceptibility of *axr1-12* and *axr2-1* mutants to necrotrophic fungi might be a consequence of impairment of SA or ET/JA defense. The expression patterns of *PR1* and *PDF1-2*, marker genes of SA and JA/ET signaling, respectively (Glazebrook, 2005), were examined in WT and mutants after pathogen infection. Ten-day-old MS-grown seedlings of WT Col-0, and the *axr1-12* and *axr2-1* mutants were inoculated with water (mock) or with a spore suspension

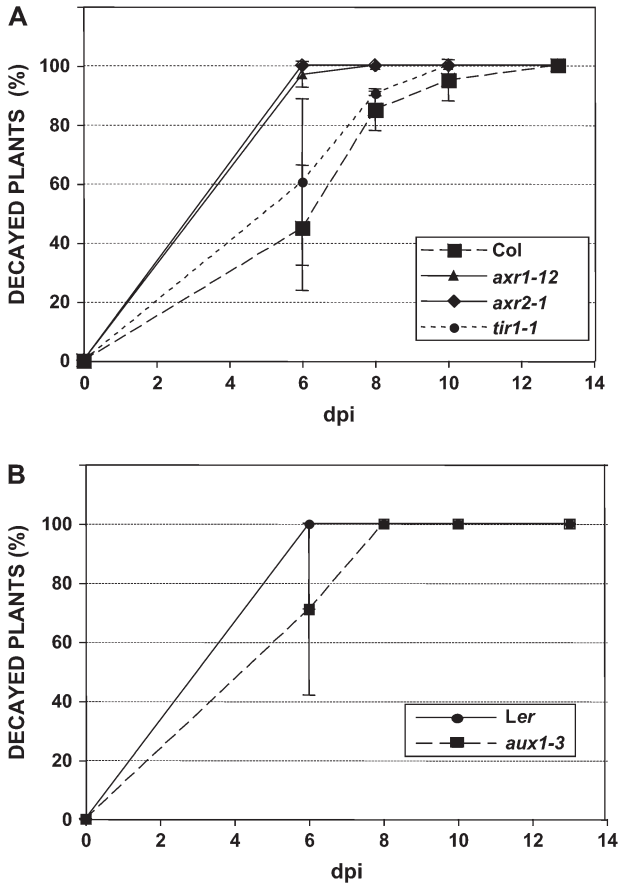

Figure 2. Enhanced Susceptibility of Auxin Signaling Mutants to *Botrytis cinerea*.

(A and B) Percentage decay of plants of WT genotypes (Col-0 and L*er*), *NahG* plants, and *axr1-12*, *axr2-1*, *tir1-1*, and *aux3-1* mutants, at different days post inoculation (dpi) with 5×10^4 spores ml^{-1} of the fungus. Data represented are the mean of three independent experiments ± SD.

of *P. cucumerina* (4×10^5 spores ml^{-1}) and total RNA extracted from seedlings at 3 and 5 dpi. The progression of the infection was followed in a proportion of seedlings to ensure the course of disease and hypersusceptibility of *NahG*, *axr1-12*, and *axr2-1* mutants prior to Northern analysis (Figure 3A). Probing of a Northern-blot showed that expression of *PR1* and *PDF1.2* mRNAs was induced to the same or higher extent in the *axr1-12* and *axr2-1* mutants than in WT plants (Figure 3B). By contrast, induction of *PR1* mRNA upon infection was impaired in *NahG* plants that are blocked in SA accumulation, whereas the expression profile of *PDF1.2* mRNA in these plants was similar to that observed in the WT plants (Figure 3B).

Two branches of the JA signaling pathway have been described to control antagonistically the response to pathogen infection and wounding (Lorenzo et al., 2004). The fine-tuning regulation of these responses depends on the balance of activation of ERF1 and MYC2 transcriptional factors (Lorenzo et al., 2004). Thus, MYC2 mutants (e.g. *jin1*) are

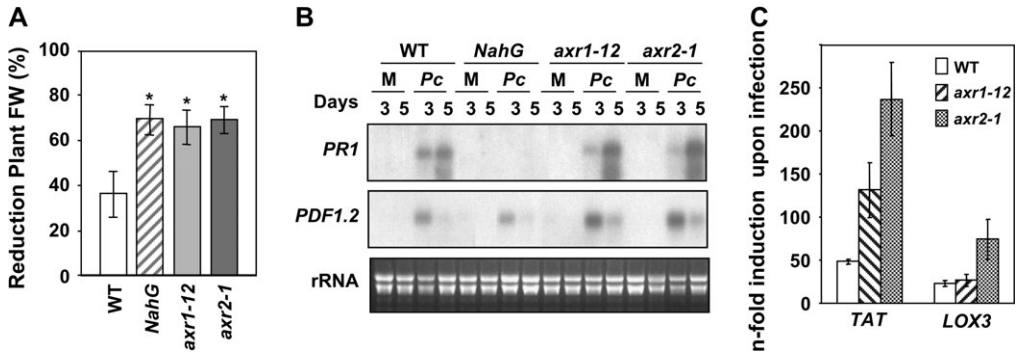

Figure 3. Defense Response of Auxin Signaling Mutants to *P. cucumerina* Infection.

(A) Reduction in plant fresh weight (FW) caused by *P. cucumerina* in WT (Col-0), *NahG*, *axr1-12*, and *axr2-1* plants, 10 d after inoculation with 4×10^5 spores ml^{-1}. Asterisks indicate data statistically significant different from WT ($P > 0.99$; *t*-test).

(B) Northern-blot analysis of *PR1* and *PDF1.2* expression in WT and *NahG* plants and *axr1-12* and *axr2-1*mutants, 3 and 5 d after mock inoculation (M) or infection with *P. cucumerina* (*Pc*). 7 μg of total RNA were loaded per lane and the blot was hybridized with the indicated probes. Ethidium bromide stained rRNA is included as loading control. Data values represent one of two independent experiments that gave similar results.

(C) qRT–PCR analysis of the expression of the JA-regulated *TAT* and *LOX3* upon plant inoculation with the necrotroph *P. cucumerina.*RNA samples from WT plant and *axr1-12* and *axr2-1* mutants, 5 d after mock inoculation (M) or infection with *P. cucumerina* (Pc) were used for the analysis. Values are represented as *n*-fold induction of gene expression in *Pc* samples compared to M samples from each genotype analyzed. Data correspond to the average (± SD) of two replicates.

impaired in the wound-responsive, JA-regulated signaling pathway, but show an enhanced activation of the JA/ET defensive pathway upon pathogen infection (Lorenzo et al., 2004). To exclude the possibility that the enhanced susceptibility observed in the *axr1-12* and *axr2-1* mutants was the result of a defect in the activation of the wound-responsive branch of JA signaling, the expression of two marker genes, *TAT* and *LOX3*, of this pathway (Lorenzo et al., 2004) was analyzed by qRT–PCR in these mutants and WT plants. As showed in Figure 3C, upon *P. cucumerina* infection, the expression of both genes was induced to a higher extent in the *axr1-12* and *axr2-1* mutants than in WT, probably reflecting enhanced fungal colonization observed in the mutants. These results suggest that activation of SA, JA/ET, and JA defensive pathways upon *P. cucumerina* fungal infection is not impaired in the *axr1-12* and *axr2-1* mutants. We therefore reasoned that the enhanced susceptibility of these mutants to *P. cucumerina* may be due to a more specific defect in the auxin response. The expression of the *AXR1* and *AXR2* genes in WT plants and the *axr1-12* and *axr2-1* mutants upon *P. cucumerina* infection was also tested by Northern-blot analysis but no significant changes in expression were observed compared to mock inoculated plants (data not shown).

Pharmacological Inhibition of Auxin Transport Leads to Increased Necrotrophic Infection

Extensive mining of publicly available *Arabidopsis* transcriptome data (www.genenvestigator.ch.org) revealed that a significant portion (~65%) of auxin signal transduction-related genes encoding AUX/IAA, ARFs, or TIR/ABP are down-regulated upon *Arabidopsis* infection with *B. cinerea* (Table S1). The repressed genes include the auxin receptor *TIR1*, the majority of characterized *AUX/IAA* genes encoding transcriptional repressors, such as *AXR2/IAA7* and *AXR3/IAA17*, and a significant number of ARF-encoding genes (Hagen and Guilfoyle, 2002; Dharmasiri et al., 2005a, 2005b). These data are consistent with enhanced susceptibility of *Arabidopsis axr1-12* and *axr2-1* mutants to necrotrophic fungi observed here (Figures 1 and 2) and point to a general transcriptional repression of the auxin response that may contribute to disease development. To test this hypothesis, we treated WT, *axr1-12*, *axr2-1*, and *NahG* plants with different concentrations (1 and 5 μM) of the auxin transport inhibitor TIBA (2,3,5-Triiodobenzoic acid; Geldner et al., 2001). The plants were then sprayed with *P. cucumerina* spores (4×10^5 spores ml^{-1}). The susceptibility of TIBA-treated and non-treated (control) plants was determined at 10 dpi. As shown in Figure 4A, there was a higher reduction in plant FW caused by fungal infection of WT plants treated with TIBA than in non-treated plants. The level of susceptibility of WT plants treated with 5 μM TIBA was similar to that observed in the control *axr1-12* and *axr2-1* mutants and *NahG* plants (Figure 4A). Significantly, TIBA treatment of *axr1-12* and *axr2-1* mutants and *NahG* plants did not result in further enhancement of susceptibility to *P. cucumerina* compared to untreated plants of the same lines (Figure 4A). These data show that loss of auxin transport phenocopies the hypersusceptibility of *axr1-12* and *axr2-1* mutants to necrotrophic fungi.

The expression of *PR1* and *PDF1.2* mRNAs was analyzed on a Northern-blot of the non-treated (control) and TIBA-treated plants after mock inoculation or *P. cucumerina* infection. Upon fungal infection, the expression of *PR1* in TIBA-pretreated WT plants was higher than in the control samples, whereas

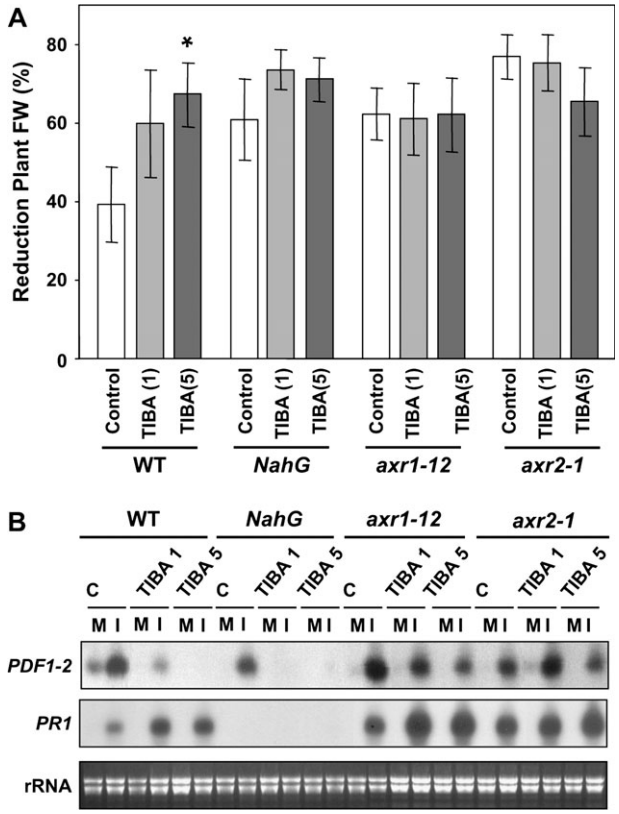

Figure 4. Effects of the Auxin Transport Inhibitor TIBA on *Arabidopsis* Defense Response to *P. cucumerina*.

(A) Reduction in plant fresh weight (FW) caused by *P. cucumerina* in WT (Col-0), *NahG*, *axr1-12* and *axr2-1* plants non-treated (C) or TIBA-treated (1 μM (TIBA (1)) or 5 μM (TIBA (5))). FW reduction was calculated 10 d after inoculation of plants with a suspension of 4×10^5 spores $ml^{-1}$of the fungus. Asterisks indicate data significantly different from the corresponding non-treated (C) dataset ($P > 0.99$; *t*-test).

(B) Northern-blot analysis of the effect of TIBA treatment on *PR1* and *PDF1.2* expression after *P. cucumerina* infection. Total RNA was isolated from plants non-treated (C), or treated with TIBA 1 μM (TIBA 1) and 5 μM (TIBA 5) at 7 d after mock-inoculation (M) or inoculation with *P. cucumerina* (I). 7 μg of total RNA were loaded per lane, the blot was hybridized with the indicated probes and rRNA was used as loading control. Data values represent one of three independent experiments with similar results.

expression of *PDF1.2* was lower in the TIBA-treated than control plants (Figure 4B). A similar pattern of expression of the *PR1* and *PDF1-2* genes was detected in the inoculated *axr1-12* and *axr2-1* mutants, irrespective of TIBA pretreatment (Figure 4B). As expected, induction of *PR1* upon fungal infection was blocked in *NahG* plants (Figure 4B). However, a repression of fungal-induced expression of *PDF1.2* was also observed in the *NahG* line (Figure 4B). The expression level of the JA-regulated genes *TAT* and *LOX3* was also tested by qRT–PCR in WT plants and both genes were found to be induced upon *P. cucumerina* infection in TIBA-treated WT plants (data not shown). These data suggest that inhibition of auxin signaling by blocking auxin transport affects the activation of SA and JA/ET signaling pathways upon necrotrophic fungal infection. The enhanced expression of SA-regulated *PR1* in TIBA-treated WT plants is in line with the proposed negative cross-talk between the SA and auxin signaling (Wang et al., 2007). We also tested the effect of exogenous treatments of WT plants with different concentrations (1–10 μM) of the natural auxin IAA before or after *P. cucumerina* inoculation, but no significant changes in plant susceptibility were detected (data not shown).

P. cucumerina Infection Leads to Stabilization of Heat Shock-Induced AXR3-GUS Protein

The enhanced susceptibility of *axr1-12* and *axr2-1* mutants (Figures 1 and 2) and TIBA-treated WT plants (Figure 4) to the necrotrophic fungi tested, as well as the global down-regulation of auxin response genes upon *B. cinerea* infection (Table S1), suggest that *Arabidopsis* infection with virulent necrotrophs causes a repression of auxin signaling. This repression could be in part mediated by reduced degradation of some AUX/IAA repressors (Quint and Gray, 2006; Navarro et al., 2006; Wang et al., 2007). To test this hypothesis, we examined expression of the reporter gene *HS::AXR3NT–GUS* encoding a fusion between the amino terminus (NT) of the auxin response repressor AXR3/IAA17 and GUS (β-glucuronidase) driven by a heat-shock (HS)-inducible promoter. After heat shock treatment, the stability of AXR3NT–GUS protein, measured as GUS activity, declines in IAA-treated plants whereas it continues to increase in SA-treated plants (Wang et al., 2007). Moreover, flg22 treatment of plants caused enhanced stability of AXR3NT–GUS protein compared to non-treated plants (Navarro et al., 2006). Leaves of 10-d-old WT (Col-0) and transgenic *HS::AXR3NT-GUS* seedlings grown in MS medium were mock-inoculated or inoculated with a spore suspension of *P. cucumerina* (4×10^5 spores ml^{-1}). Two days later, the plants were subjected to a heat shock treatment (37°C for 2 h) and the level of AXR3NT–GUS fusion protein determined (Gray et al., 2001). As previously described, GUS activity was detected in the roots but not leaves of HS-treated *HS::AXR3NT–GUS* seedlings (Figure 5; Gray et al., 2001). Notably, GUS staining was detected in the leaves of *HS::AXR3NT–GUS* seedlings inoculated with *P. cucumerina* and HS-exposed, but not in the mock inoculated, HS-exposed seedlings (Figure 5). No GUS staining was detected in HS-exposed WT plants (Figure 5). These results suggest that a stabilization of the AXR3 transcriptional repressor protein occurs in leaves upon *P. cucumerina* inoculation that might contribute to inhibition of the plant auxin response.

Role of SCF (Skp1–Cullin–F-box) E3-Ubiquitin Ligase Complexes in Restriction of Fungal Necrotroph Infection

An effective *Arabidopsis* auxin response relies on the removal of AUX/IAA family of TF repressors through auxin-stimulated binding by SCF^{TIR1} complexes and targeting to the ubiquitin–proteasome pathway (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). The enhanced susceptibility to necrotrophic

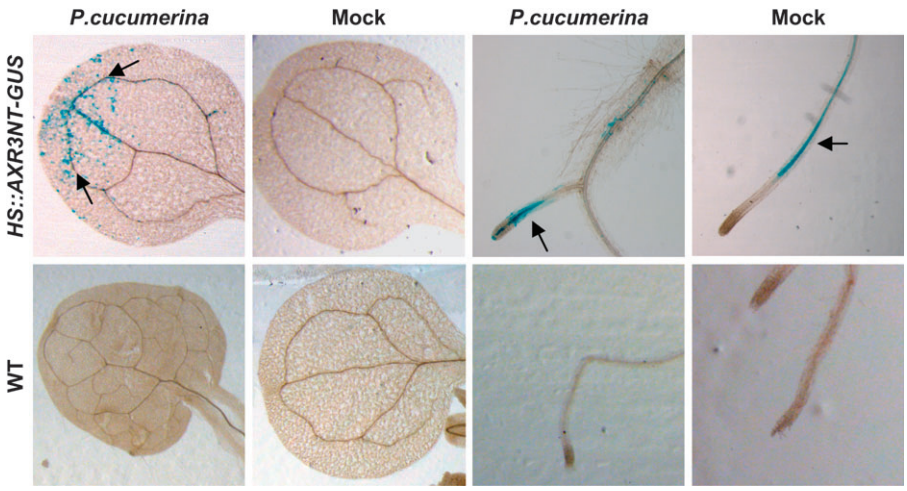

Figure 5. Stabilization of AXR3 Transcriptional Factor Repressor Protein upon Necrotrophic Fungal Infection.

Ten -day-old seedlings from WT (Col-0) plants and *HS::AXR3NT–GUS* transgenic plants were either mock-inoculated with water or with a spore suspension (4×10^5 spores ml^{-1}) of *P. cucumerina* 48 h latter a heat shock treatment was done and GUS activity was then determined immediately. Representative pictures of leaves and roots of the indicated genotypes are shown. Data values represent one of two independent experiments that gave similar results. Arrows indicate areas of GUS staining in the analyzed tissues.

fungi of the *axr2-1* mutant that has a stabilized AXR2 repressor protein, and of the *axr1-12* and *axr6-1* mutants that are impaired in two components (AXR1/RUB1 and AXR6/Cullin1) of the SCF E3-ubiquitin ligase complexes and are defective in IAA/AUX degradation (Gray et al., 2001; Hellmann et al., 2003), suggest that ubiquitin-mediated protein degradation is an important component of plant resistance to necrotrophs. Since *Arabidopsis SGT1b*, encoding a molecular co-chaperone, contributes to the auxin response controlled by SCF^{TIR1} (Gray et al., 2001, 2003), we explored whether mutations in *SGT1b* also affect resistance to *P. cucumerina*. Alongside, we tested the responses of an *Arabidopsis rar1* mutant lacking a SGT1/HSP90-interactor RAR1 (Shirasu and Schulze-Lefert, 2003) that is important for many *R* gene-mediated resistance responses to *P. syringae* bacteria and the obligate biotrophic oomycete *H. parasitica* (Muskett et al., 2002; Tornero et al., 2002; Holt et al., 2005), and a *rar1 sgt1b* mutant combination (Muskett et al., 2002). Ten-day-old MS-grown seedlings of the *rar1-10* and *sgt1b-1* single mutants and *rar1-10 sgt1b-1* double mutant in accession L*er* were sprayed with a suspension spore of *P. cucumerina* (4×10^5 spores ml^{-1}). As shown in Figure 6A, the reduction in plant FW caused by fungal infection was similar in the mutants and WT L*er*. The response of an *sgt1a-1* mutant defective in the second *Arabidopsis SGT1* gene *SGT1a* (Azevedo et al., 2006) was also similar to its WT parental accession Ws-0 (Figure 6A). Thus in contrast to *AXR1*, *AXR6*, and *AXR2*, *SGT1* and *RAR1* do not help to restrict necrotrophic fungal infection. We then tested resistance of the Col-0 double mutants *rar1-21 axr1-3* and $sgt1b^{eta3}$ *axr1-3* to *P. cucumerina* ($sgt1b^{eta3}$ is phenotypically a null *sgt1b* mutant in Col-0 that expresses a truncated SGT1b protein; Gray et al., 2003; Noël et al., 2007). No significant differences were observed in the reduction of plant FW compared to the highly susceptible *axr1-3* single mutant (Figure 6B). Therefore, while the activities of AXR1 and AXR2 which are intimately linked to SFC E3 ubiquitin ligase functions are needed for full restriction of necrotroph infection, *SGT1b* and *RAR1* do not appear to contribute to SCF-related processes in resistance to these pathogens. By contrast, isolate-specific (gene-for-gene) resistance mediated by *RPP4* to the obligate oomycete pathogen *H. parasitica* is strongly disabled in *rar1* and moderately compromised in *sgt1b* mutants (Holt et al., 2005). We therefore tested whether the mutants disabling SCF E3-ubiquitin ligase activities affect *RPP4*-mediated resistance. Wild-type, *axr1-3*, *axr1-12*, *axr2-1*, and *axr6-1* mutants were spray inoculated with conidiospores (1×10^4 spores mL^{-1}) of *H. parasitica* isolate Emwa1 (recognized by *RPP4*; Holt et al., 2005) and the extent of pathogen infection measured by the production of conidiospores on leaves at 5 dpi. As shown for *axr1-3* (Figure 7A), none of the mutants tested displayed increased susceptibility to *H. parasitica.* This was confirmed by monitoring the extent of pathogen hyphal growth in leaves stained with lactophenol-trypan-blue (Figure 7B). As expected, we observed a strong impairment of *RPP4* resistance in *rar1-21* and a weak defect in $sgt1b^{eta3}$ single mutants (Figure 7A) that correlated with increased colonization of leaves compared to WT Col-0 (Figure 7B). We then tested the phenotypes of the *rar1-21 axr1-3* and $sgt1b^{eta3}$ *axr1-3* double mutants in response to *H. parasitica* Emwa1 inoculation and observed an increase in susceptibility to *H. parasitica* in $sgt1b^{eta3}$ *axr1-3* at the level of sporulation (Figure 7A) and oomycete colonization of leaf tissues (Figure 7B). The genetic dispensability of *AXR1* and *AXR2* in *RPP4* resistance to *H. parasitica* suggests that SCF-mediated ubiquitination does not play a major role in the restriction of biotrophic pathogens, in contrast to its measurable contribution to resistance to necrotrophic fungi. The requirement for *SGT1b* in oomycete resistance

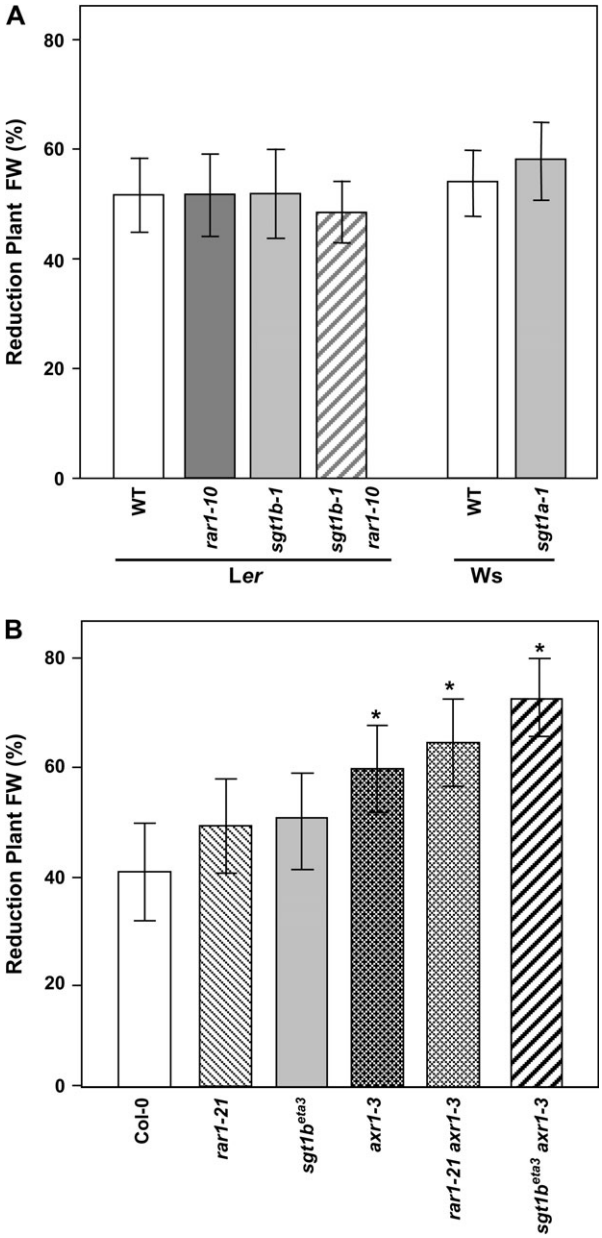

Figure 6. Resistance to *P. cucumerina* of *rar1* and *sgt1* Mutants.

Reduction in plant fresh weight (FW) caused by *P. cucumerina* in WT (L*er*, Ws) plants and *rar1-10*, *sgt1b-1*, *rar1-10 sgt1b-1* and *sgt1a-1* mutants **(A)**, and in WT (Col-0) plants, single mutants *rar1-21*, *sgt1b*^{eta3} and *axr1-3* mutants, and the double mutants *rar1-21 axr1-3* and *sgt1b*^{eta3} *axr1-3* **(B)**. Plants were sprayed with 4×10^5 spores ml^{-1} of *P. cucumerina* and the reduction of plant FW determined 10 d after inoculation. Asterisks indicate data significantly different from WT ($P > 0.99$; *t*-test). Data values represent one of four independent experiments with similar results.

and the genetic additivity of *sgt1b*^{eta3} *axr1-3* mutants in susceptibility point to a minor but detectable contribution of *AXR1* to oomycete resistance that is only seen if *SGT1b* function is disabled.

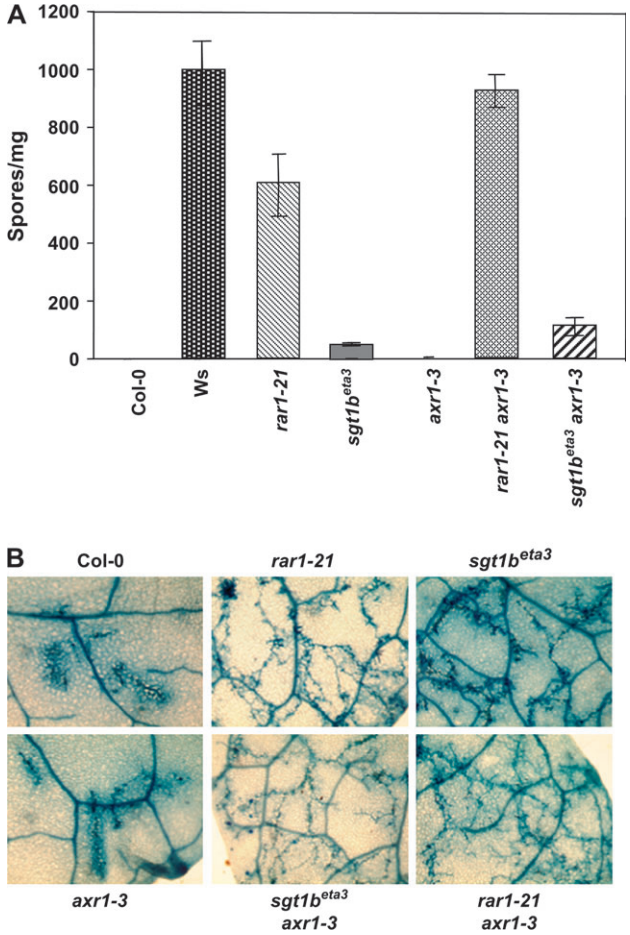

Figure 7. Resistance of Auxin Signaling and *sgt1b* Mutants to the Biotrophic Oomycete Pathogen *H. parasitica*.

(A) Numbers of spores mg^{-1}leaf FW were counted in WT plants (Col-0 and Ws, resistance and susceptible genotypes, respectively), in the single mutants *rar1-21*, *sgt1b*^{eta3}and *axr1-3*, and in double mutants *rar1-21 axr1-3* and *sgt1b*^{eta3} *axr1-3*, 5 d after inoculation with 1×10^4 spores ml^{-1}of *H. parasitica* isolate Emwa1. Data values represent the average of three replicate samples in one of three independent experiments with similar results.

(B) Trypan-blue staining of representative leaves of the indicated genotypes 7 d after *H. parasitica* inoculation.

Inhibition of Proteasome Activity Impairs Resistance to *P. cucumerina*

We took a pharmacological approach to test whether proteasome-mediated protein degradation plays a role in plant resistance to necrotrophs. Ten-day-old MS-grown seedlings of WT (Col-0), *axr1-12*, *axr2-1*, and *NahG* were treated for 24 h with water (control) or different sub-lethal concentrations (2 and 10 μM) of the proteasome inhibitor MG132 (Ramos et al., 2001). Plants were then transferred to MG132-free MS plates and either mock-inoculated or infected with *P. cucumerina* (4×10^5 spores ml^{-1}). Susceptibility was then determined at 10 dpi. The reduction in plant FW caused by fungal infection was higher in WT seedlings treated with 10 μM MG132 than in

control (untreated) seedlings and the extent of susceptibility was similar to that observed in control (untreated) *axr1-12*, *axr2-1*, or *NahG* seedlings (Figure 8). As with the auxin transport inhibitor (TIBA; Figure 4), treatment of the *axr1-12*, *axr2-1*, and *NahG* seedlings with MG132 did not lead to enhanced susceptibility to *P. cucumerina* compared to untreated seedlings (Figure 8). These results indicate that proteasome activity is required for effective mobilization of *Arabidopsis* resistance to *P. cucumerina*.

DISCUSSION

Auxin affects many aspects of development and growth in healthy plants (Woodward and Bartel, 2005; Quint and Gray, 2006). The auxin response has emerged more recently as an important factor in promoting *P. syringae* infection of *Arabidopsis* (Navarro et al., 2006) that can be repressed at multiple levels by the defence hormone SA (Wang et al., 2007). We present evidence here that supports a role of SCF-mediated ubiquitination and, more specifically, auxin signaling in promoting resistance to fungal necrotrophs (Figure 9). We find that the auxin signaling mutants *axr1*, *axr2*, and *axr6*, which are impaired in auxin-mediated AUX/IAA degradation through SCF^{TIR1}-targeted ubiquitination (Gray et al., 2001; Hellmann et al., 2003), allow more infection of leaves by the necrotrophic fungi *P. cucumerina* and *B. cinerea* (Figures 1, 2, and 9). The defective degradation of AUX/IAA transcriptional repressor proteins in auxin signaling mutants affect the activation of Auxin Response Factors (ARFs) and the expression of auxin-responsive genes (Hagen and Guilfoyle, 2002; Figure 9). Both TIBA inhibition of auxin transport (Geldner et al., 2001) and MG132 proteasome activity (Ramos et al., 2001) phenocopy the signaling defects of *axr1-12* and *axr2-1* mutants, leading to an enhanced susceptibility to necrotrophic fungi, such as *P. cucumerina* (Figures 4, 8, and 9). Also, infection by virulent necrotrophic fungi, such as *P. cucumerina*, causes increased stabilization of heat shock-induced AXR3–GUS protein (Figure 5) that is normally destabilized through SCF^{TIR1}-targeted ubiquitination (Gray et al., 2001), and a global down-regulation of auxin response genes (Table S1), suggesting that *Arabidopsis* infection with virulent necrotrophs causes a repression of auxin signaling (Figure 9). The contrasting effects of auxin on the progression of disease caused by hemi-biotrophic

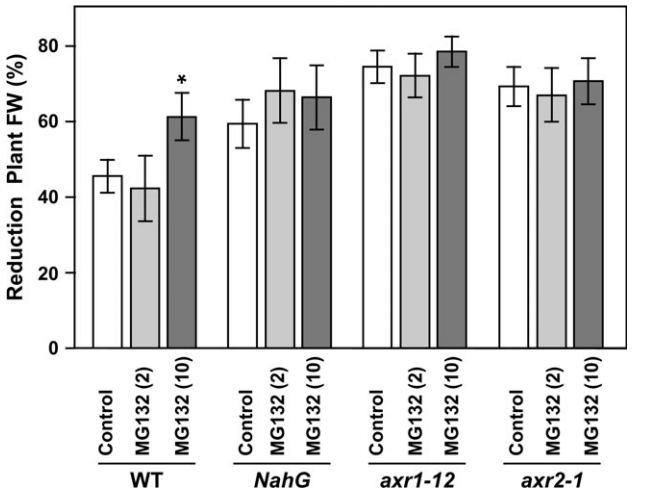

Figure 8. Effects of Proteasome Inhibitor MG132 on *Arabidopsis* Resistance to *P. cucumerina* Infection.

Reduction of plant fresh weight (FW) caused by *P. cucumerina* in WT (Col-0) and *NahG* plants and the *axr1-12*, *axr2-1* mutants, previously non-treated (C) or treated with 2 μM (2) or 10 μM (10) of the proteasome inhibitor MG132. FW reduction was calculated 10 d after inoculation of plants with a suspension of 4×10^5 spores ml^{-1} of the fungus. Asterisks indicate data significantly different from the corresponding non-treated (C) dataset ($P > 0.99$; *t*-test). Data values represent one of three independent experiments with similar results.

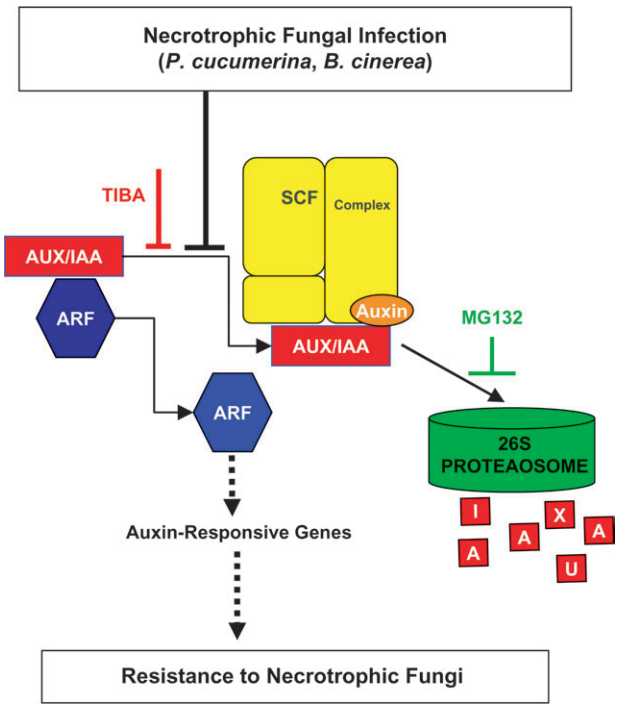

Figure 9. Scheme of Auxin Signaling Function in the Regulation of *Arabidopsis* Resistance to Necrotrophic Fungi.

Auxin binding to SCF complex leads to enhanced removal of members of the AUX/IAA family of transcriptional factor (TF) repressors by the SCF E3-ubiquitin ligase proteasome (26S) pathway (Kepinski and Leyser, 2005). The degradation of AUX/IAA leads to the activation of Auxin Response Factors (ARFs) and the expression of auxin-responsive genes, which, in turn, positively regulate *Arabidopsis* resistance to necrotrophic fungi. The auxin-mediated degradation of AUX/IAA is blocked in the *axr1*, *axr2*, and *axr6* mutants. Both TIBA inhibition of auxin transport and MG132 proteasome activity phenocopy the signaling defects of *axr* mutant, leading to an enhanced susceptibility to necrotrophic fungi (e.g. *P. cucumerina*). During infection of wild-type plants with the necrotrophic fungi *B. cinerea* and *P. cucumerina*, a moderate suppression of the auxin response occurs that may facilitate plant colonization by these pathogens.

bacteria (Navarro et al., 2006; Wang et al., 2007) and necrotrophic fungi (this work) suggest that auxin signaling is an integral component of the complex hormone network that modulates plant responses to different pathogens and pests (Glazebrook, 2005; Ferry et al., 2004).

The enhanced susceptibility of auxin response mutants to infection by necrotrophs suggests that auxin perception and/or signaling might connect closely to the modulation of plant cell death programs, since colonization by necrotrophs is aided by factors that promote cell death (Govrin and Levin, 2000). A challenge is to distinguish direct from indirect hormone effects. For example, the *axr1* and *axr2* mutants are known to be impaired also in JA/ET signaling pathways (Tiryaki and Staswick, 2002; Woodward and Bartel, 2005), and the enhanced susceptibility of *axr1* to different isolates of the soil-borne pathogen *Pythium* spp. correlated with a defect in JA signaling (Tiryaki and Staswick, 2002). Analysis of other JA pathway response mutants, such as *coi1-1* and *jar1-1*, also showed that JA plays a key role in *Arabidopsis* resistance to *Pythium* sp., *P. cucumerina*, and *B. cinerea* (Adie et al., 2007; Berrocal-Lobo et al., 2002; Thomma et al., 1998, 1999). Moreover, SA can negatively regulate auxin signaling (Wang et al., 2007). Importantly, in our study, we found that the expression patterns of *PR1* and *PDF1-2* that are marker genes of the SA and JA/ET signaling pathways, respectively (Glazebrook, 2005), and of *TAT* and *LOX3* that are marker genes of the wound-inducible, JA-regulated signaling pathway (Lorenzo et al., 2004), were not impaired in the *axr1-12* and *axr2-1* mutants upon *P. cucumerina* infection (Figure 3). These results suggest that the enhanced susceptibility of *axr1* and *axr2* plants to necrotrophic fungi is unlikely to be accounted simply by repression of the SA, JA/ET, or JA defense pathway. Enhanced and reduced expression of *PR1* and *PDF1.2*, respectively, in TIBA-treated WT plants (Figure 4) are consistent with a proposed negative cross-talk between SA and both the auxin and ET/JA pathways (Thomma et al., 1998; Wang et al., 2007). A recent comprehensive transcriptomic analysis of auxin response in *Arabidopsis* has revealed that auxin regulates in a complex manner genes associated with the biosynthesis, catabolism, and signaling pathways of other phytohormes (Paponov et al., 2008). The characterization of this complex signaling interaction that determines the fine control of plant resistance to pathogens is a future challenge in the plant immunity field.

We did not observe increased susceptibility to necrotrophs in the auxin response mutants *tir1-1*, *aux3-1*, and *sgt1b*^{eta3} (Figures 1, 2, and 6). The lack of phenotype in *tir1-1* and *aux3-1* may be explained by functional redundancy in *Arabidopsis*. TIR1 is one of four *Arabidopsis* auxin binding proteins identified (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005) and AUX1 is one of several auxin efflux importers expressed in the *Arabidopsis* genome (Woodward and Bartel, 2005). Hence, the auxin resistance phenotypes of these single mutants are comparatively weak (Woodward and Bartel, 2005). It is possible that *sgt1b* defects in SCF E3-ubiquitin ligase functions are also compensated for by the presence of *SGT1a*, which, although less penetrant, has intrinsic SGT1 activity (Azevedo et al., 2006). However, the partial loss of *RPP4* resistance observed in *sgt1b* mutants to the obligate biotrophic pathogen *H. parasitica* combined with genetic dispensability of *AXR1*, *AXR2*, and *AXR6* in resistance to this pathogen and an opposite trend in these mutants in response to *P. cucumerina* suggests different mechanisms are being engaged in defence responses to biotrophs and necrotrophs. Since, in yeast and plants, SGT1 has multiple sites of action inside the cell (Azevedo et al., 2006; Noël et al., 2007; Dubacq et al., 2002; Catlett and Kaplan, 2006), we think that an SGT1 function other than assisting SCF E3-ubiquitin ligases predominates in *R* gene-triggered resistance to oomycetes. Such an SGT1 activity might be as a co-chaperone in combination with HSP90 in protein complex assembly and/or maturation or influence the HSC70 chaperone machinery in controlling protein steady-state levels or localization (Azevedo et al., 2006; Noël et al., 2007). The additive loss of *RPP4* resistance observed in the *sgt1b*^{eta3} *axr1-3* double mutant (Figure 7) may signify a role for SGT1-assisted SCF-mediated ubiquitination in the absence of *R* gene-triggered immunity, after a certain degree of plant–oomycete compatibility is established. By contrast, SCF-mediated ubiquitination and proteolysis, that do not genetically engage *SGT1b*, are clearly important for resistance to fungal necrotrophs.

The positive contribution of auxin signaling to defense against necrotrophic fungi may render this pathway vulnerable to manipulation by pathogens and it is notable that *Arabidopsis* infection with virulent necrotrophic fungi such as *B. cinerea* (Table S1) or *P. cucumerina* (C. Sánchez-Rodriguez and A. Molina, unpublished results) cause a general down-regulation in the expression of auxin response genes (Figure 9). Repression of auxin-regulated genes was also described in the interaction between *Nicotiana benthamiana* and *B. cinerea* (El Oirdi and Bouarab, 2007). A down-regulation of auxin response genes may, in part, be mediated by the stabilization of auxin transcriptional factor repressors such as AXR3 (Figure 5) or AXR2 that, in turn, would result in reduced activities of ARF TFs (Hagen and Guilfoyle, 2002; Figure 9). During necrotroph infection of wild-type plants, we think it is likely that a moderate suppression of the auxin response by necrotrophs is countered by endogenous promotion of the auxin response that limits pathogen colonization. The results presented here and those published previously (Wang et al., 2007; Navarro et al., 2006) emphasize the fine control of plant defences to necrotrophic and biotrophic pathogens through the differential engagement and balance of hormone response systems.

METHODS

Biological Materials and Growth Conditions

The *Arabidopsis* wild-type accessions used in this study were Col-0, L*er*, and Ws-0. The auxin response mutants *axr1-12*, *axr1-3*, *axr2-1*, *axr6-1*, and *tir1-1* (in Col-0 background) and

aux1-3 (in L*er* background) have been described previously (Pickett et al., 1990; Leyser et al., 1993; Ruegger et al., 1998; Nagpal et al., 2000; Hellmann et al., 2003). The mutants *rar1-10*, *sgt1b-1*, and *rar1-10 sgt1b-1* (in Col-0), and *sgt1a-1* (in Ws-0) have been reported previously (Muskett et al., 2002; Azevedo et al., 2006). Double mutant lines were made between *rar1-21* (Tornero et al., 2002) or *sgt1b*^{eta3} (Gray et al., 2003) and *axr1-3* (Lincoln et al., 1990) all in accession Col-0, by crossing the single mutants and selecting homozygous double mutant combinations in F_2 progeny through allele-specific PCR (primer combinations are available on request). The *HS::AXR3NT–GUS* plants were kindly provided by Dr Mark Estelle (Indiana University, IN, USA).

Plants were grown in growth chambers under a 10 h light/14 h dark photoperiod, 70% relative humidity, 22°C day and 20°C night temperatures, and a light intensity of ~150 μE m^{-2} s^{-1}, as described previously (Berrocal-Lobo et al., 2002; Llorente et al., 2005). For plant growth on soil, seeds were sown in pots containing a mixture of organic substrate and vermiculite (3:1 v/v) and irrigated with water once a week (Berrocal-Lobo et al., 2002). For plants growth on Murashige-Skoog (MS) medium, seeds were surface-sterilized and sown on plates containing MS medium solidified with 0.8% (w/v) phytoagar (Sigma), as reported previously (Llorente et al., 2005).

The fungal pathogens *Plectosphaerella cucumerina* and *Botrytis cinerea* were kindly provided by Dr B. Mauch-Mani (University of Fribourg, Switzerland) and Dr R. Raposo (INIA, Spain), respectively. Spores from *P. cucumerina* and *B. cinerea* were collected as reported (Berrocal-Lobo et al., 2002; Berrocal-Lobo and Molina, 2004). *Hyaloperonospora parasitica* isolate (Emwa1) was maintained on the genetically susceptible *Arabidopsis* accession Ws-0, as described (Feys et al., 2005).

Plant Infection with Pathogens

Four-week-old soil-grown plants or 10-d-old MS medium-grown plants were used for the experiments with necrotrophic fungi. Inoculation of MS medium-grown plants with *P. cucumerina* and *B. cinerea* was done by spraying MS plates containing plants with 1.5 ml of 4×10^5 and 5×10^4 spores ml^{-1}, respectively (Berrocal-Lobo and Molina, 2004; Llorente et al., 2005). Mock inoculations were done with sterile water containing an equivalent amount of glycerol to the fungal spore suspension used for infection (< 0.01%). Disease caused by *P. cucumerina* was determined by measuring the percent fresh weight reduction (FW ± standard error) caused by the fungal infection (Berrocal-Lobo and Molina, 2004). Progression of *B. cinerea* infection was followed macroscopically by viewing the disease symptoms and the percent decay at different dpi was estimated. At least 15 plants per genotype were inoculated in multiple independent repeats. For inoculation of soil-grown plants with *P. cucumerina*, three leaves per plant were drop inoculated with 5 μl of a spore suspension (4×10^5 spores ml^{-1}) of the fungus or with water. After inoculation, plants were kept under the same growth conditions and the average disease rating (± SD) was measured at different dpi, as reported (Llorente et al., 2005). Disease rating (DR) was: 0, no symptoms; 1, chlorosis; 2, necrosis; 3, severe tissue maceration. A minimum of 20–30 plants per genotype were inoculated in at least three independent experiments, and the DR means and standard deviations estimated at different dpi.

Inoculation with *H. parasitica* was done by spraying 2-week-old plants grown on soil with a conidiosphore suspension (1×10^4 spores ml^{-1}) of isolate Emwa1. The extent of pathogen sporulation was quantified as described before (Feys et al., 2005). Lactophenol trypan-blue staining of leaves was done, as described (Feys et al., 2005). Statistical analysis of the data in disease resistance experiments was performed using a two-tailed Student's *t*-test assuming equal variances, with α = 0.05.

Plant Pharmacological Treatments

Treatments with the auxin transport inhibitor TIBA (2,3,5-Triiodobenzoic acid, Sigma-Aldrich, USA), IAA (3-Indolacetic acid, Sigma-Aldrich) or the proteasome inhibitor MG132 (Z-Leu-Leu-al, Sigma-Aldrich) were performed on 10-d-old MS-grown seedlings. For the TIBA and IAA treatments, seedlings were transferred to MS plates containing different concentrations of the chemicals and, 24 h later, they were sprayed with a suspension (4×10^5 spores mL^{-1}) of *P. cucumerina* or water, and the reduction plant FW was determined. For MG132 experiments, 10-d-old MS-grown seedlings were sprayed with water or different concentrations of MG132 (2 or 10 μM). After 5 h, plants were transferred to chemical-free MS plates, inoculated with a suspension (4×10^5 spores mL^{-1}) of *P. cucumerina* or water and the reduction in plant FW was determined as previously indicated.

Gene Expression Analysis

For Northern-blot analyses, total RNA was purified and blotted on Hybond-N+ membranes (Amersham, UK), as reported (Berrocal-Lobo et al., 2002). Probes were labeled with 50 μCi of α-^{32}P-dATP. The *PR1*, *PDF1.2*, and β-tubulin probes and the hybridization conditions have been previously described (Berrocal-Lobo et al., 2002). The *AXR1* probe (201 pb) was amplified using the oligonucleotides 5′-GTTTGTTCCGATGTTGGGG-3′ and 5′-GGTCTCAGCTGAACTGTC-5′. The *AXR2* probe (221 pb) was amplified using the oligonucleotides 5′-CGTTTGCGCATTATGAAGGG-3′ and 5′-CTGCCCTATATACCCAT-3′. At least 12 plants per genotype were inoculated in each experiment performed and the experiment repeated at least twice. The expression of 61 auxin regulated genes (Hagen and Guilfoyle, 2002) upon pathogen and hormone treatment was analyzed using the Genevestigator Meta-Analyzer Tools (www.genevestigator.ethz.ch/at/). qRT–PCR analyses were performed, as described previously (Hernandez-Blanco et al., 2007). Ubiquitin (UBQ, AT5G25760) expression was used to normalize the transcript level in each sample. Oligonucleotides used for cDNA amplification were designed with Primer Express (version 2.0; Applied

Biosystems): At1g17420 (LOX3), 5′-GCGGAGATTGTTGAAGCG-TTT-3′ and 5′-GCCCCACACCTATTTCTACGGT-3′; At2g24850 (TAT), 5′-TTCGCAAATACGATCTTCTCCC-3′ and 5′-GTTGATGATTACCA-TTGCGACG-3′; UBQ (At5g25760), 5′-AAAGGACCTTCGGAGAC-TCCTTACG-3′ and 5′-GGTCAAGAATCGAACTTGAGGA-GGTT-3′.

Reporter Gene Activity Assay

Wild-type (Col-0) and *HS::AXR3NT–GUS* plants were grown on MS plates, as indicated above. Ten-day-old seedlings were mock treated with water or inoculated with a suspension (2×10^6 spores mL^{-1}) of *P. cucumerina* spores. After 48 h, plants were heat shocked at 37°C for 2 h. GUS activity was assayed by staining seedlings in 5-bromo-4chloro-3indolyl-beta-D-glucoronic acid solution, as previously described (Cao et al., 1994).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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REFERENCES

AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R.A., and Mengiste, T. (2006). Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to *Botrytis* infection. Plant J. **48,** 28–44.

Adie, B.A., Pérez-Pérez, J., Pérez-Pérez, M.M., Godoy, M., Sánchez-Serrano, J.J., Schmelz, E.A., and Solano, R. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. Plant Cell. **19,** 1665–1681.

Audenaert, K., De Meyer, G.B., and Hofte, M.M. (2002). Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol. **128,** 491–501.

Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D., and Parker, J.E. (2002). Regulatory role of *SGT1* in early *R* gene-mediated plant defenses. Science. **295,** 2077–2080.

Azevedo, C., Betsuyaku, S., Peart, J., Takahashi, A., Noël, L., Sadanandom, A., Casais, C., Parker, J., and Shirasu, K. (2006). Role of SGT1 in resistance protein accumulation in plant immunity. Embo J. **25,** 2007–2016.

Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K., and Schulze-Lefert, P. (2002). The RAR1 interactor SGT1, an essential component of R gene- triggered disease resistance. Science. **295,** 2073–2076.

Berrocal-Lobo, M., and Molina, A. (2004). *Ethylene-Response Factor-1* mediates *Arabidopsis* resistance to the soilborne fungus *Fusarium oxysporum*. Mol. Plant Microbe. Interact. **17,** 763–770.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. **29,** 23–32.

Cao, H., Bowling, S.A., Gordon, S., and Dong, X. (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell. **6,** 1583–1592.

Catlett, M.G., and Kaplan, K.B. (2006). Sgt1b is a unique co-chaperone that acts as a client adaptor to link Hsp90 to Skp1p. J. Biol. Chem. **281,** 33739–33748.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host–microbe interactions: shaping the evolution of the plant immune response. Cell. **124,** 803–814.

de Torres-Zabala, M., Truman, W., Bennett, M.H., Lafforgue, G., Mansfield, J.W., Rodríguez-Egea, P., Bögre, L., and Grant, M. (2007). *Pseudomonas syringae* pv. *tomato* hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. EMBO J. **26,** 1434–1443.

del Pozo, J.C., Dharmasiri, S., Hellmann, H., Walker, L., Gray, W.M., and Estelle, M. (2002). AXR1-ECR1-dependent conjugation of RUB1 to the Arabidopsis Cullin AtCUL1 is required for auxin response. Plant Cell. **14,** 421–433.

Devoto, A., Muskett, P.R., and Shirasu, K. (2003). Role of ubiquitination in the regulation of plant defence against pathogens. Curr. Opin. Plant Biol. **6,** 307–311.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005a). The F-box protein TIR1 is an auxin receptor. Nature. **435,** 441–445.

Dharmasiri, N., Dharmasiri, S., Weijers, D., Lechner, E., Yamada, M., Hobbie, L., Ehrismann, J.S., Jürgens, G., and Estelle, M. (2005b). Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell. **9,** 109–119.

Dubacq, C., Guerois, R., Courbeyrette, R., Kitagawa, K., and Mann, C. (2002). Sgt1b contributes to cyclic AMP pathway activity and physically interacts with the adenylyl cyclase Cyr1p/Cdc35p in budding yeast. Eukaryotic Cell. **1,** 568–582.

El Oirdi, M., and Bouarab, K. (2007). Plant signalling components EDS1 and SGT1 enhance disease caused by the necrotrophic pathogen *Botrytis cinerea*. New Phytol. **175,** 131–139.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. (2003). *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. Plant J. **35,** 193–205.

Ferry, N., Edwards, M.G., Gatehouse, J.A., and Gatehouse, A.M. (2004). Plant–insect interactions: molecular approaches to insect resistance. Curr. Opin. Biotechnol. **15,** 155–161.

Feys, B.J., Wiermer, M., Bhat, R.A., Moisan, L.J., Medina-Escobar, N., Neu, C., Cabral, A., and Parker, J.E. (2005). Arabidopsis

SENESCENCE-ASSOCIATED GENE 101 stabilizes and signals within an ENHANCED DISEASE SUSCEPTIBILITY 1 complex in plant innate immunity. Plant Cell. **17,** 2601–2613.

Geldner, N., Friml, J., Stierhof, Y.D., Jürgens, G., and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature. **413,** 425–428.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43,** 205–227.

Glickmann, E., Gardan, L., Jacquet, S., Hussain, S., Elasri, M., Petit, A., and Dessaux, Y. (1998). Auxin production is a common feature of most pathovars of Pseudomonas syringae. Mol. Plant Microbe Interact. **11,** 156–162.

Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. **10,** 751–757.

Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. Genes Dev. **13,** 1678–1691.

Gray, W.M., Kepinski, S., Rouse, D., Leyser, O., and Estelle, M. (2001). Auxin regulates SCF(TIR1)-dependent degradation of AUX/IAA proteins. Nature. **414,** 271–276.

Gray, W.M., Muskett, P.R., Chuang, H.W., and Parker, J.E. (2003). Arabidopsis SGT1b is required for SCFTIR1-mediated auxin response. Plant Cell. **15,** 1310–1319.

Hagen, G., and Guilfoyle, T. (2002). Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Mol. Biol. **49,** 373–385.

Hellmann, H., Hobbie, L., Chapman, A., Dharmasiri, N., del Pozo, C., Reinhardt, D., and Estelle, M. (2003). Arabidopsis AXR6 encodes CUL1 implicating SCF E3 ligases in auxin regulation of embryogenesis. EMBO J. **22,** 3314–3325.

Hernández-Blanco, C., et al. (2007). Impairment of cellulose synthases required for Arabidopsis secondary cell wall formation enhances disease resistance. Plant Cell. **19,** 890–903.

Holt, B.F., 3rd, Belkhadir, Y., and Dangl, J.L. (2005). Antagonistic control of disease resistance protein stability in the plant immune system. Science. **309,** 929–932.

Holt, B.F., 3rd, Hubert, D.A., and Dangl, J.L. (2003). Resistance gene signaling in plants: complex similarities to animal innate immunity. Curr. Opin. Immunol. **15,** 20–25.

Kepinski, S., and Leyser, O. (2005). The *Arabidopsis* F-box protein TIR1 is an auxin receptor. Nature. **435,** 446–451.

Laurie-Berry, N., Joardar, V., Street, I.H., and Kunkel, B.N. (2006). The *Arabidopsis thaliana* JASMONATE INSENSITIVE 1 gene is required for suppression of salicylic acid-dependent defenses during infection by *Pseudomonas syringae*. Mol. Plant Microbe Interact. **19,** 789–800.

Leyser, H.M., Lincoln, C.A., Timpte, C., Lammer, D., Turner, J., and Estelle, M. (1993). Arabidopsis auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. Nature. **364,** 161–164.

Lincoln, C., Britton, J.H., and Estelle, M. (1990). Growth and development of the *axr1* mutants of Arabidopsis. Plant Cell. **2,** 1071–1080.

Liu, Y., Schiff, M., Serino, G., Deng, X.W., and Dinesh-Kumar, S.P. (2002). Role of SCF ubiquitin-ligase and the COP9 signalosome in the *N* gene-mediated resistance response to *Tobacco mosaic virus*. Plant Cell. **14,** 1483–1496.

Llorente, F., Alonso-Blanco, C., Sánchez-Rodriguez, C., Jorda, L., and Molina, A. (2005). ERECTA receptor-like kinase and heterotrimeric G protein from Arabidopsis are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant J. **43,** 165–180.

Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R. (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcriptional factor that essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell. **16,** 1938–1950.

Manulis, S., Haviv-Chesner, A., Brandl, M.T., Lindow, S.E., and Barash, I. (1998). Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of Erwinia herbicola pv. gypsophilae. Mol. Plant Microbe Interact. **11,** 634–642.

Maor, R., Haskin, S., Levi-Kedmi, H., and Sharon, A. (2004). In planta production of indole-3-acetic acid by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*. Appl. Environ. Microbiol. **70,** 1852–1854.

Marchant, A., Kargul, J., May, S.T., Muller, P., Delbarre, A., Perrot-Rechenmann, C., and Bennett, M.J. (1999). AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. EMBO J. **18,** 2066–2073.

Mauch-Mani, B., and Mauch, F. (2005). The role of abscisic acid in plant–pathogen interactions. Curr. Opin. Plant Biol. **8,** 409–414.

Muskett, P., and Parker, J. (2003). Role of *SGT1* in the regulation of plant *R* gene signalling. Microbes Infect. **5,** 969–976.

Muskett, P.R., Kahn, K., Austin, M.J., Moisan, L.J., Sadanandom, A., Shirasu, K., Jones, J.D.G., and Parker, J.E. (2002). Arabidopsis *RAR1* exerts rate-limiting control of *R* gene- mediated defenses against multiple pathogens. Plant Cell. **14,** 979–992.

Nagpal, P., Walker, L.M., Young, J.C., Sonawala, A., Timpte, C., Estelle, M., and Reed, J.W. (2000). AXR2 encodes a member of the Aux/IAA protein family. Plant Physiol. **123,** 563–574.

Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fuijoka, S., Arai, Y., Sekimata, K., Takatsuto, S., Yamaguchi, I., and Yoshida, S. (2003). Brassinosteroid function in a broad range of disease resistance in tobacco and rice. Plant J. **33,** 887–898.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science. **312,** 436–439.

Noël, L.D., Cagna, G., Stuttmann, J., Wirthmüller, L., Betsuyaku, S., Witte, C.-P., Bhat, R., Pochon, N., Colby, T., and Parker, J.E. (2007). Interaction between SGT1 and cytosolic HSC70 chaperones regulates Arabidopsis immune responses. Plant Cell. **19,** 4061–4076.

Nürnberger, T., and Kemmerling, B. (2006). Receptor protein kinases–pattern recognition receptors in plant immunity. Trends Plant Sci. **11,** 519–522.

Paponov, I.A., Paponov, M., Teale, W., Menges, M., Chakrabortee, S., Murray, J.A.H., and Palme, K. (2008). Comprehensive transcriptome analysis of auxin responses in Arabidopsis. Mol. Plant. **1,** 321–337.

Pickett, F.B., Wilson, A.K., and Estelle, M. (1990). The *aux1* Mutation of Arabidopsis Confers Both Auxin and Ethylene Resistance. Plant Physiol. **94,** 1462–1466.

Quint, M., and Gray, W.M. (2006). Auxin signaling. Curr. Opin. Plant Biol. **9,** 448–453.

Ramos, J.A., Zenser, N., Leyser, O., and Callis, J. (2001). Rapid degradation of auxin/indoleacetic acid proteins requires conserved amino acids of domain II and is proteasome dependent. Plant Cell. **13,** 2349–2360.

Robert-Seilaniantz, A., Navarro, L., Bari, R., and Jones, J.D. (2007). Pathological hormone imbalances. Curr. Opin. Plant Biol. **10,** 372–379.

Ruegger, M., Dewey, E., Gray, W.M., Hobbie, L., Turner, J., and Estelle, M. (1998). The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grr1p. Genes Dev. **12,** 198–207.

Shirasu, K., and Schulze-Lefert, P. (2003). Complex formation, promiscuity and multi-functionality: protein interactions in disease-resistance pathways. Trends Plant Sci. **8,** 252–258.

Thomma, B.P.H.J., Eggermont, K., Pennickx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl Acad. Sci. U S A. **95,** 15107–15111.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.J., and Broekaert, W.F. (1999). Requirement of functional ethylene-insensitive 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. **121,** 1093–1101.

Tornero, P., Merritt, P., Sadanandom, A., Shirasu, K., Innes, R.W., and Dangl, J.L. (2002). *RAR1* and *NDR1* contribute quantitatively to disease resistance in Arabidopsis, and their relative contributions are dependent on the *R* gene assayed. Plant Cell. **14,** 1005–1015.

Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2007). Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. Proc. Natl Acad. Sci. U S A. **104,** 1075–1080.

Tiryaki, I., and Staswick, P.E. (2002). An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant axr1. Plant Physiol. **130,** 887–894.

Vandeputte, O., Oden, S., Mol, A., Vereecke, D., Goethals, K., El Jaziri, M., and Prinsen, E. (2005). Biosynthesis of auxin by the gram-positive phytopathogen *Rhodococcus fascians* is controlled by compounds specific to infected plant tissues. Appl. Environ. Microbiol. **71,** 1169–1177.

Wang, D., Pajerowska-Mukhtar, K., Culler, A.H., and Dong, X. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. Curr. Biol. **17,** 1784–1790.

Woodward, A.W., and Bartel, B. (2005). Auxin: regulation, action, and interaction. Ann. Bot. **95,** 707–735.