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Signaling Pathways That Regulate the Enhanced Disease Resistance of *Arabidopsis* "*Defense, No Death*" Mutants

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***Arabidopsis dnd1* and *dnd2* mutants lack cyclic nucleotide-gated ion channel proteins and carry out avirulence or resistance gene–mediated defense with a greatly reduced hypersensitive response (HR). They also exhibit elevated broad-spectrum disease resistance and constitutively elevated salicylic acid (SA) levels. We examined the contributions of NPR1, SID2 (EDS16), NDR1, and EIN2 to *dnd* phenotypes. Mutations that affect SA accumulation or signaling (*sid2, npr1*, and *ndr1*) abolished the enhanced resistance of *dnd* mutants against *Pseudomonas syringae* pv. *tomato* and *Hyaloperonospora parasitica* but not *Botrytis cinerea*. When SA-associated pathways were disrupted, the constitutive activation of *NPR1*-dependent and *NPR1*-independent and SA-dependent pathways was redirected toward *PDF1.2*-associated pathways. This *PDF1.2* overexpression was downregulated after infection by *P. syringae*. Disruption of ethylene signaling abolished the enhanced resistance to *B. cinerea* but not *P. syringae* or *H. parasitica*. However, loss of *NPR1, SID2*, *NDR1*, or *EIN2* did not detectably alter the reduced HR in *dnd* mutants. The susceptibility of *dnd ein2* plants to *B. cinerea* despite their reduced-HR phenotype suggests that cell death repression is not the primary cause of *dnd* resistance to necrotrophic pathogens. The partial restoration of resistance to *B. cinerea* in *dnd1 npr1 ein2* triple mutants indicated that this resistance is not entirely *EIN2* dependent. The above findings indicate that the broad-spectrum resistance of *dnd* mutants occurs due to activation or sensitization of multiple defense pathways, yet none of the investigated pathways are required for the reduced-HR phenotype.**

Additional keywords: Arabidopsis thaliana, AtCNGC2, AtCNGC4, *HLM1, P. syringae* pv. *tomato* DC3000, *RPS2*.

Plants have numerous defenses against pathogen attack, some of which are constitutive while others are induced by contact with the pathogen. Specific recognition of pathogens can occur via direct or indirect interaction of the products of host resistance (*R*) genes with corresponding pathogen avirulence (*avr*) gene products (Jones and Dangl 2006; Nimchuk et al. 2003). This "gene-for-gene" recognition rapidly induces an array of host defense responses, through signaling pathways that include cellular ion fluxes, production of reactive oxygen intermediates, mitogen-activated protein kinase cascades, and accumulation of salicylic acid (SA), with contributions from many signaling proteins (Glazebrook 2005; Hammond-Kosack and Parker 2003; Jones and Dangl 2006; Nimchuk et al. 2003). Compatible interactions in which host or pathogen lack the cognate *R-avr* gene exhibit similar, albeit slower and weaker, defense-associated changes in gene expression (Lucas 1998; Tao et al. 2003). It is of interest to understand the signaling mechanisms that activate inducible plant defense responses.

A characteristic feature of *avr-R*–mediated resistance is the hypersensitive response (HR)—the programmed cell death of a small number of host cells at the site of pathogen attack (Greenberg and Yao 2004; Heath 2000). Although the HR has been hypothesized to limit access of biotrophs to host resources, several studies have indicated that the HR can be separated from other aspects of *avr-R*–mediated resistance (Bendahmane et al. 1999; del Pozo and Lam 1998; Jakobek and Lindgren 1993; Kohm et al. 1993; Yu et al. 1998, 2000). The HR apparently can contribute to defense through death of the host cell or by contributing to the activation of defense in adjacent cells and to the activation of systemic acquired resistance (SAR) throughout the plant (Heath 2000).

We previously isolated *Arabidopsis dnd1* and *dnd2* mutants that exhibit a "defense, no death" phenotype (Yu et al. 1998, 2000). These plants carry out *avr-R*-mediated defense responses despite substantial absence of the HR but also exhibit constitutively elevated SA levels, reduced plant size, and elevated broad-spectrum disease resistance (Yu et al. 1998, 2000). The *dnd1* and *dnd2/hlm1* mutations carry stop codons that disrupt the cyclic nucleotide-gated ion channel proteins AtCNGC2 and AtCNGC4, respectively (Balague et al. 2003; Clough et al. 2000; Jurkowski et al. 2004). A separate *Arabidopsis cpr22* mutation caused fusion of two other cyclic nucleotide-gated ion channel proteins, AtCNGC11 and AtCNGC12 (Yoshioka et al. 2006). The *cpr22* plants exhibit constitutive defense signaling and Ca^{2+}-dependent programmed cell death; however, unlike the *dnd/hlm* mutants, they still develop a normal HR, and single-gene knockouts of AtCNGC11 or AtCNGC12 do not confer *dnd*-like phenotypes (Balague et al. 2003; Clough et al. 2000; Jurkowski et al. 2004; Urquhart et al. 2007; Yoshioka et al. 2001, 2006). Impacts of these ion channel mutations on defense are not surprising given the importance of ion fluxes in plant defense signaling (Nurnberger and Scheel 2001); however, the means by which the *dnd* and other CNGC mutants alter defense remain unclear.

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AtCNGC2 and AtCNGC4 are more closely related to each other than to other *Arabidopsis* CNGCs (Maser et al. 2001) but the two genes are functionally nonredundant, in that loss of either can cause *dnd* phenotypes. They may, however, form a heterotetramer ion channel, as is known to occur with animal CNGC channels (Zhong et al. 2003). Study of AtCNGC2 and AtCNGC4 has demonstrated conductance of Ca^{2+} and K^+ but not Na^+ by AtCNGC2 (Ali et al. 2007; Hua et al. 2003; Leng et al. 1999, 2002; Tornero and Dangl 2001), and conductance of K^+ and Na^+ by AtCNGC4 (Balague et al. 2003). AtCNGC2 and AtCNGC4 have different binding affinities for calmodulin isoforms, suggesting differential regulation of channel activity (Kohler and Neuhaus 2000). Additionally, expression of these genes is differentially regulated, because *AtCNGC2* is constitutively expressed regardless of treatment while *AtCNGC4* is induced by treatment with avirulent *Xanthomonas* spp. or with methyl-jasmonate (Balague et al. 2003). Studies using transgenic *dnd1* and *dnd2* plants expressing bacterial salicylate hydroxylase (*nahG*$^+$), which catabolizes SA, suggested that SA is required for the elevated resistance of *dnd* mutants but not for the loss of HR (Clough et al. 2000; Jurkowski et al. 2004). *PAD4* is also required for elevated resistance in *dnd1* and *dnd2/hlm1* but not for other phenotypes of these mutants (Jirage et al. 2001).

A number of components of plant defense pathways have been revealed by analysis of *Arabidopsis* mutants with increased disease susceptibility. *NDR1*, for example, is required for the function of many R proteins that possess coiled-coil, nucleotide-binding site leucine-rich repeat domains (NB-LRR), whereas many R proteins with an N-terminal domain homologous to Toll and the interleukin-1 receptor (TIR-NB-LRR) require *EDS1* and *PAD4* (Aarts et al. 1998; Feys et al. 2001), defining at least two separate pathways for defense signaling. The existence of a third pathway is indicated by the finding that the *RPP7* and *RPP8* genes for resistance to *Hyaloperonospora parasitica* activate defenses independently of *EDS1* and *NDR1* (McDowell et al. 2000). Two very important classes of mutants with enhanced disease susceptibility include *eds5/sid1* and *eds16/sid2*, which are impaired in SA accumulation (Nawrath and Metraux 1999; Rogers and Ausubel 1997; Volko et al. 1998), and *npr1/nim1*, which fail to respond to exogenously applied SA (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997). SA is involved in *avr-R*–mediated defenses and it is required for establishment of SAR and for basal resistance to some virulent pathogens (Cao et al. 1994; Nawrath and Metraux 1999). Mutant *ndr1* plants exhibit a partial reduction in SA accumulation after infection (Shapiro and Zhang 2001), whereas *EDS16/SID2* encodes isochorismate synthase, a central protein in SA biosynthesis whose absence largely eliminates SA production (Wildermuth et al. 2001). *NPR1* acts downstream of SA to mediate activation of defense genes (Cao et al. 1994; Delaney et al. 1995; Pieterse and Van Loon 2004) and also influences SA levels, which are often elevated in *npr1* plants (Ryals et al. 1996; Shah et al. 1997). However, some SA-dependent defense responses are independent of *NPR1* (Bowling et al. 1997; Glazebrook et al. 1996; Rate et al. 1999).

Analysis of *Arabidopsis* mutants impaired in jasmonic acid (JA) or ethylene biosynthesis or perception has revealed that these two signaling molecules act in concert to induce plant defenses against necrotrophic pathogens (Balbi and Devoto 2008; Knoester et al. 1999; Lorenzo et al. 2003; Penninckx et al. 1998; Staswick et al. 1998; Thomma et al. 1999). For example, the resistance of *Arabidopsis* against the pathogens *Pseudomonas syringae* and *H. parasitica* is known to be mediated primarily through SA-mediated signaling pathways rather than JA or ethylene pathways whereas, in contrast, defense against *Botrytis cinerea* is mediated primarily through JA or ethylene pathways (Balbi and Devoto 2008; Feys and Parker 2000; Pieterse and Van Loon 2004; Spoel et al. 2003; Thomma et al. 1999); there is also a small contribution to defense against *B. cinerea* from basal SA accumulation not involving *SID2*-mediated SA biosynthesis (Ferrari et al. 2003; Govrin and Levine 2002). There is evidence of crosstalk between ethylene and JA responses. For example, expression of some JA-responsive genes is antagonized by ethylene (Ellis and Turner 2001; Rojo et al. 1999) and promotion of ozone-induced cell death by ethylene is antagonized by JA (Overmyer et al. 2000, 2003; Tuominen et al. 2004). There is also complex and biologically significant crosstalk between SA-dependent and JA- or ethylene-dependent defense pathways that, for example, can lead to NPR1-mediated suppression of JA signaling and defenses (Balbi and Devoto 2008; Feys and Parker 2000; Pieterse and Van Loon 2004; Spoel et al. 2003). Crosstalk between pathways leading to defense and stress responses likely serves to fine-tune plant responses to multiple biotic and abiotic stresses.

In the present study we used epistasis analysis to examine the contributions of *NPR1-*, *SID2-*, *NDR1-*, and *EIN2*-associated pathways to expression of the distinct defense phenotypes that arise in *dnd1* and *dnd2* mutants. Introduction of *npr1, ndr1, sid2,* or *ein2* impacted some but not all of the *dnd* phenotypes, and unanticipated redirection of defense signaling was observed.

RESULTS

Morphology of double mutants carrying *dnd1* or *dnd2*.

Morphologically, *dnd1* and *dnd2* plants exhibit dwarf rosettes compared with wild-type Columbia plants (Yu et al. 1998, 2000). We introduced mutations that perturb *R* gene-mediated signaling (*ndr1*), SA-mediated defense signaling (*sid2* and *npr1*), or ethylene-mediated defense signaling (*ein2*) into the *dnd1* and *dnd2* backgrounds. The resulting plant lines were grown in numerous independent experiments, and a representative example of the reproducibly altered rosette morphology that was observed for some genotypes is provided in Figure 1. Homozygous *npr1*, *sid2*, *ndr1*, and *ein2* single-mutant plants were similar to wild-type Columbia in size and appearance. None of these mutations, when introduced into the *dnd1* or *dnd2* backgrounds, completely reversed the dwarf phenotype. However, the *ndr1* and the *sid2* mutations slightly but consistently relieved the dwarf rosette size of *dnd1* and *dnd2* plants (Fig. 1A and B). In contrast, *dnd1 npr1* lines exhibited an exacerbation of the dwarf rosette phenotype (Fig. 1A). The *dnd1 npr1* plants also displayed macroscopic spontaneous lesions in the absence of pathogen and a wrinkled leaf phenotype (Fig. 1C). Unlike the effects seen in the *dnd1* background, introduction of *npr1* into the *dnd2* genetic background partially relieved the dwarf rosette phenotype, and *dnd2 npr1* plants did not exhibit spontaneous lesions or wrinkled leaves (Fig. 1D). Introduction of the *ein2* mutation into the *dnd1* and *dnd2* backgrounds did not alter the dwarf phenotype (Fig. 1A and 1D). Triple-mutant *dnd1 npr1 ein2* and *dnd2 npr1 ein2* plants were similar in size to *dnd1* and *dnd2* single-mutants, respectively, although, like *npr1 ein2* plants, their color was a more pale green than wild-type or *dnd* plants (Fig. 1D). These triple mutants exhibited ruffled leaf edges.

NPR1-independent expression of β-glucanase-2.

The *npr1-1* genetic background used to construct double mutants in this study contains a β-glucanase (GUS) transcriptional reporter fusion (*BGL2::GUS*); *npr1-1* plants fail to induce *BGL2::GUS* expression in response to exogeneous application of SA (Cao et al. 1994). We noted strong GUS staining in *dnd1 npr1* and *dnd2 npr1* plants in the absence of pathogens

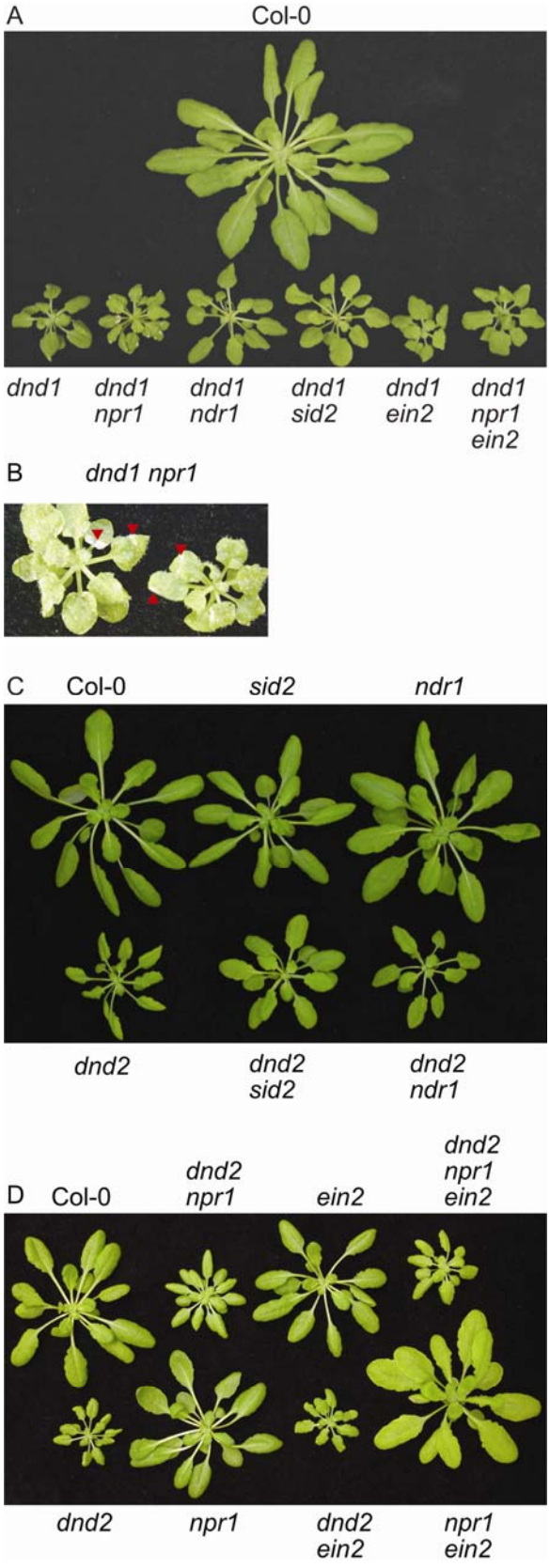

Fig. 1. Effect of various mutations on the rosette morphology of *dnd1* and *dnd2* plants. **A,** Two-month-old *dnd1* and double mutant plants. **B,** Spontaneous lesions on 2-month-old *dnd1 npr1* plants. Some lesions are indicated with red arrows. **C,** Five week old *dnd2*, *dnd2 sid2*, and *dnd2 ndr1* plants. **D,** Five-week-old *dnd2* plant with double mutants *dnd2 npr1* and *dnd2 ein2*, and triple mutant *dnd2 npr1 ein2*.

and without SA application, showing that mutations in *dnd1* and *dnd2* activate *BGL2* (*PR-2*) via an *NPR1*-independent pathway (Fig. 2A and B).

SA production in *dnd npr1* and *dnd sid2* double mutants.

Leaves of *dnd1* and *dnd2* mutants accumulated high levels of SA (Jurkowski et al. 2004; Yu et al. 1998). We measured SA for *dnd1* and *dnd2* mutants carrying mutations in *npr1*, *sid2*, *ndr1*, or *ein2*. As expected, levels of total SA in the *dnd1* or *dnd2* background were markedly reduced when the *sid2* mutation, which significantly impairs SA biosynthesis, was present (Fig. 3). A much smaller effect was seen for free SA (Fig. 3), and we noted that the *dnd* mutants exhibited *SID2*-independent production of SA. In both the *dnd1* and *dnd2* backgrounds, the presence of the *npr1* mutation correlated with a large increase in SA to levels higher than those seen for *npr1*, suggesting impacts on SA feedback regulation (discussed below). The effect of the *ndr1* mutation was less clear. In one of two experiments, levels of both conjugated and free SA were lower in *dnd1 ndr1* than in *dnd1*; however, plants in the other experiment showed little to no effect of the *ndr1* mutation on total or free SA in either the *dnd1* or *dnd2* background. As expected, the *ein2* mutation had little to no effect on SA levels in either *dnd1* or *dnd2* (Fig. 3).

HR phenotype of *dnd* plants is not relieved by *npr1*, *ndr1*, *sid2*, or *ein2*.

The *dnd* mutants were isolated in a mutant screen for plants that failed to exhibit the HR in response to high titer of *P. sy-*

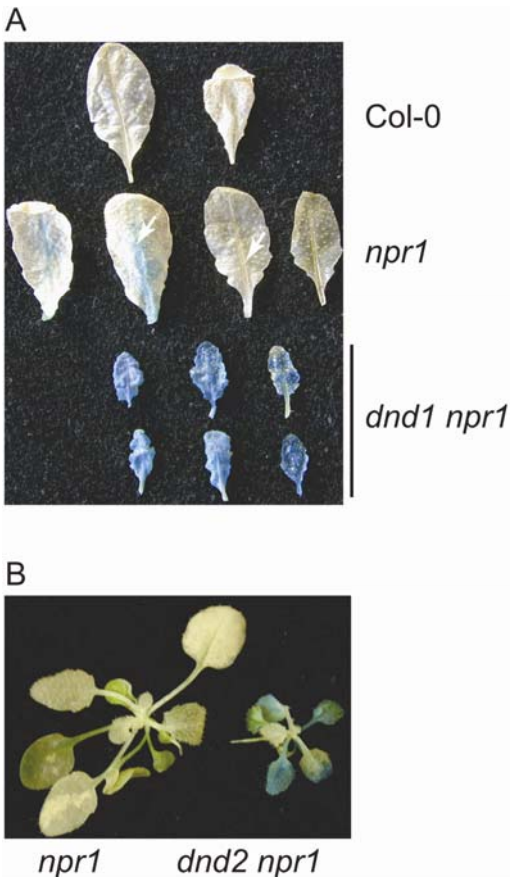

Fig. 2. β-Glucanase (GUS) expression phenotypes of single and double mutants. Mutants *npr1*, *dnd1 npr1*, and *dnd2 npr1* carry the *BGL2* promoter region fused to the β-glucuronidase reporter gene. **A,** Constitutive expression of *BGL2::GUS* in leaves of noninoculated *dnd1 npr1* plants. **B,** Constitutive expression of *BGL2::GUS* in noninoculated *dnd2 npr1* plants.

ringae pv. *glycinea* race 4 expressing *avrRpt2* (Yu et al. 1998, 2000). We conducted a similar assay by inoculating *dnd1* and *dnd2* double mutants and single-mutant parents with *P. syringae* pv. *glycinea* race 4 expressing *avrRpt2* at 1×10^8 CFU/ml and assessing HR at 24 h. As expected, wild-type Columbia and the mutants *npr1*, *sid2*, *ein2*, and *npr1 ein2* exhibited a strong HR, whereas a weak or intermediate HR was observed for *ndr1* mutants. Mutant plants of *dnd1*, *dnd2*, and their double mutants with *npr1*, *sid2*, *ein2*, and *ndr1*, as well as the triple mutants *dnd1 npr1 ein2* and *dnd2 npr1 ein2*, did not show an HR (Table 1). Thus, mutation of *NPR1*, *SID2*, *NDR1*, or *EIN2* did not relieve the loss-of-HR phenotype of the *dnd* mutants.

Distinct impacts of *npr1*, *sid2*, and *ndr1* on growth of *P. syringae* pv. *tomato*.

Impacts of the *npr1*, *sid2*, *ein2*, and *ndr1* mutations on the defense responses of *dnd* mutants were further examined by measuring the growth of virulent *P. syringae* pv. *tomato* DC3000 (DC3000) or avirulent DC3000 expressing *avrRpt2* (Fig. 4). The sections of Figure 4 identify instances in which there were significant differences between host genotypes in the amount of bacterial growth observed, as determined by analysis of variance (ANOVA) for the combined data from three or more independent experiments. As previously reported, populations of both virulent and avirulent DC3000 were restricted in leaves of *dnd1* and *dnd2* mutants relative to wild-type Columbia (Yu et al. 1998, 2000).

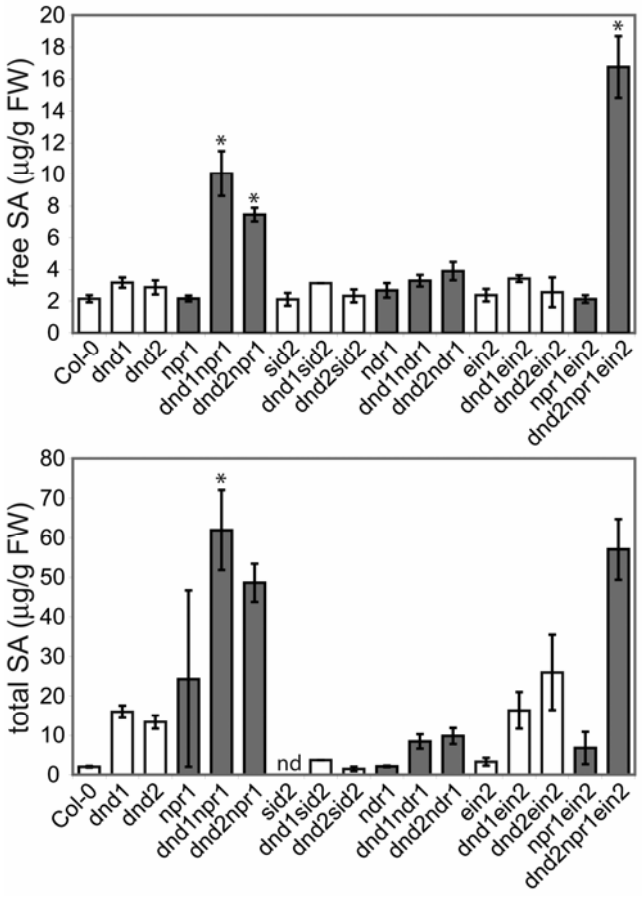

Fig. 3. Salicylic acid (SA) levels in single, double, and triple mutants. Free (unconjugated) and total SA in leaves of 4-week-old plants. Four replicates were measured for each genotype within each experiment; error bars represent standard error of the mean. Similar genotypes are grouped by shading; asterisks denote plant lines for which SA levels were significantly different from the parental line that is left-most within the similarly shaded group of bars (analysis of variance $P < 0.05$; Tukey test). Similar results were obtained in a second, independent experiment; nd = not detectable.

The ability of *dnd1* and *dnd2* plants to restrict bacterial growth was compromised by *npr1*; levels of both virulent and avirulent DC3000 were significantly higher in leaves of *dnd1 npr1* and *dnd2 npr1* than in *dnd1* and *dnd2*, respectively (Fig. 4A and B). Double mutants carried bacterial populations similar to those found in the *npr1* single mutant except in the case of *dnd2 npr1* plants inoculated with virulent bacteria (Fig. 4A and B), suggesting that *NPR1*-independent defense pathways partially contribute to the enhanced resistance of *dnd2* plants to virulent DC3000.

Restriction of growth of virulent and avirulent *P. syringae* pv. *tomato* DC3000 was entirely dependent on *SID2* for both *dnd1* and *dnd2*. Interestingly, growth of DC3000 expressing *avrRpt2* was higher in leaves of *dnd1 sid2* and *dnd2 sid2* than in leaves of *sid2* alone (Fig. 4C and D).

The *ndr1* mutation, which impairs resistance mediated by *RPS2*, *RPM1*, and *RPS5* (Century et al. 1995), disrupted the ability of both *dnd1* and *dnd2* to restrict growth of DC3000 expressing *avrRpt2* (Fig. 4E and F). Intriguingly, the *ndr1* mutation also impacted resistance against virulent DC3000 in *dnd2* plants. DC3000 growth was similar in *dnd1* and *dnd1 ndr1* plants (Fig. 4E). However, in *dnd2 ndr1* plants, DC3000 population sizes were reproducibly intermediate between those found in *dnd2* leaves and those in *ndr1* leaves (Fig. 4F). The elevated restriction of virulent DC3000 caused by *dnd2* exhibits a partial dependence on *NDR1* that is not seen for *dnd1*.

Impairment of ethylene responses by the *ein2* mutation did not detectably alter the ability of *dnd1* or *dnd2* to restrict bacterial growth. In *dnd1 ein2* and *dnd2 ein2* leaves, virulent and avirulent *P. syringae* pv. *tomato* DC3000 numbers were restricted to levels similar to those in *dnd1* and *dnd2* leaves, respectively (Fig. 4G and H). In a separate set of experiments that focused on triple mutants, although *dnd1* plants did restrict growth of avirulent DC3000 to a greater extent than *dnd1 ein2*, loss of *EIN2* again did not observably alter bacterial growth for the other host and bacterial genotypes (Fig. 4I

Table 1. Hypersensitive response (HR) of *dnd1* and *dnd2* mutants in combination with mutations in *npr1, ndr1, sid2*, and *ein2*[a]

Genotype (no. of experiments)	HR (*avrRpt2*$^+$)	HR (no *avr*)
Col-0 (3)	+	–
dnd1 (3)	–	–
dnd2 (2)	–	–
npr1 (3)	+	–
dnd1 npr1 (1)	–	–
dnd2 npr1 (3)	–	–
ndr1 (3)	±	–
dnd1 ndr1 (2)	–	–
dnd2 ndr1 (2)	–	–
sid2 (3)	+	–
dnd1 sid2 (2)	–	–
dnd2 sid2 (2)	–	–
ein2 (2)	+	–
dnd1 ein2 (1)	–	–
dnd2 ein2 (2)	–	–
npr1 ein2 (3)	±	–
dnd1 npr1 ein2 (1)	–	–
dnd2 npr1 ein2 (2)	–	–

[a] One-month-old plants were vacuum infiltrated with *Pseudomonas syringae* pv. *glycinea* race 4 at 10^8 CFU/ml expressing *avrRpt2* (*avrRpt2*$^+$) or carrying an empty plasmid vector (no avirulence [no *avr*]). Summarized scores from multiple experiments using a 0-to-5 scale: 0 to 1.9 = –, 2 to 2.9 = ±, 3 to 3.9 = +, and 4 to 5 = ++.

and J). As one way to address whether the *NPR1*-independent defenses of *dnd* plants are activated through JA or ethylene pathways, we constructed *dnd npr1 ein2* triple mutants but saw no further effect. With virulent and avirulent DC3000 bacteria, leaf population levels were similar in *dnd npr1 ein2* triple mutants and *dnd npr1* double mutants (Fig. 4I and J).

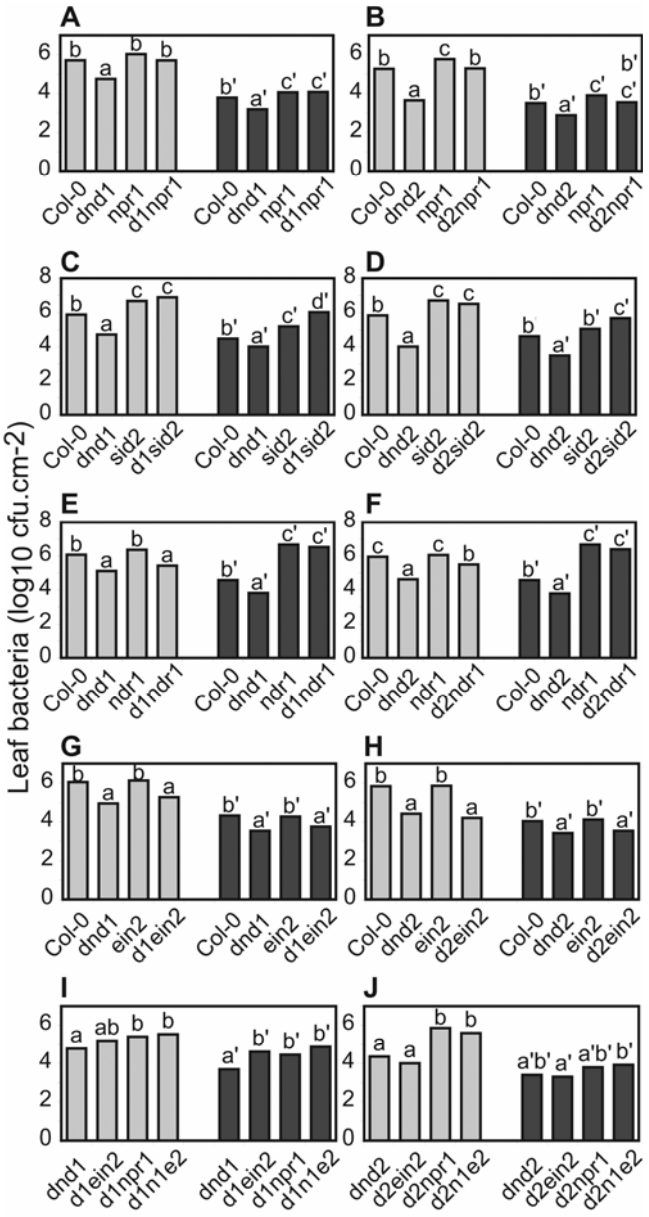

Fig. 4. Bacterial populations in leaves of single, double, and triple mutant plants. Leaf bacterial populations were determined 3 days after plants (4 to 5 weeks old) were inoculated by vacuum infiltration with *Pseudomonas syringae* pv. *tomato* DC3000 (light gray bars) at 5×10^4 CFU/ml or DC3000 + *avrRpt2* (dark bars). Least squares means from the indicated number of independent experiments are presented. Within each set of four genotypes treated with the same bacterial strain, bars marked with the same letter were not significantly different (analysis of variance $P < 0.05$). Data are from a total of 17 experiments, and wild-type Columbia was included in 16 of these experiments. Genotypes tested in comparison with wild-type and single-mutant controls were **A,** *dnd1 npr1* (three experiments); **B,** *dnd2 npr1* (four experiments); **C,** *dnd1 sid2* (three experiments); **D,** *dnd2sid2* (four experiments); **E,** *dnd1 ndr1* (three experiments); **F,** *dnd2 ndr1* (three experiments); **G,** *dnd1 ein2* (five experiments); **H,** *dnd2 ein2* (seven experiments); **J,** *dnd1 npr1 ein2* (four experiments); and **K,** *dnd2 npr1 ein2* (three experiments). In figure labels, genotypes are abbreviated as follows: d1 = *dnd1*; d2 = *dnd2*; d1n1e2 = *dnd1npr1ein2*; and d2n1e2 = *dnd2npr1ein2*.

Altered chlorotic responses to *P. syringae* pv. *tomato* in *dnd1* double mutants.

Pathogen population size and disease damage to the host (symptoms such as chlorosis and cell death) do not always correlate. We monitored the development of disease symptoms in *dnd1* double mutants inoculated by vacuum infiltration with either virulent *P. syringae* pv. *tomato* DC3000 or DC3000 expressing *avrRpt2*. As previously observed in many laboratories, wild-type Columbia plants inoculated with virulent DC3000 first exhibited chlorosis approximately 3 days after inoculation (not shown), and more successfully limited disease damage relative to immunocompromised genotypes such as *sid2* or *npr1* (Fig. 5). Note that, in two other experiments, *sid2* plants inoculated with DC3000 expressing *avrRpt2* exhibited more evident chlorosis on their leaves than is shown in the experiment of Figure 5. As expected from previous studies, *dnd1* plants inoculated with either virulent or avirulent DC3000 remained asymptomatic up to and beyond 7 days after inoculation, indicating significant resistance even in the absence of an *avr-R* interaction (Fig. 5).

Although bacterial growth in double-mutant *dnd1 npr1* plants was high, as in *npr1* mutants (Fig. 4), disease symptoms of *dnd1 npr1* plants were more like *dnd1* plants, with only minimal chlorosis or other disease symptoms after inoculation with either virulent or avirulent DC3000 (Fig. 5). In contrast,

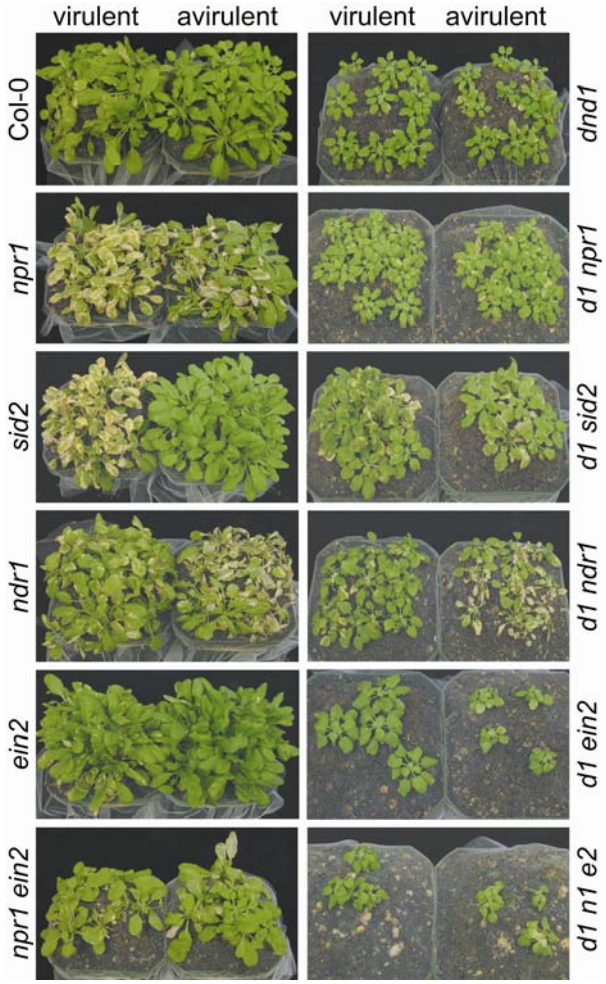

Fig. 5. Disease symptoms in double and triple mutants. Two-month-old *dnd1* double or triple mutant plants 7 days after inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 ± *avrRpt2* (2×10^5 CFU/ml) by vacuum infiltration. Chlorosis was evident 3 days postinoculation in susceptible lines. This experiment was repeated three times with similar results.

dnd1 sid2 plants, like *sid2* plants, developed chlorosis after inoculation with virulent or avirulent DC3000 (Fig. 5). These results suggest that *dnd1* mutants suppress symptom development in an *NPR1*-independent manner; however, this suppression may require elevated SA levels. The *ndr1* plants exhibited chlorosis (similar to wild-type plants) when inoculated with virulent DC3000 but severity of chlorosis was significantly greater when inoculated with DC3000 expressing *avrRpt2* (Fig. 5) (Century et al. 1995). The pattern of chlorosis seen for *dnd1 ndr1* plants in response to virulent and avirulent DC3000 was comparable with that seen for *ndr1* (Fig. 5).

The *dnd1* mutants and the *dnd1 ein2* double mutants were quite similar in overall symptom development in response to either virulent or avirulent DC3000 (Fig. 5). Likewise, *dnd1 npr1* and *dnd1 npr1 ein2* plants showed similar symptom development. This is consistent with our overall observations that *ein2* has minimal impact on *dnd* phenotypes. Double-mutant *npr1 ein2* plants developed much less chlorosis than *npr1* single mutants, providing a particularly pronounced example of the previous observation that ethylene insensitivity can enhance the disease tolerance of *Arabidopsis*, tomato, soybean, and *Nicotiana* spp. to *P. syringae* and other bacteria (Bent et al. 1992; Hoffman et al. 1999; Knoester et al. 1998; Lund et al. 1998).

SA-dependent, *NPR1*-independent PR-1 expression.

Replicated RNA blot analyses were conducted to assess how *npr1*, *sid2*, *ndr1*, and *ein2* mutations alter *dnd*-associated expression of *PR-1* and *PDF1.2*, standard marker genes for SA-dependent and JA- or ethylene-dependent defense responses, respectively. As previously reported, both *dnd1* and *dnd2* exhibited constitutive *PR-1* expression in the absence of pathogens and greater *PR-1* expression in response to avirulent *P. syringae* pv. *tomato* (Fig. 6) (Jurkowski et al. 2004; Yu et al. 1998). Also as expected, single mutants *npr1*, *sid2*, and *ndr1* failed to show substantial levels of *PR-1* gene expression 24 h after inoculation whereas *ein2* plants resembled wild-type Columbia (Fig. 6). In double mutants, the constitutive *PR-1* gene expression of *dnd1* plants was reduced but not eliminated by *npr1*, and no further induction was seen in response to avirulent DC3000 (Fig. 6). Expression of *PR-1* was not detected for *dnd1 sid2* plants even when inoculated with virulent or avirulent DC3000 (Fig. 6). Taken together, these data suggest that *dnd1* mutation results in the activation of an SA-dependent, *NPR1*-independent pathway leading to *PR-1* expression.

The *dnd1 ndr1* plants retained constitutive *PR-1* gene expression similar to the *dnd1* single mutant (Fig. 6). No additional *PR-1* induction was seen in infected *dnd1 ndr1* plants (Fig. 6). In replicated experiments involving *ein2*, *PR-1* gene expression was essentially unchanged between *dnd1* and *dnd1 ein2* plants or between *dnd1 npr1* and *dnd1 npr1 ein2* plants, providing another instance where *ein2* had little or no effect on *dnd* phenotypes (Fig. 6).

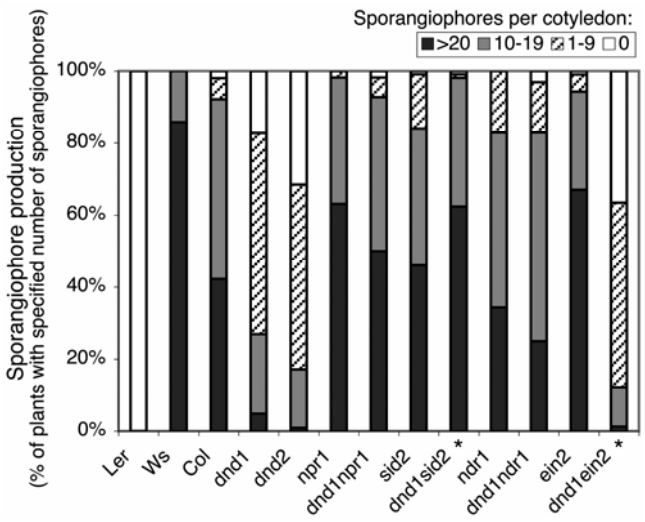

Fig. 7. Production of *Hyaloperonospora parasitica* sporangiophores on cotyledons of *dnd1* double mutants 7 days after inoculation with Emco5 isolate. Data from two separate experiments are combined (total number of plants tested for each genotype ranged between 44 and 61). Asterisks designate double mutants that were significantly different from the corresponding non-*dnd1* single mutant (e.g., *dnd1 sid2* compared with *sid2*), determined by analysis of variance ($P < 0.05$; Tukey test). Sporangiophore production on *dnd1* was significantly different from all other genotypes except *dnd1 ein2*.

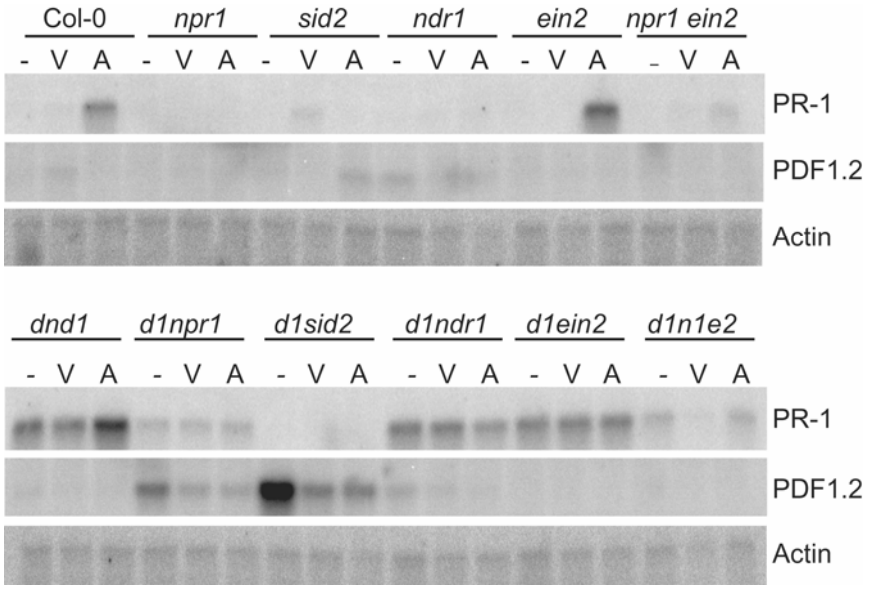

Fig. 6. *PR-1* and *PDF1.2* expression in *dnd1* double and triple mutants. Month-old plants either were not inoculated (–) or were inoculated by vacuum infiltration with 2×10^6 CFU of *Pseudomonas syringae* pv. *tomato* DC3000 (V) or DC3000 *avrRpt2* (A) per milliliter, and leaf tissue for RNA extraction was collected 24 h later. Actin served as a loading control. Similar results were obtained in independent experiments.

PDF1.2 expression in *dnd1* is promoted if SA pathways are blocked and is suppressed by *P. syringae* pv. *tomato* DC3000 infection.

In light of the crosstalk that can occur between SA and JA or ethylene defense signaling, we also investigated how *dnd* and the other mutations altered expression of *PDF1.2*. As expected from previous reports, RNA blot analyses did not reveal notable *PDF1.2* expression in Columbia or in *npr1*, *sid2*, *ndr1*, or *ein2* mutants before or after inoculation with virulent or avirulent DC3000 (Fig. 6). We found that *PDF1.2* expression was also minimal in *dnd1*, *dnd1 ndr1*, and *dnd1 ein2* plants (Fig. 6). However, although *dnd1* plants did not exhibit constitutive *PDF1.2* expression, substantial levels of *PDF1.2* RNA were reproducibly observed in noninoculated *dnd1 npr1* and *dnd1 sid2* plants (Fig. 6). The observed *PDF1.2* expression was partially *EIN2* dependent, because it was reduced in *dnd1 npr1 ein2* plants. The constitutive defense signaling of *dnd* mutants is apparently directed to JA or ethylene pathways when SA- or NPR1-mediated pathways are blocked. Of equal interest, in multiple replicates, the strong *PDF1.2* expression in noninoculated *dnd1 npr1* and *dnd1 sid2* plants was significantly reduced when plants were inoculated with either virulent *P. syringae* pv. *tomato* or avirulent *P. syringae* pv. *tomato* expressing *avrRpt2* (Fig. 6).

Requirement for *NPR1*, *SID2*, and *NDR1* for *dnd1* resistance to virulent *H. parasitica.*

To further evaluate the disease resistance phenotypes of *dnd1* double mutants, we inoculated seedlings with an isolate of the oomycete downy mildew pathogen *H. parasitica* (Emco5). The Landsberg *erecta* allele of *RPP8* confers resistance to Emco5, whereas Columbia plants are susceptible to Emco5 and carry a nonfunctional allele of *RPP8* (McDowell et al. 1998). Columbia *dnd1* plants exhibited strong resistance to this virulent isolate of *H. parasitica*, as expected (Yu et al. 1998), although this resistance was not as effective as that conferred by *RPP8* (Fig. 7; c.f. Ler). This strong level of "compatible interaction resistance" to Emco5 by *dnd1* plants was significantly compromised by introduction of *npr1*, *sid2*, or *ndr1* into the *dnd1* genotype (Fig. 7). Notably, *dnd1 sid2* plants supported significantly higher sporangiophore production than did *sid2* plants (Fig. 7). In contrast, the *dnd1 ein2* mutant retained the disease resistance phenotype of the *dnd1* parental line. These data indicate that *NPR1*, *SID2*, and *NDR1,* but not *EIN2*, are necessary for *dnd1*-mediated resistance to virulent *H. parasitica*.

Loss of resistance to *B. cinerea* in *dnd ein2* is reversed by *npr1*.

To evaluate JA- or ethylene-mediated defense capacity in *dnd1* and *dnd2* mutants, we inoculated plants with the necrotrophic fungal pathogen *B. cinerea*. Govrin and Levine (2000) previously reported that the loss-of-HR *dnd1* plants do not support *B. cinerea* growth; we also observed less *B. cinerea* growth on *dnd* mutants relative to wild-type plants (Fig. 8). We noted a striking susceptibility to *B. cinerea* in *dnd1 ein2* and *dnd2 ein2* plants compared with *dnd1* and *dnd2*, respectively (Fig. 8), indicating a dependence on ethylene signaling for *dnd1*- and *dnd2*-mediated resistance to *B. cinerea*. Note that *dnd ein2* double mutants retain the defective HR of *dnd* mutants (Table 1).

Independently replicated RNA blot analyses showed strong *PDF1.2* expression in *dnd1* and *dnd2* plants challenged with *B. cinerea* (Fig. 9). No induction of *PDF1.2* expression over that for mock-inoculated materials was seen for *dnd1 ein2* or *dnd2 ein2* plants infected with *B. cinerea*; however, elevated expression was seen in the *dnd1* and *dnd2* backgrounds in the presence of the *npr1*, *sid2*, and *ndr1* mutations (Fig. 9). Interestingly, although inoculated *dnd1 ein2* and *dnd2 ein2* exhibited minimal *PDF1.2* expression, expression was elevated in *dnd1 npr1 ein2* and *dnd2 npr1 ein2* mutants (Fig. 9). These triple mutants carrying a defective *EIN2* were substantially more resistant to *B. cinerea* than *ein2* singles or *dnd ein2* double mutants (Fig. 8).

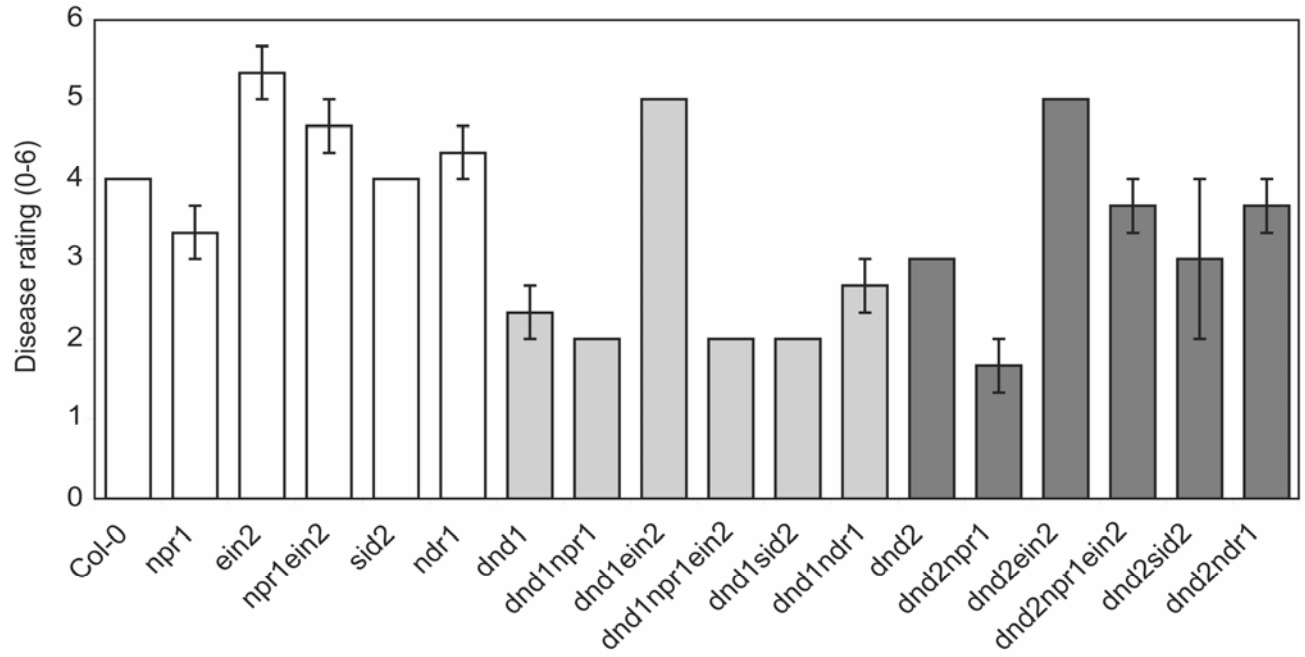

Fig. 8. Disease ratings of *dnd1* double and triple mutants inoculated with *Botrytis cinerea*. Two-month-old plants were inoculated with *B. cinerea* at 2×10^5 spores/ml and rated for disease symptoms 3 days postinoculation. Means ± standard error (SE) of disease ratings from three independent experiments are shown for all lines except *dnd1 npr1 ein2*, which lacks SE data because only two replicates were completed. Asterisks identify severity scores that were significantly different from the left-most line with the same shading (*Col-0, dnd1*, or *dnd2*), determined by analysis of variance ($P < 0.05$; Tukey test). Disease rating scale: 0 = no disease to 6 = extensive disease.

DISCUSSION

Plants carrying mutations in *DND1* (*AtCNGC2*) or *DND2* (*AtCNGC4, HLM1*) show multiple phenotypes, including reduced or absent HR, dwarfing, enhanced resistance to virulent and avirulent pathogens, elevated SA levels, and constitutive expression of defense marker genes (Balague et al. 2003; Clough et al. 2000; Govrin and Levine 2000; Jirage et al. 2001; Jurkowski et al. 2004; Yu et al. 1998, 2000). It has been unclear how these cyclic nucleotide-gated ion channels and mutation of these channels are tied into normal defense pathways. Here, we identify some of the well-characterized defense pathways that mediate the enhanced resistance of *dnd1* and *dnd2* mutants, and show that plant defense pathways are activated in interesting ways in *dnd* double and triple mutants. Our findings reinforce the concept that plant defense is controlled by regulatory networks rather than linear pathways and that specific elements of the plant response (e.g., pathogen growth restriction, SA production, expression of defense-associated genes, disease lesions, HR, and dwarfing) are regulated in overlapping but partially separable ways.

The enhanced resistance of *dnd1* and *dnd2* plants to virulent and avirulent *P. syringae* pv. *tomato*, and of *dnd1* plants to virulent *H. parasitica*, was dependent on *NPR1* and required SA synthesized through the *SID2*-encoded isochorismate synthase, indicating that SA signaling mediated through *NPR1* is an important contributor to the enhanced resistance of these mutants. *SID2*-deficient *dnd1* and *dnd2* plants still carried higher levels of SA than were found in *sid2* single mutants, presumably due to SA production via a second isochorismate synthase or the phenylalanine ammonium lyase (PAL) pathway (Wildermuth et al. 2001). Notably, the constitutive *PR-1* expression of *dnd1* plants was reduced in *dnd1 npr1* plants but was undetectable in *dnd1 sid2*. Together with the observation that *dnd1 npr1* and *dnd2 npr1* plants showed activation of the *PR-2* promoter–GUS construct, this suggests that *NPR1*-independent, SA-dependent pathways leading to *PR* gene expression are activated in *dnd1* and *dnd2*. Activation of *NPR1*-independent SA-dependent pathways has been previously observed (Clarke et al. 2000; Greenberg 2000; Nandi et al. 2003; Shah et al. 1999, 2001). We found that resistance to *P. syringae* pv. *tomato* and to *H. parasitica*, and constitutive expression of *PR-1*, appear to be dependent specifically on SA produced via SID2, suggesting that SID2 activity may be required to produce SA in appropriate cellular locations or to sufficient levels for defense activation.

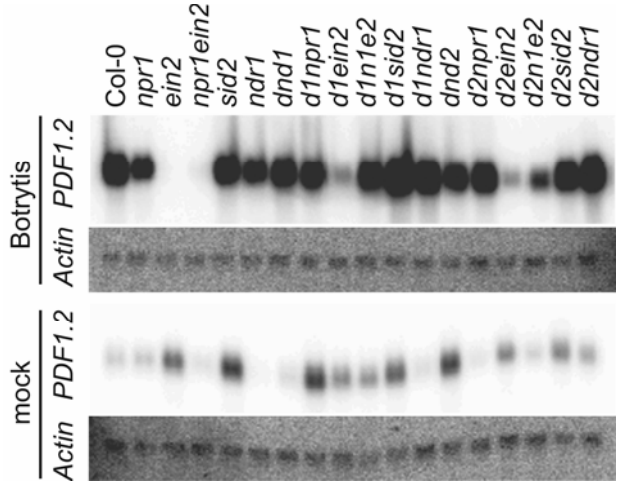

Fig. 9. *PDF1.2* expression in *dnd1* double mutants inoculated with *Botrytis cinerea*. Two-month-old plants were inoculated with *B. cinerea* at 2×10^5 spores/ml. Sample order is the same for both blots. Tissue samples were collected from *Botrytis cinerea*–inoculated or mock-inoculated plants 3 days postinoculation. Actin served as a loading control. Experiment was performed twice with similar results.

Although *NPR1*-independent pathways were activated in *dnd1* and *dnd2* plants, they were not effective in defense against virulent *P. syringae* pv. *tomato* and *H. parasitica* or avirulent *P. syringae* pv. *tomato*, because these pathogens were no less successful on *dnd1* or *dnd2* plants mutated at *NPR1* or *SID2* than on *npr1* and *sid2* single mutants. Interestingly, *dnd1 sid2* and *dnd2 sid2* plants supported higher populations of avirulent *P. syringae* pv. *tomato* than did *sid2* plants, and *dnd1 sid2* plants were more susceptible to *H. parasitica* than *sid2* plants (Figs. 4 and 7; H. W. Jung and J. T. Greenberg, *unpublished results*).

The *dnd1* mutants show reduced symptom development when inoculated with *P. syringae*. This phenotype was maintained in *dnd1 npr1* plants but lost in *dnd1 sid2* and *dnd1 ndr1* plants, suggesting that SA is required for *dnd1*-mediated suppression of symptom development via an *NPR1*-independent pathway. Because *ndr1* plants are impaired in SA accumulation after inoculation (Shapiro and Zhang 2001), the observation that *dnd1 ndr1* plants show chlorotic symptoms similar to *ndr1* plants is consistent with a requirement for SA for *dnd1*-mediated disease symptom suppression.

The *dnd1 npr1* and *dnd2 npr1* mutants showed increased SA levels compared with *dnd1* and *dnd2*, presumably due to the loss of feedback regulation of SA accumulation by *NPR1* (Delaney et al. 1995; Shah et al. 1997; Wildermuth et al. 2001). Similar increases in SA levels due to *npr1* have been reported for other constitutive defense mutants, including *ssi2* (Shah et al. 2001) and the *cpr* mutants (Clarke et al. 2000). Interestingly, *dnd1 npr1* and *dnd2 npr1* plants responded differently to this increase in SA. Although *dnd2 npr1* plants showed a slight size increase compared with *dnd2* single mutants, *dnd1 npr1* plants showed exacerbated dwarfing compared with *dnd1*, as well as spontaneous lesion formation. *NPR1* may repress or promote cell death depending on the cellular context: for example, *NPR1* represses the HR but promotes spontaneous cell death in the lesion mimic mutant *agd2* (Rate and Greenberg 2001) and promotes lesion development in the *hrl1* lesion mimic mutant (Devadas et al. 2002). Evidently, *NPR1* suppresses lesion formation in *dnd1* but not *dnd2*.

Small differences between *dnd1* and *dnd2* mutants were also observed in experiments with virulent *P. syringae* pv. *tomato* DC3000, where the *npr1* mutation entirely disrupted the elevated resistance of *dnd1* but only partially disrupted *dnd2* resistance, and the *ndr1* mutation partially disrupted the elevated resistance of *dnd2* but not *dnd1*. Although the phenotypic impacts on the plant caused by loss of the DND1/CNGC2 and DND2/CNGC4 ion channels is overall quite similar, these results point to subtle differences in pathways activated in *dnd1* as opposed to *dnd2* mutants.

Returning to the discussion of plant morphologies, triple-mutant studies with *dnd1* showed that introduction of *ein2* into the *dnd1 npr1* mutants prevented the development of spontaneous lesions and restored them to a rosette size similar to *dnd1*, suggesting that ethylene signaling is involved in lesion formation in these plants. Ethylene has previously been implicated as a regulator of ozone-induced lesion formation in *Arabidopsis* (Rao et al. 2002; Tuominen et al. 2004).

NDR1 is required for resistance mediated by the genes *RPM1*, *RPS2*, and *RPS5* (Century et al. 1995); therefore, it was not surprising to see an *NDR1* requirement for the enhanced resistance of *dnd1* and *dnd2* to DC3000 expressing *avrRpt2*, recognized by *RPS2*-expressing plants. However, *dnd2* also showed a partial requirement for *NDR1* for resistance to virulent DC3000. Although *dnd1* plants did not show a statistically

significant *NDR1* requirement for resistance to virulent *P. syringae* pv. *tomato*, mutation of *ndr1* eliminated *dnd1* resistance to virulent *H. parasitica*. Evidently, the enhanced resistance of both *dnd1* and *dnd2* to certain virulent pathogens requires *NDR1*. However, other phenotypes of the *dnd1* and *dnd2* mutants, including SA accumulation, dwarfing, and constitutive *PR-1* expression, showed only a partial *NDR1* requirement or no requirement for *NDR1*. This mirrors previous findings for the role of *PAD4* in the *dnd* phenotypes: both *dnd1* and *dnd2* have previously been shown to require *PAD4* for resistance to virulent *P. syringae*, although *dnd1* and *dnd2* SA levels, constitutive *PR-1* expression, and rosette size were unaffected by the *pad4* mutation (Jirage et al. 2001). The requirement for both *PAD4* and *NDR1*, considered to define separate signaling pathways downstream of distinct groups of *R* genes (Aarts et al. 1998; Feys et al. 2001), in the enhanced resistance of *dnd1* and *dnd2* to *P. syringae* and *H. parasitica* suggests that these mutants activate multiple defense pathways. However, it is important to note that measurements of SA levels in *dnd1* and *dnd2* plants impaired in either *PAD4* (Jirage et al. 2001) or *NDR1* (this article) were performed on noninoculated plants. Because both *PAD4* and *NDR1* are involved in accumulation of SA postinoculation (Jirage et al. 1999; Shapiro and Zhang 2001; Zhou et al. 1998), it is also possible that impaired disease resistance in *dnd pad4* or *dnd ndr1* double mutants is simply due to impaired SA accumulation upon infection. If so, this would explain the apparent uncoupling of SA accumulation and *PR-1* expression from enhanced resistance seen in *dnd1 ndr1* plants.

In other lesion-mimic mutants, similar uncoupling of resistance from phenotypes such as *PR* gene expression and SA accumulation has been seen (Clarke et al. 1998; Greenberg and Yao 2004; Yoshioka et al. 2006). The *cpr22* mutant, which results from a fusion of two cyclic nucleotide-gated ion channel genes (Yoshioka et al. 2006), provides a particularly relevant example. Epistasis analyses indicated that the enhanced resistance of *cpr22* to virulent *H. parasitica* and *P. syringae* pathogens required functional *NDR1, PAD4*, and *EDS1* genes, whereas other phenotypes such as stunting, constitutive *PR-1* expression, spontaneous lesions, and SA accumulation were independent of *NDR1, PAD4*, and *EDS1* (Yoshioka et al. 2006).

None of the defense mutations introduced into *dnd1* or *dnd2* led to restoration of the HR in response to challenge with avirulent *P. syringae* pv. *tomato* DC3000. In other mutants that lack the HR, alteration of SA signaling or SAR induction has been shown to restore the HR: the HR was restored in *agd2* mutants that lacked a functional NPR1 gene (Rate and Greenberg 2001), and introduction of the *npr1* mutation, depletion of SA by *nahG*, or induction of SAR restored the HR in the *hrl1* mutant (Devadas and Raina 2002). As previously mentioned, *dnd1 npr1* plants showed spontaneous lesions not seen for *dnd1*, and these were suppressed by introduction of *ein2*. However, an HR in response to inoculation with avirulent pathogen was still absent. Like the HR, normal rosette size was not restored by introduction of any of the defense mutations introduced into *dnd1* or *dnd2*. Slight size increases were seen when *sid2* or *ndr1* were introduced into *dnd1* or *dnd2*, or *npr1* into *dnd2*. It has previously been shown that expression of the bacterial salicylate hydroxylase gene *nahG*$^+$ in *dnd1* and *dnd2* only partially relieves the dwarf phenotype, suggesting, as do the results reported here, that other factors beside the level of SA affect rosette size in these mutants (Clough et al. 2000; Jurkowski et al. 2004). These two aspects of the *dnd* phenotypes, rosette size and lack of HR, are clearly affected by mechanisms or pathways beyond those that were explicitly examined in the present study.

EIN2 is important for defense against *B. cinerea* but is relatively uninvolved in resistance to *P. syringae* pv. *tomato* DC3000 and *H. parasitica*, SA accumulation, and *PR-1* expression (Balbi and Devoto 2008; Pieterse and Van Loon 2004; Thomma et al. 1999). This was also true in *ein2* double mutants with *dnd1* or *dnd2*. Previous work has suggested that the enhanced resistance of *dnd1* to *B. cinerea* is due to its deficient programmed cell death response (Govrin and Levine 2000). Although altered programmed cell death may be a contributing factor to the elevated resistance of *dnd* mutants to *B. cinerea*, the ethylene pathway is more significant. The *dnd1 ein2* and *dnd2 ein2* plants still had a deficient HR in response to *P. syringae* pv. *tomato*, yet were highly susceptible to *B. cinerea*.

The JA or ethylene defense pathways that are induced by wounding, herbivory, and necrotrophic pathogens are often monitored by tracking *PDF1.2* expression, and *PDF1.2* expression shows *EIN2*-dependence (Balbi and Devoto 2008; Penninckx et al. 1998; Pieterse and Van Loon 2004; Thomma et al. 1998). In the present study, *PDF1.2* was expressed after challenge with *B. cinerea* except in *ein2*, *dnd1 ein2* and *dnd2 ein2* lines, as might be predicted. Interestingly, we observed constitutive expression of *PDF1.2* in *dnd1 npr1* and *dnd1 sid2* plants that is absent in *dnd1, npr1*, or *sid2* single mutants. Apparently, the constitutively elevated defense activation in *dnd* mutants is channeled preferentially toward NPR1- and SA-dependent pathways but is channeled toward *PDF1.2*-associated pathways when SA-associated pathways are not available. This *PDF1.2* overexpression in *dnd1 npr1* and *dnd1 sid2* plants was downregulated after inoculation with virulent or avirulent DC3000. Although cross-talk between JA and SA pathways is partially understood (Balbi and Devoto 2008; Beckers and Spoel 2006; De Vos et al. 2006; Dong 2004; Pieterse and Van Loon 2004; Spoel et al. 2003), the mechanism that directs this defense signaling toward and away from PDF1.2 pathways in plants carrying *dnd* mutations is not known and could be examined through future study both of host factors and of pathogen effectors.

Possibly related to the preceding matter, *RPS2*-mediated defense was operational in *dnd sid2* double mutants (DC3000 grew less if it expressed *avrRpt2*) (Fig. 4C and D) but growth of DC3000 expressing *avrRpt2* was substantially higher in leaves of *dnd1 sid2* and *dnd2 sid2* than in leaves of *dnd1*, *dnd2*,or *sid2* alone. The virulence contribution of AvrRpt2 may be greater when *dnd* mutations are present in the *sid2* background, or the redirection of *dnd*-activated defense signaling toward *PDF1.2*-associated pathways may prevent effective activation of *R*-gene-mediated defenses that can otherwise operate in a *sid2* mutant.

Of further interest, *dnd1 npr1 ein2* and *dnd2 npr1 ein2* triple mutants inoculated with *B. cinerea* showed restoration of resistance and of *PDF1.2* expression that was absent in *dnd1 ein2* and *dnd2 ein2*. Mutation of *NPR1* presumably allows activation of *EIN2*-independent JA or ethylene defense pathways and reduces damage from *B. cinerea* by releasing the suppression of JA or ethylene responses mediated by *NPR1* (Spoel et al. 2003). Together with the observation that mutation of genes from several different defense pathways impairs the enhanced resistance of *dnd* mutants, the above findings suggest that the loss of cyclic nucleotide-gated ion channels in *dnd1* and *dnd2* plants, rather than activating a particular defense pathway, produces a generalized defense activation signal. This is consistent with recent findings (Ali et al. 2007) suggesting that NO may be the or one of the relevant signals. The signal derived from loss of *DND/CNGC* ion channels is preferentially transduced through SA-mediated pathways, is directed to JA or ethylene pathways if the SA pathways are disrupted, and can be further redirected if both SA and JA or ethylene pathways are disrupted, or upon pathogen infection.

MATERIALS AND METHODS

Growth conditions.

Unless noted otherwise, all plants were grown in 9-h photoperiods at 22°C. Light intensity was in the range of 100 to 180 μE. Plants were grown on Sunshine Mix no. 1 and irrigated from below with distilled water.

Generation of double and triple mutants.

Unless specifically noted, plant lines referenced by a lowercase gene symbol were homozygous for the mutant allele. All double and triple mutants described here were created using *dnd1* or *dnd2* as the pollen-recipient plant. The mutant alleles used were *dnd1-1* (Yu et al. 1998), *dnd2-1* (Jurkowski et al. 2004), *npr1-1* (Cao et al. 1994), *ein2-1* (Guzman and Ecker 1990), *ndr1-1* (Century et al. 1995), *sid2-2* (*eds16-1*) (Dewdney et al. 2000), and *npr1-1 ein2-1* (Clarke et al. 2001). All mutants were generated in the *Arabidopsis thaliana* Columbia genetic background. The *dnd1-1* mutation was confirmed with a dCAPS marker (*Mbo*I restriction site) using the primer pair 5′-TGCAGGCAGTGTTTTGGTTA and 5′-ATGAGATTAAGAG CAAAACCCGA. The *dnd2-1* mutation was confirmed with a dCAPS marker (*Nla*III restriction site) using the primer pair 5′-TCCAAATGGGTTCGAGCAT and 5′-GCAATCTTGAAC TGAATCC. Mutants carrying the *npr1-1* mutation were identified by screening respective F_2 populations with a previously described CAPS marker (Cao et al. 1997). Mutant lines containing the *ein2-1* allele were identified by plating F_2 seed on half-strength MS plates containing 10 μM 1-amino-cyclopropane-1-carboxylic acid (ACC) and allowing the seedlings to germinate in the dark for 3 to 4 days. Seedlings that displayed ethylene insensitivity were transplanted into soil. All dwarf plants exhibiting ethylene insensitivity were subsequently sequenced at the *ein2-1* allele. Triple mutants of *dnd1 npr1 ein2* and *dnd2 npr1 ein2* were first selected on half-strength MS plates containing 10 μM ACC, sequenced at *ein2-1*, and then checked for homozygosity at *npr1-1* by polymerase chain reaction. The *ndr1-1* mutation was detected using the primer pair 5′-AATCTACTACGACGATGTCCAC and 5′-GTAACCGATG GCAACTTTCAC. The *sid2-2* mutation was detected using the primer pair 5′-TTACGGTAATCGCGGAAGAG and 5′-AAGC TTGCAAGAGTGCAACA.

Plant growth and histochemical GUS assay.

Plant growth characteristics such as rosette size relative to control genotypes were noted for multiple plants in each of numerous experiments across multiple years; single representative plants are shown in Figure 1. The histochemical GUS assay was performed as described (Cao et al. 1994).

Pathogen assays.

To determine bacterial growth in leaves, 1-month-old plants were inoculated with DC3000 carrying either *avrRpt2* or the empty pVSP61 vector at 5×10^4 CFU/ml by vacuum infiltration. At 3 days postinoculation (dpi), homogenized leaf tissue was dilution-plated on selective media as previously described (Yu et al. 1998). For each experiment, four leaf samples were taken per genotype. Each leaf sample comprised a total of four leaf discs taken from two plants.

For observation of disease symptoms, 2-month-old plants were inoculated with DC3000 carrying either *avrRpt2* or the empty pVSP61 vector at 2×10^5 CFU/ml by vacuum infiltration; symptoms were observed 3 dpi. HR assays were performed by vacuum-infiltrating 2-month-old plants with *P. syringae* pv. *glycinea* race 4 carrying either *avrRpt2* or the empty pVSP61 vector at 10^8 CFU/ml; tissue collapse was scored 24 h postinoculation using a 0-to-5 scale in which 0 = no collapse, 1 = minor damage to less than 5% of leaves, 2 = some watersoaked or collapsed tissue present on 5 to 35% of leaves, 3 = 35 to 75% of leaves watersoaked or collapsed, 4 = widespread coalescing areas of collapsed leaf tissue, and 5 = total collapse of all leaves. Combined average of scores from multiple experiments were summarized for Table 1 as 0 to 1.9 = –, 2 to 2.9 = ±, 3 to 3.9 = +, and 4 to 5 = ++. Additional experiments with DC3000 and the plant lines from this study used spray inoculation of 2 to 3-week-old seedlings as per Tornero and Dangl (2001); however, the results were variable (poorly reproducible within our lab and too often failing to reproduce published results from other labs). Therefore, data for those experiments are not reported. *H. parasitica* Emco5 assays were performed as previously described (McDowell et al. 2000). Sporangiophore counts per seedling were grouped into four categories prior to ANOVA tests (Fig. 7). For *B. cinerea* assays, due to leaf size differences between genotypes, whole-plant phenotype tests (spray inoculation) were chosen over lesion size measurement on detached leaves (droplet inoculation; whole-plant disease phenotypes correlate with detached-leaf phenotypes) (Denby et al. 2004; Govrin and Levine 2000; Mengiste et al. 2003). *B. cinerea* cultures were grown on potato dextrose agar at room temperature for 7 to 10 days. Spores were scraped from the agar surface and resuspended in potato dextrose broth at 2×10^5 spores/ml. Two-and-a-half-month-old plants were lightly sprayed with the spore suspension; domes were placed over the plants to maintain high humidity, and disease assessments were made 7 dpi. The pot label identifying the genotype was obscured until plants had been rated for disease symptoms. Disease rating scale used was 0 = no detectable lesions; 1 = small rare lesions, no fungal growth visible; 2 = lesions on up to 10% of leaves, little to no fungal growth visible; 3 = significant necrosis of leaves (10 to 30% of leaves) and visible fungal growth; 4 = extensive fungal growth, with death of 30 to 60% of leaves; 5 = extensive fungal growth, with death of 60 to 80% of leaves; and 6 = fungus overgrew plants and less than 10% of green leaves remained. Inoculation conditions were optimized to provide a wide spread of scores between the most and least susceptible genotypes.

Northern blot analysis.

RNA isolation was conducted using either mini-to-midi RNA isolation kits (Invitrogen) or RNeasy kits (Qiagen) following the manufacturer's instructions. Northern blots were probed as previously described (Jurkowski et al. 2004). All RNA blot findings are based on independent biological replicates and, in most cases, were performed three or more times.

Quantification of SA.

Both free and total (including conjugated) SA were quantified from noninoculated leaf tissue of 4-week-old plants as described (Vanacker et al. 2001). Between 0.2 and 0.5 g of leaf tissue per sample was utilized. The experiment was performed twice, using entirely independent materials and in two separate years.

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