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Arabidopsis DND2, a Second Cyclic Nucleotide-Gated Ion Channel Gene for Which Mutation Causes the "*Defense, No Death*" Phenotype

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A previous mutant screen identified *Arabidopsis dnd1* and *dnd2* "*defense, no death*" mutants, which exhibit loss of hypersensitive response (HR) cell death without loss of gene-for-gene resistance. The *dnd1* phenotype is caused by mutation of the gene encoding cyclic nucleotide-gated (CNG) ion channel AtCNGC2. This study characterizes *dnd2* plants. Even in the presence of high titers of *Pseudomonas syringae* expressing *avrRpt2*, most leaf mesophyll cells in the *dnd2* mutant exhibited no HR. These plants retained strong *RPS2*-, *RPM1*-, or *RPS4*-mediated restriction of *P. syringae* pathogen growth. Mutant *dnd2* plants also exhibited enhanced broad-spectrum resistance against virulent *P. syringae* and constitutively elevated levels of salicylic acid, and pathogenesis-related (PR) gene expression. Unlike the wild type, *dnd2* plants responding to virulent and avirulent *P. syringae* exhibited elevated expression of both salicylate-dependent *PR-1* and jasmonate and ethylene–dependent *PDF1.2*. Introduction of *nahG*$^+$ (salicylate hydroxylase) into the *dnd2* background, which removes salicylic acid and causes other defense alterations, eliminated constitutive disease resistance and PR gene expression but only weakly impacted the HR$^-$ phenotype. Map-based cloning revealed that *dnd2* phenotypes are caused by mutation of a second CNG ion channel gene, *AtCNGC4*. Hence, loss of either of two functionally nonredundant CNG ion channels can cause *dnd* phenotypes. The *dnd* mutants provide a unique genetic background for dissection of defense signaling.

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*The *e*-Xtra logo stands for "electronic extra" and indicates that Figure 7 appears in color on-line.

Mutations that alter plant defenses have been employed by many researchers to understand the molecular basis of defense signal transduction and plant disease resistance. *Arabidopsis* has been a valuable species for this type of research, not only for cloning plant genes involved in disease resistance but also for building integrated models that describe defense signal transduction pathways and their interrelationships. A number of excellent reviews of this subject are available (Dangl and Jones 2001; Dong 2001; Glazebrook 2001; McDowell and Dangl 2000; Zhang and Klessig 2001).

Arabidopsis dnd (*defense, no death*) mutants have been isolated and characterized (Chan et al. 2003; Clough et al. 2000; Govrin and Levine 2000; Jirage et al. 2001; Yu et al. 1998, 2000). The *dnd1* mutant has similarities to other *Arabidopsis* constitutive defense mutants including *cpr*, *cim*, *lsd*, and *acd* lines (Bowling et al. 1994; Dietrich et al. 1994; Greenberg and Ausubel 1993; Maleck et al. 2002). These mutants exhibit elevated levels of salicylic acid (SA), constitutive expression of pathogenesis-related (PR) genes, and increased levels of resistance to virulent pathogens (Glazebrook 2001). However, *dnd1* plants differ from most "constitutive defense" mutants in that, in addition to the above phenotypes, they produce little or no hypersensitive response (HR) cell death in response to avirulent *Pseudomonas syringae* (Yu et al. 1998). Despite this loss of the HR, *dnd1* mutants retain strong *R* gene-mediated disease resistance. *DND1* encodes the cyclic nucleotide-gated (CNG) ion channel AtCNGC2 (Clough et al. 2000; Maser et al. 2001). Loss of this ion channel is recessive and causes the *dnd1* mutant phenotypes (Clough et al. 2000). The *Arabidopsis agd2*, *acd6*, *hrl1,* and *hlm1* mutants have also been reported to exhibit a reduced HR (Balague et al. 2003; Devadas and Raina 2002; Devadas et al. 2002; Rate and Greenberg 2001; Rate et al. 1999). *Arabidopsis* HR$^-$ mutants are useful tools for the study of defense activation and cell-death pathways.

The HR is a rapid and localized programmed cell death of host cells that occurs at the site of infection of an avirulent pathogen (Heath 2000). Gene-for-gene (*avr/R*-mediated) defense activation usually but not always causes HR cell death, and the HR is therefore closely associated with *R* gene-mediated disease resistance (Beers and McDowell 2001; Heath

2000; Richberg et al. 1998). Depending on the pathogen, the HR may help limit pathogen growth by promoting plant cell wall reinforcement, by eliminating host functions necessary for pathogen multiplication (e.g., for biotrophic fungi and viruses), by depriving the pathogen of nutrients or by fostering release of antimicrobial enzymes or other antimicrobial metabolites (Dixon 1994; Richael 1999; Richberg et al. 1998). The HR also promotes release of elicitors that activate defense responses in surrounding cells and systemically throughout the plant (Alvarez et al. 1998; Dorey 1997; Heath 2000; Ryals et al. 1997). However, the relative role of the HR remains unclear, as it has been shown in numerous studies that HR cell death is not always required for *avr/R*-mediated disease resistance (Bendahmane et al. 1999; del Pozo and Lam 1998; Kohm et al. 1993; Yu et al. 1998, 2001). Biochemical and genetic studies have identified SA, reactive oxygen species, and nitric oxide as key regulators of HR induction (Beers and McDowell 2001; Heath 2000; Richberg et al. 1998). There is evidence that HR cell death promotion or inhibition may depend as much on the balance among these compounds and among the proteins that regulate their presence as on their absolute concentration (Delledonne et al. 2001; Klessig et al. 2000). Some genes that are not *R* genes but which modulate *avr-R* gene signal transduction have been identified (Aarts et al. 1998; Muskett et al. 2002; Tornero et al. 2002), but there is ample need for further identification of the genes and physiological conditions that control HR cell death.

In many but not all plant species, salicylic acid plays important roles as an inducer of systemic acquired resistance (SAR), PR gene expression, and other defense-related responses (Delaney 1994; Dong 2001; Gaffney 1993; Ryals et al. 1996). Plants expressing the bacterial gene *nahG*$^+$, whose salicylate hydroxylase product degrades SA to catechol, do not develop SAR, are delayed in expressing an HR, and show enhanced susceptibility to virulent pathogens and decreased resistance to normally avirulent pathogens (Chivasa and Carr 1998; Delaney 1994; Gaffney 1993; Rao and Davis 1999). We have previously observed that *Arabidopsis dnd1* mutants expressing *nahG*$^+$ lose their elevated resistance to virulent and avirulent pathogens, potentially confirming the role of elevated SA in those phenotypes (Clough et al. 2000). However, *dnd1* plants that express *nahG*$^+$ still exhibit some dwarfing and retain the loss-of-HR phenotype, suggesting that SA-independent phenomena also contribute to *dnd1* phenotypes (Clough et al. 2000). In light of recent studies, results obtained using *nahG*$^+$ must be interpreted with caution (Heck et al. 2003; vanWees and Glazebrook 2003).

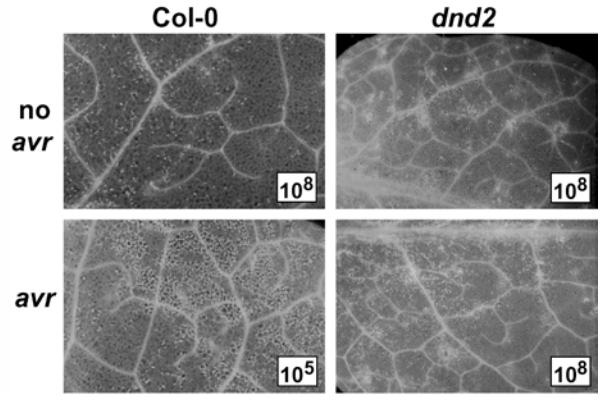

Fig. 1. *dnd2* plants are defective in execution of the hypersensitive response (HR) in response to avirulent *P. syringae*. Leaves sampled 24 h after inoculation. Leaf cells that have undergone the HR are visible as autofluorescent flecks (leaf veins are constitutively autofluorescent). Note that the Col-0/*avr* leaf in this figure (the HR$^+$ positive control) was inoculated with 5×10^5 CFU/ml, a 400-fold lower titer of bacteria than the other three leaves, which were inoculated with 2×10^8 CFU/ml of bacteria. Col/*avr* inoculated at 2×10^8 CFU/ml exhibit confluent autofluorescence and total collapse of leaf tissue 24 h after inoculation (not shown). no *avr* = inoculated with *Pseudomonas syringae* pv. *glycinea* R4 pVSP61 (no cloned *avr* gene); *avr* = inoculated with *P. syringae* pv. *glycinea* R4 pV288 (expressing *avrRpt2*).

In addition to SA-dependent pathways, SA-independent pathways are also activated in response to various pathogens (Conrath et al. 2002; Dong 1998, 2001; Pieterse and van Loon 1999). At least some of these SA-independent pathways require the signaling molecules jasmonic acid (JA) and ethylene. These signaling pathways had previously been characterized in relation to wounding responses (Hildmann et al. 1992) but now have been associated with some aspects of disease resistance. When activated, these pathways can elicit an induced systemic resistance (ISR) of moderate strength that is effective against a different spectrum of pathogens than SAR (Conrath et al. 2002; Penninckx et al. 1996; Pieterse and van Loon 1999; Ton et al. 2002). The *PDF1.2* gene is expressed during ISR but not SAR and serves as a widely used marker for ISR and JA and ethylene–mediated defense signaling (Dong 2001; Penninckx et al. 1996).

The mutant screen that produced the *dnd1* line also yielded other quantitative loss-of-HR mutants (Yu et al. 2000). Among these was the *dnd2* mutant line, which together with the *dnd1* mutant exhibited the strongest suppression of HR (Yu et al. 2000). In the present study, we used a map-based cloning approach to reveal that *DND2* encodes a CNG ion channel, *AtCNGC4*. As we completed map-based cloning of *DND2*, Balague and colleagues (2003) reported that the *Arabidopsis hlm1* mutant also carries a mutation in *AtCNGC4*. The present work also reports our molecular and physiological characterization of *dnd2* plants.

RESULTS

Microscopic observation demonstrates that *dnd2* plants are HR$^-$.

The plant line originally called Y3 but subsequently referred to as *dnd2* was isolated in a screen for *Arabidopsis* mutants that exhibit an altered HR following inoculation with *P. syringae* that express *avrRpt2* (Yu et al. 2000). This line bore superficial resemblance to the *Arabidopsis dnd1* mutant line identified in the same screen. Genetic mapping (presented below; Yu et al. 2000) indicated that two separate genes are mutated in *dnd1* and *dnd2*. Both *dnd* lines exhibit a dwarf rosette phenotype and dramatic reduction of the macroscopically visible HR produced not only in response to *P. syringae* that express *avrRpt2* but also to *P. syringae* expressing *avrRpm1* or *avrB* (Yu et al. 2000). Disruption of the HR controlled by more than one *avr/R* combination implied that the *dnd2* line carries a relatively general alteration of defense responses.

To confirm the absence of HR cell death in *dnd2* mutants, leaf tissues were examined by fluorescence microscopy to detect autofluorescence 24 h after pathogen inoculation. Wild-type Col-0 and *dnd2* were inoculated with low and high concentrations of *P. syringae* pv. *glycinea* R4 that is nonvirulent on *Arabidopsis* Col-0, or *P. syringae* pv. *glycinea* R4 expressing *avrRpt2*, which elicits the HR on *Arabidopsis* plants that express *RPS2*. Figure 1 depicts the results for *dnd2* plants inoculated with the high bacterial titer and for control Col-0 plants inoculated at high or low bacterial titer as noted. *P. syringae* pv. *glycinea* R4 (no *avr* gene) applied at the low concentration (5×10^5 CFU/ml) elicited only a background of isolated fluorescent flecks in Col-0 or *dnd2* leaves (data not shown). When plants were inoculated with 2×10^8 CFU/ml of

P. syringae pv. *glycinea* R4 (no *avr*), a similar or slightly higher background of isolated fluorescent cells was observed (Fig. 1, upper panels). When *P. syringae* pv. *glycinea* R4 expressing *avrRpt2* was applied at 5×10^5 CFU/ml, an elevated number of isolated fluorescent cells was observed in wild-type Col-0 (on average, roughly 2 to 20% of all cells; Fig. 1, lower left panel), as expected for the wild-type HR at this level of pathogen. The *dnd2* plants did not show this elevated rate of cell death. Inoculation with the high 2×10^8 CFU/ml concentration of *P. syringae* pv. *glycinea* R4 expressing *avrRpt2* induced the expected strong HR in Col-0 (confluent autofluorescence and macroscopically visible tissue collapse; data not shown). However, most *dnd2* leaves displayed only the background of rare fluorescent cells at a rate similar to that observed in the *P. syringae* pv. *glycinea* R4 controls, even when subjected to 2×10^8 CFU/ml of *P. syringae* pv. *glycinea* R4 expressing *avrRpt2* (Fig. 1, lower right panel). An increase in autofluorescent cell death relative to the no-*avr* control was observed in approximately one in ten *dnd2* leaves inoculated with 2×10^8 CFU/ml of *P. syringae* pv. *glycinea* R4 expressing *avrRpt2* and then only in isolated areas that represented a fraction of the inoculated tissue.

In separate experiments, HR cell death was monitored using Evans blue dye to stain dead cells. Results were consistent with the data from fluorescence detection. The majority of the *dnd2* leaves examined showed little or no HR cell death over that observed in negative controls, but in a minority of leaves, patches of stained cells could be observed in a subset of the inoculated tissue (data not shown). Hence, as was previously observed for *dnd1* mutants (Yu et al. 1998), loss of HR in *dnd2* plants is widespread even at high pathogen titers but is quantitative rather than complete.

The *dnd2* mutant retains gene-for-gene resistance.

To test whether the suppression of HR in *dnd2* mutants is associated with a loss of disease resistance, the in planta growth of *P. syringae* pv. *tomato* DC3000 (virulent on *Arabidopsis* Col-0) and *P. syringae* pv. *tomato* DC3000 expressing *avrRpt2* (avirulent on *Arabidopsis* Col-0) was monitored in Col-0 and *dnd2* plants. The *dnd2* mutant limited the growth of *P. syringae* pv. *tomato* DC3000 *avrRpt2* similar to the Col-0 control plants, suggesting that *dnd2* plants retain gene-for-gene resistance in the *avrRpt2/RPS2* pathway (Fig. 2). Similar results were observed for both *avrRpm1* and *avrB/RPM1* pathways (Fig. 2 and data not shown). Resistance mediated by *avrRPS4/RPS4* is intermediate in Col-0, and we noted that this resistance was enhanced reproducibly in *dnd2* plants (Fig. 2 and data not shown). The fact that restriction of growth of avirulent *P. syringae* pv. *tomato* DC3000 was substantially stronger than the restriction of virulent *P. syringae* pv. *tomato* DC3000 in *dnd2* plants confirms that gene-for-gene–mediated defense induction was operative despite the general absence of the HR.

The *dnd2* line exhibits elevated resistance to virulent pathogens.

Growth of virulent *P. syringae* pv. *tomato* DC3000 was reduced in *dnd2* plants, relative to the bacterial growth observed in wild-type Col-0 (Fig. 2). Disease symptoms were also reduced when *dnd2* plants were inoculated with *P. syringae* pv. *tomato* DC3000; necrotic lesions and surrounding chlorosis were observed in Col-0 leaves four to six days after inoculation, while leaves of the *dnd2* mutants were uniformly free of disease (data not shown). This is consistent with the observed reductions in bacterial growth and suggests that *dnd2* plants are in a state resembling SAR. The gene-for-gene resistance against avirulent *P. syringae* was stronger than the constitutive SAR-like resistance against virulent *P. syringae* (Fig. 2).

***PR-1* gene expression is elevated yet inducible in *dnd2* plants.**

A number of *Arabidopsis* constitutive defense mutants, such as the *dnd1*, *cpr*, *cim*, and *acd* mutants, have been shown to exhibit constitutively elevated expression of some PR genes (Glazebrook 2001). We examined PR gene expression in *dnd2* plants. When blots of total RNA from leaf samples were hybridized with the *PR-1* gene probe, no significant expression was observed in noninoculated Col-0 plants, but noninoculated *dnd2* mutants constitutively expressed *PR-1* (Fig. 3 and data not shown). In Col-0, levels of *PR-1* mRNA were induced 24 h after infection by *P. syringae* pv. *tomato* DC3000 and were induced to a greater extent in response to *P. syringae* pv. *tomato* DC3000 expressing *avrRpt2* (Fig. 3 and data not shown). This trend of PR gene induction being stronger during incompatible responses than during compatible responses has been observed in numerous plant-pathogen interactions. In three of five independent experiments, mutant *dnd2* plants exhibited a substantial increase in *PR-1* gene expression following inoculation with avirulent *P. syringae*, beyond the elevated basal levels of expression. In *dnd2* plants inoculated with virulent *P. syringae* pv. *tomato* DC3000 (and with DC3000 *avrRpt2*$^+$ in two of the five experiments), *PR-1* remained high relative to wild-type Col-0 but exhibited minimal or no elevation relative to noninoculated *dnd2* plants. Overall, constitutively elevated resistance in *dnd2* plants was associated with constitutively elevated PR gene expression, and the gene-for-gene signal transduction that leads to stronger elevation of PR gene expression remained functional.

The *dnd2* mutants have enhanced salicylic acid production.

SA has been shown to induce PR gene expression, and elevated levels of SA (endogenous or applied) can lead to broad-spectrum resistance (Ryals et al. 1996). To investigate possible mechanisms for the elevated resistance and constitutively expressed PR genes in the *dnd* mutants, we analyzed the SA content of leaf samples from *dnd2* plants. Levels of endogenous free SA and SA-glucoside from wild-type Col-0 and *dnd2* plants were determined. Figure 4 illustrates the high levels of free SA and SA-glucoside present in noninoculated *dnd2* plants as opposed to noninoculated wild-type Col-0 plants. Constitutively high SA levels provide at least a partial mecha-

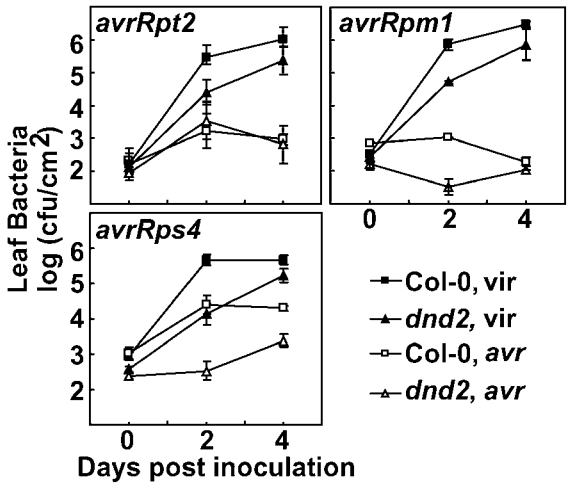

Fig. 2. The suppression of the hypersensitive response in *dnd2* plants does not lead to loss of gene-for-gene resistance, but the *dnd2* plants restrict growth of virulent bacteria to a greater extent than do wild-type plants. The growth of *Pseudomonas syringae* pv. *tomato* DC3000 carrying no avirulence gene (vir) or *avrRpt2*, *avrRpm1*, or *avrRPS4* was assessed in *Arabidopsis* Col-0 and *dnd2* plants.

nistic explanation for the observed defense elevation state of *dnd2* plants.

Expression of *nahG* in *dnd2* plants removes the constitutive PR gene expression and elevated resistance to virulent pathogens but not the loss of HR.

To place the *dnd2* mutation with respect to SA-mediated defense signaling pathways, *dnd2* plants containing the bacterial salicylate hydroxylase gene (*nahG*$^+$) were generated. Expression of *nahG*$^+$ has been shown to effectively remove SA from plants (Gaffney 1993), but very recent work suggests that *nahG*$^+$ expression can block plant defense responses through SA-independent mechanisms as well (Heck et al. 2003; van Wees and Glazebrook 2003; discussed below). In the present study, homozygous *dnd2* plants were either crossed to the well-characterized *Arabidopsis* Col-0 *nahG*$^+$ plant line B15 (Lawton et al. 1995) and progeny lines homozygous for the *dnd2* mutation and carrying the *nahG*$^+$ gene were then identified. or homozygous *dnd2/dnd2* plants were transformed with the 35S-*nahG*$^+$ construct originally used to make line B15. The effect on the growth of virulent and avirulent *P. syringae* in *dnd2* plants was then tested. Expression of *nahG*$^+$ disrupted both the elevated partial resistance against a normally virulent pathogen as well as the gene-for-gene resistance against an avirulent pathogen (Fig. 5A). The loss of resistance to virulent and avirulent *P. syringae* correlated with a loss of constitutive *PR-1* gene expression of *dnd2* plants expressing *nahG*$^+$ (Fig. 5B).

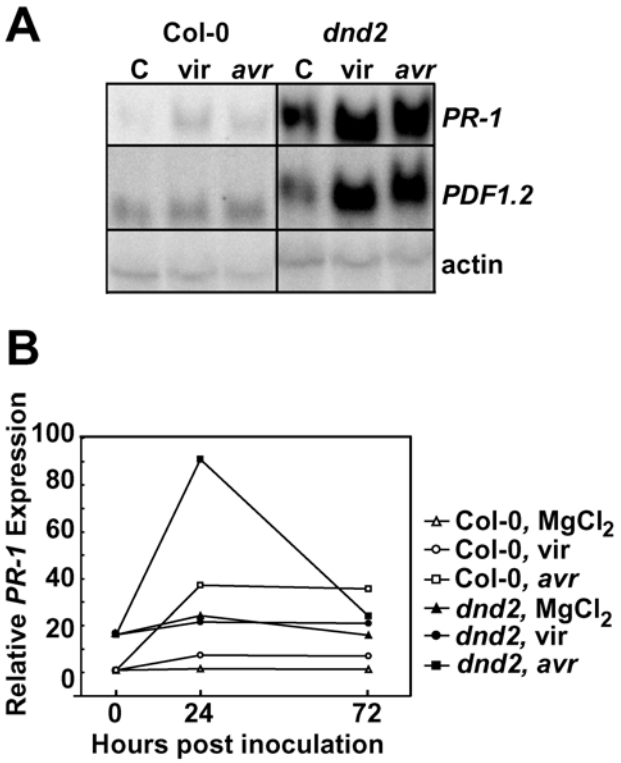

Fig. 3. *dnd2* plants exhibit constitutive *PR-1* expression and pathogen-inducible *PDF1.2* and *PR-1* expression. Plants were inoculated with either *Pseudomonas syringae* pv. *tomato* DC3000 carrying no avirulence gene (vir) or *avrRpt2* (*avr*) or were mock-inoculated ($MgCl_2$). **A,** Northern blot from one experiment, probed successively with *PR-1*, *PDF1.2*, and actin (loading control). RNA was extracted 24 h after inoculation. **B,** *PR-1* expression in a second, independent experiment. Blot was probed successively with *PR-1* and *β-ATPase* (loading control), and the phosphorimager readout was quantified. Relative *PR-1* mRNA levels were calculated by normalizing to *β-ATPase* mRNA level for that sample, with all samples then normalized to the noninoculated Col-0 t_0 sample.

While removal of SA, other *nahG*$^+$ effects, or both greatly impacted the disease resistance phenotypes of *dnd2* plants, other *dnd2* phenotypes were not strongly affected. When rosette diameters were compared, homozygous *dnd2/nahG*$^+$ plants were significantly larger than *dnd2* plants but significantly smaller than wild-type Col-0 or *nahG*$^+$ plants (data not shown). The observation that *dnd2/nahG*$^+$ plants were still "semidwarfed" in leaf and rosette size despite SA removal indicated that factors beyond high SA levels affect the size of mutant *dnd2* plants.

Removal of SA from *dnd2* plants also only partially relieved the HR$^-$ phenotype. As previously reported (Yu et al. 1998), Col-0 plants developed a strong HR within 24 h after inoculation with *P. syringae* expressing *avrRpt2*, while the HR was delayed in Col-0/*nahG*$^+$ plants and was apparent at 48 h but not 24 h. The *dnd2* and *dnd2/nahG*$^+$ plant lines exhibited little or no macroscopically detectable HR at 24 h. In four independent experiments, *dnd2/nahG*$^+$ plants examined at 48 h either continued to show almost no HR or developed mild HR symptoms in only some leaves. Removal of SA via *nahG*$^+$ did not restore homozygous *dnd2* mutants to a HR$^+$ phenotype. As an example, Figure 5C shows the results of one experiment that used four independent homozygous *dnd2/nahG*$^+$ lines obtained via transformation of a *dnd2* parent. At 48 h, the HR-associated tissue collapse was marginally more severe in two or three of the *dnd2/nahG*$^+$ lines relative to *dnd2* controls yet was still notably less severe than in Col-0 plants (Fig. 5C). In two of the *dnd2/nahG*$^+$ lines shown in Figure 5C, SA and SA-glucoside levels were measured. The *nahG*$^+$ transgene reduced SA levels to the limits of accurate detection in line a, but in *dnd2/nahG*$^+$ line b, the salicylate levels remained elevated as for *dnd2* (the average SA + salicylic acid-glucoside (SAG) content per g fresh weight was 4.0 μg/g for line b and 0.5 μg/g for line a). This correlated with the failure of the *nahG*$^+$ construct to alter HR responses in line b as opposed to the other *nahG*$^+$ lines. But even in cases, such as line a, when salicylate levels were significantly reduced by expression of the *nahG*$^+$ transgene, the HR was only restored to *dnd2* mutant plants to a minor extent (Fig. 5C).

JA-associated gene expression is activated in *dnd2* plants.

In light of the above observation that some *dnd2* phenotypes are SA-independent, we examined the expression of the ethylene and JA–associated indicator gene *PDF1.2*. As also reported by others, *PDF1.2* expression in wild-type Col-0 was minimal before and after infection by virulent or avirulent *P. syringae*. Noninoculated *dnd2* plants also did not exhibit much *PDF1.2* expression (Fig. 3). Intriguingly and in contrast to Col-0, *dnd2* plants exhibited substantial *PDF1.2* expression upon treatment with virulent or avirulent *P. syringae* (Fig. 3).

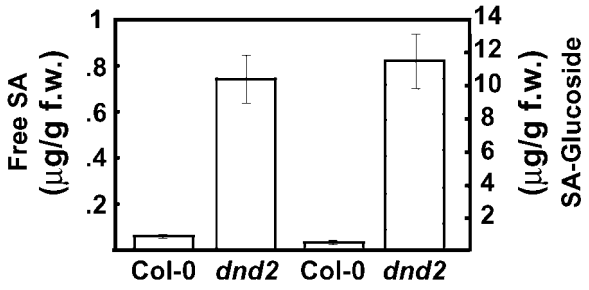

Fig. 4. *dnd2* plants exhibit elevated levels of salicylic acid (SA). Open bars (left y-axis) report free SA; hatched bars (right y-axis) report glucoside-conjugated forms of SA in Col-0 and *dnd2* plants.

***DND2* encodes a cyclic nucleotide-gated ion channel, *AtCNGC4* (*HLM1*).**

In order to isolate and characterize the gene responsible for the *dnd2* phenotypes, a genetic mapping project was carried out. Crosses of *dnd2* to wild-type Col-0 and No-0 ecotypes yielded F_1 individuals that exhibited an HR^+ phenotype and were wild type in size. An F_2 population from a *dnd2* × No-0 cross segregated for HR^+:HR^- plants in a ratio that was consistent with a 3:1 ratio ($\chi^2 = 0.57$, $P = 0.45$), indicating that a single recessive locus is responsible for the HR^- phenotype of *dnd2* plants. The dwarfed phenotype cosegregated with the HR^- phenotype. Crosses between homozygous *dnd1/dnd1* and *dnd2/dnd2* plants also yielded F_1 individuals that exhibited an HR^+ phenotype and wild-type size and appearance, indicating that these two mutant lines carry recessive mutations in separate genes.

The *dnd2* phenotypes showed linkage to markers *LFY3*, nga129, and nga76, all of which map to the lower arm of chromosome 5, and were not linked to markers on any other chromosomes (data not shown). Approximately 1,100 F_2 plants were prescreened for recombination events between flanking genetic markers nga129 and *LFY3* (Fig. 6). Fine-mapping of the *DND2* genetic interval was facilitated by screening an additional 500 F_2 plants, using seven newly developed PCR-based markers (discussed below). Additional fine-mapping of 30 F_2 lines that carried a crossover in this region delineated *DND2* to the genetic interval between markers K19P17-H1 and MDK4-A, with three and one recombination events, respectively (Fig. 6). Based on the relatively small size of this genetic interval (approximately 200 kb), a candidate gene approach was then taken to identify the *DND2* gene.

Since *DND1* encodes a CNG ion channel (*AtCNGC2*), we began our candidate gene approach by sequencing *AtCGNC4* (At5g54250), a CNG ion channel family member located in the *DND2* genetic interval. Sequence analysis revealed that *dnd2* plants contain a G to A point mutation at tryptophan (amino acid 89) leading to a premature stop codon in the first putative transmembrane-spanning domain of *AtCGNC4*. Sequence data were verified by developing a dCAPS marker that detects the *dnd2-1* mutation (discussed below). In order to confirm mapping and sequencing data, *dnd2-1* plants were transformed with a binary vector containing wild-type *AtCNGC4*, driven by its native promoter. T_1 *dnd2-1* plants transformed with the *AtCNGC4* construct exhibited rosettes that were comparable in size to that of wild-type Col-0 plants, whereas *dnd2-1* plants (nontransformed) and *dnd2-1* lines transformed with the empty vector exhibited dwarf rosettes (Fig. 7). In addition to size complementation, HR assays performed on T_1 lines demonstrated functional complementation of *dnd2-1* plants by *AtCNGC4* (Fig. 7). All *dnd2* + *AtCNGC4* T_1 lines tested exhibited an HR in response to avirulent *P. syringae*.

The above data demonstrate that the mutant phenotypes of *dnd2* plants are caused by disruption of the gene encoding *AtCNGC4*. Recently, Balague and associates (2003) described the *Arabidopsis hlm1* mutants in which *AtCNGC4* is mutated and phenotypes similar to *dnd2* are observed. The *dnd2* and *hlm1* mutations define the same gene, which previously had been designated *AtCNGC4.*

DISCUSSION

An earlier screen to identify unknown components of the *avrRpt2-RPS2* disease resistance pathway of *Arabidopsis* produced mutants defective in the *RPS2*-mediated HR, most notably the well-characterized *dnd1* mutant (Clough et al. 2000; Yu et al. 1998, 2000). In this study, we sought to characterize the *dnd2* mutant line. We found that *dnd2* plants exhibit similar morphologies and disease phenotypes to *dnd1* plants. Map-based cloning revealed that *dnd2* phenotypes are due to loss of the CNGC4 cyclic nucleotide-gated ion channel protein, which is a strikingly similar result to the previous discovery that *dnd1* phenotypes are caused by loss of CNGC2 (Clough et al. 2000). Like the *dnd1* mutants, *dnd2* plants are dwarfed and fail to develop the HR in response to avirulent *P. syringae* yet carry out effective gene-for-gene resistance. Mutant *dnd1* and *dnd2* plants also exhibit constitutively high expression of *PR-1,* elevated levels of SA, and display an elevated "SAR-like" resistance in response to virulent pathogens. The gene-for-gene interactions that we tested typically signal for HR cell death, but the HR is somehow blocked in *dnd* plants. HR sup-

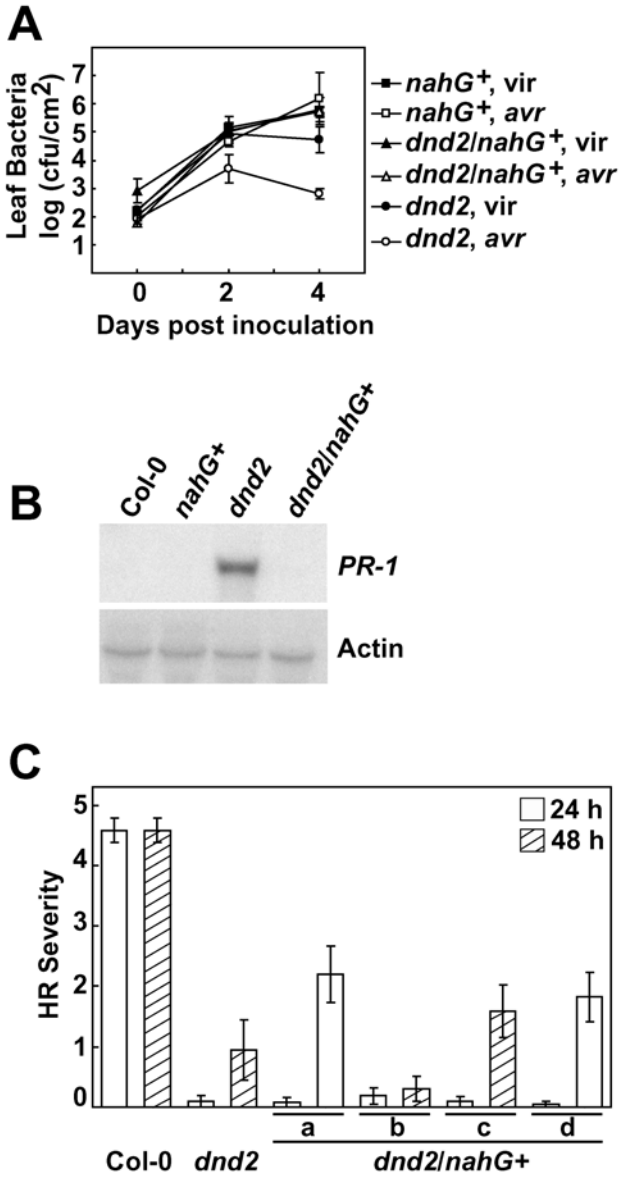

Fig. 5. A, Degradation of salicylic acid (SA) in *dnd2* plants by *nahG*$^+$ leads to the loss of disease resistance. Growth of *Pseudomonas syringae* pv. *tomato* DC3000 carrying no avirulence gene (vir) or *avrRpt2* (*avr*) was assessed in *nahG*$^+$, *dnd2* and *dnd2/nahG*$^+$ plants. **B,** *dnd2* plants that express *nahG*$^+$ lose constitutive *PR-1* expression. RNA was isolated from noninoculated 1-month-old wild-type Col-0, *nahG*$^+$, *dnd2*, and *dnd2/nahG*$^+$ plants. **C,** Hypersensitive response (HR) development in four independent homozygous *dnd2/nahG*$^+$ lines (a, b, c, d) obtained via transformation of a *dnd2* parent. Note that SA and SA-glucoside levels were not effectively reduced in transgenic *dnd2/nahG*$^+$ line b. HR severity was rated 24 and 48 h after inoculation with *P. syringae* pv. *glycinea* R4 *avrRpt2*$^+$.

pression and the other phenotypes are apparently caused by loss of CNG channel function, but the effect may be direct or relatively indirect. HR suppression may be the result of the constitutively activated defenses, but this would have to be via a relatively novel mechanism, as many other *Arabidopsis* mutants that exhibit constitutive defenses do not have the HR$^-$ phenotype. Alternatively, the lesion that primarily causes HR disruption might secondarily lead to activated defenses. How the loss of CNG channels in *dnd1*, *dnd2*, and *hlm1* mutants disrupts the HR remains unclear; this will be the focus of future studies.

We tested the hypothesis that SA mediates many or all phenotypes of *dnd2* plants. SA is known to induce PR genes, and the induction of these genes could lead to the induced "SAR-like" state of the *dnd2* plants. Removal of SA using the *nahG*$^+$ transgene (Gaffney 1993; Lawton et al. 1995) rendered *dnd2*/*nahG*$^+$ plants more susceptible to virulent and avirulent pathogens, as is also seen in wild-type plants expressing *nahG*$^+$ (Fig. 5A). Increased bacterial populations on *dnd2/nahG*$^+$ plants were correlated with the loss of constitutive *PR-1* gene expression (Fig. 5B). These data were consistent with data obtained for *dnd1/nahG*$^+$ lines (Clough et al. 2000) and could imply that SA is required for the resistance to *P. syringae* observed in *dnd*2 mutants. However, in light of recent findings by van Wees and Glazebrook (2003) and Heck and associates (2003), it remains possible that the loss of disease resistance in *dnd/ nahG*$^+$ lines is only partially or not at all due to elimination of SA. Catechol excess in the cell (van Wees and Glazebrook 2003) or other catechol-independent effects of *nahG*$^+$ (Heck et al. 2003) may also play a role. We interpret all *nahG*$^+$ results with caution and have initiated study of *sid2/dnd* double mutants to further address the issue.

The HR$^-$ and dwarf phenotypes of *dnd2* plants appear to be more clearly attributable to a factor other than excessive SA. The *nahG*$^+$ transgene has pleiotropic impacts, but it does remove SA. Yet removal of SA from *dnd2* plants using *nahG*$^+$ did not restore the wild-type HR; there was only some minor abatement of the HR$^-$ phenotype (Fig. 5C). The same was true for the dwarf phenotype of *dnd2* plants.

One can postulate that SA removal would have restored the HR to *dnd* mutants but other pleiotropic *nahG*$^+$ effects then made the HR go away again. This, however, seems inconsistent with data for other *Arabidopsis* mutants. The introduction of *nahG*$^+$ or *npr1* into another HR$^-$ mutant, *agd2*, caused loss of disease resistance in response to *P. syringae* expressing *avrRpm1* but restored the HR (Rate and Greenberg 2001). In an *acd6* genetic background, *nahG*$^+$ completely relieved the dwarf rosette phenotype, suppressed disease resistance to *P. syringae*, and restored the HR (Rate et al. 1999). The double mutant *acd6 npr1* was only partially susceptible to *P. syringae*, suggesting the involvement of an *NPR1*-independent pathway (Rate et al. 1999). Furthermore, introducing *nahG*$^+$ or *npr1* into the HR$^-$ background of the *hrl1* mutant completely restored the ability to elicit the HR (Devadas and Raina 2002). The HR was only restored in those *hrl1 npr1* plants that did not exhibit a lesion-mimic phenotype (Devadas and Raina 2002). The disease resistance phenotypes of *hrl1*/*nahG*$^+$ and *hrl1 npr1* double mutants were not reported (Devadas and Raina 2002; Devadas et al. 2002). Based on the above differences, *agd2*, *hrl1*, *dnd1*, and *dnd2* (*hlm1*) appear to suppress the HR at different points of the cell death signaling cascade.

The persistence of the HR$^-$ phenotype in *dnd2*/*nahG*$^+$ plants suggested that SA-independent pathways are also activated in *dnd* plants. The SA-independent defense activation pathways that signal through ethylene and JA have received considerable attention in recent years (Conrath et al. 2002; Pieterse and van Loon 1999). These pathways, which had first been implicated in wound- and insect-induced responses, also mediate activation of some pathogen-inducible genes and ISR (Conrath et al. 2002; Thomma et al. 1999). *PDF1.2* has become a standard marker for ethylene- and JA-dependent defense pathways (Penninckx et al. 1996) and was used in the present study to characterize *dnd2* plants (Fig. 3). In contrast to wild-type *Arabidopsis* Col-0, *PDF1.2* gene expression levels were significantly induced in *dnd2* plants that had been infected with either virulent or avirulent *P. syringae*. This suggests that mutation of *DND2* (*AtCNGC4*) primes the plant to respond to

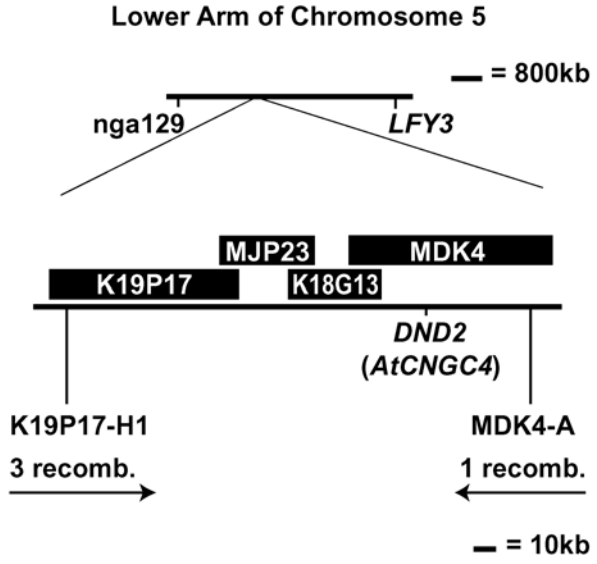

Fig. 6. Physical mapping of *DND2* locus. The top line represents the genomic region on chromosome V between markers nga129 and *LFY3*, to which the *DND2* locus was first mapped. The lower line represents approximately 200 kb from this region, delimited by the markers K19P17-H1 and MDK4A. Rectangles denote BAC clones carrying genomic DNA that spans the smaller *DND2* genetic interval. The number of recombination events identified between *dnd2* and each marker is noted.

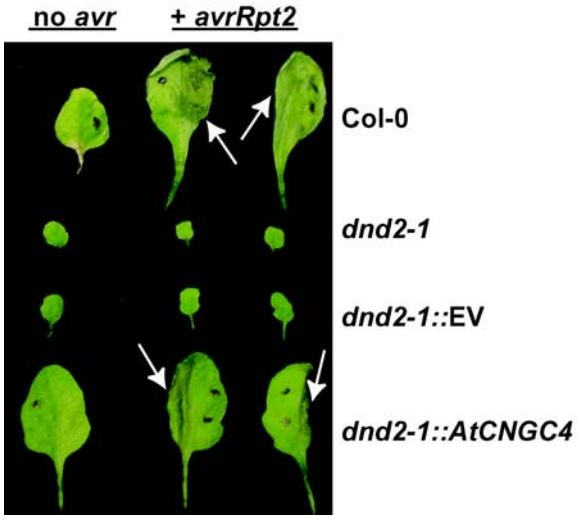

Fig. 7. Functional complementation of *dnd2-1* with *AtCNGC4*. *dnd2-1* plants were transformed with an empty vector (EV) or with *AtCNGC4* under the control of its native promoter. The photograph was taken 48 h after Col-0 and *dnd2-1*::*AtCNGC4* plants were inoculated with *Pseudomonas syringae* pv. *glycinea* R4 pVSP61 (no avr) or pV288 (*avrRpt2*) to assess the hypersensitive response (HR). *dnd2-1* and *dnd2-1*::EV plants were not inoculated, due to their small size. Arrows point to regions on Col-0 and *dnd2-1::AtCNGC4* leaves that exhibit the HR.

invading pathogens with both SA-dependent and JA- and ethylene-dependent defense responses. Activation of SA pathways has previously been shown to reduce flux through JA-dependent pathways, due in part to competition for the regulatory protein NPR1 (Heil and Bostock 2003; Spoel et al. 2003), making the dual effect of the *dnd2* mutation unusual (constitutive SA together with *P. syringae*-inducible JA pathways) (Van Wees et al. 2000). Large-scale expression profiling studies did reveal sets of genes that are activated by both SA and JA treatment (Schenk et al. 2000). However, simultaneous activation of *PR-1* and *PDF1.2*, two genes that are often used to report SA and ethylene and JA signaling, respectively, suggests that additional cell signaling networks may be activated in *dnd2* plants following infection by *P. syringae*. Balague and associates (2003) did not observe any increase in *PDF1.2* gene expression in *hlm1* plants in response to an avirulent *Xanthomonas* strain, possibly because the pathogen-induced *PDF1.2* activation in *AtCNGC4* mutants may be pathogen specific.

Following the discovery that *dnd1* phenotypes are caused by mutation of *AtCNGC2* (Clough et al. 2000), it is interesting that *AtCNGC4* is now similarly implicated in plant defense signaling (this study; Balague et al. 2003). Animal CNG ion channels are generally semiselective cation channels (Zagotta and Siegelbaum 1996). Recent studies with *Arabidopsis* CNG channels have revealed the multiple ions that these channels conduct and either regulate the levels of, are regulated by, or both. Recent studies are also revealing the multiple roles that CNG channels play in normal plant physiology. Leng and associates (2002) have shown that heterologous expression of *AtCNGC2* (*DND1*) allows cyclic nucleotide-dependent conductance of K^+ and Ca^{2+} but discriminates against Na^+, whereas *AtCNGC1* equally conducts K^+ and Na^+ in human embryonic kidney cell lines (Hua et al. 2003). Preliminary electrophysiology studies of *AtCNGC4* (*DND2*/*HLM1*) demonstrated conductance of both K^+ and Na^+ in a cyclic nucleotide-dependent fashion (Balague et al. 2003). Whole plant phenotypes have also been examined. Seedlings containing a T-DNA insertion in *AtCNGC1* exhibited greater tolerance to increasing concentrations of lead (Sunkar et al. 2000). Plants with mutations in *AtCNGC2* displayed Ca^{2+} hypersensitivity in agar media or in soil carrying physiologically normal levels of Ca^{2+}, and these plants did not exhibit K^+ or Na^+ hypersensitivity (Chan et al. 2003). Also relevant to calcium homeostasis, calmodulin-binding domains are common in CNG ion channels and calmodulin binding has been demonstrated for AtCNGC1 and AtCNGC2 (Kohler and Neuhaus 2000; Kohler et al. 1999). *AtCNGC2* (*DND1*) is a necessary component for normal plant development (Chan et al. 2003; Yu et al. 1998). An association of *AtCNGC2* with cell death events was suggested not only in studies of *dnd1* mutants (Clough et al. 2000) but also in β-glucuronidase reporter fusion studies that showed that *AtCNGC2* (*DND1*) is expressed in plant regions exhibiting dehiscence or senescence (Kohler et al. 2001). The CNG ion channel family in *Arabidopsis* contains 20 members, including *DND1* and *DND2* (*HLM1*) (Maser et al. 2001).

It is intriguing that *dnd1* and *dnd2*, mutations in two different but related ion channels, yield plants with strikingly similar phenotypes. The mutant phenotypes of homozygous mutant *dnd1* or *dnd2* plants, which carry the wild-type allele of the other *dnd* locus, suggest that *DND1* (*AtCNGC2*) and *DND2* (*AtCNGC4*) are not functionally redundant. Although the two proteins are only 56% similar, in phylogenetic trees AtCNGC2 and AtCNGC4 are more closely related to each other than to any of the other predicted CNG ion channel gene products (Kohler et al. 1999; Maser et al. 2001; Talke et al. 2003). It is possible that the two proteins form a heterotetramer channel, as demonstrated in olfactory and visual organ systems (Kaupp and Seifert 2002; Trudeau and Zagotta 2003) and that disruption of any part of this ion channel complex leads to the HR^-/constitutive-SAR phenotype. Massively parallel signature sequencing (MPSS) data indicate that the two genes are both expressed in shoot tissue (Talke et al. 2003). However, the two proteins may function independently. As also suggested in the discussion by Balague and associates (2003), localized differences in the site or timing of gene expression, channel regulation, or channel ion specificity may account for the failure of *DND1* (*AtCNGC2*) and *DND2* (*AtCNGC4*) to exhibit functional redundancy. The wild-type appearance and HR^+ phenotypes that we observed in *DND1/dnd1*, *DND2/dnd2* double heterozygote plants demonstrate that simultaneous reduction of gene dosage at both loci is not sufficient to cause obvious phenotypic defects, but complete loss of either channel protein causes *dnd* phenotypes. Preliminary data suggest that loss of both channels has an additive impact. F_2 progeny from *dnd2/dnd2* × *dnd1/dnd1* crosses included plants, appearing at very roughly a $^1/_{16}$ rate, that exhibited a pronounced extreme dwarf, "super-*dnd*" compact and curled leaf phenotype distinct from the phenotypes of *dnd1* or *dnd2* single mutants (I. Yu, G. I. Jurkowski, and A. F. Bent, *unpublished data*). This latter finding must be regarded as preliminary, because the putative *dnd1/dnd1, dnd2/dnd2* double mutant plants grew extremely poorly, were generally infertile, and were not studied further. The main point remains, however, that loss of either relatively similar protein causes similar phenotypes, yet the proteins are not functionally redundant. The identification of two separate CNG ion channel-encoding genes for which a mutation causes loss of the HR raises the testable hypothesis that mutations in some of the other 18 putative CNG ion channel genes may result in similar defense phenotypes.

It remains to be determined whether or not wild-type CNG ion channels play a relatively direct regulatory role in plant defense activation in nonmutant plants. Regardless, the disruption of ion-mediated cellular regulation caused by the *dnd1* or *dnd2* mutations offers a useful tool for further dissection of the events that control plant defense activation and generation of HR cell death.

MATERIALS AND METHODS

Plant growth conditions.

Arabidopsis plants were planted in Sunshine Mix #1 (Sun Gro Horticulture, Bellevue, WA, U.S.A.) and were grown in growth chambers or growth rooms at 22°C under short-day conditions (9-h light/15-h dark). Plants were fertilized by bottom watering with dilute Miracle-Gro (Scotts-Miracle-Gro Products, Marysville, OH, U.S.A.) on a weekly basis. A lesion mimic phenotype has been observed in some batches of *dnd2* plants, but its appearance is environmentally dependent and occurs very rarely in our growth conditions. The *dnd2* plants used in the present studies did not exhibit macroscopically visible lesion mimic phenotypes.

Macroscopic and microscopic HR assays.

P. syringae pv. *glycinea* Race 4 carrying *avrRpt2* (pV288) or vector alone (pVSP61) was used (Kunkel et al. 1993; Yu et al. 1993). Leaves were inoculated with 5×10^5 or 2×10^8 CFU per ml of *P. syringae* pv. *glycinea* R4 pV288 or *P. syringae* pv. *glycinea* R4 pVSP61, using 1-ml plastic syringes with no needle. For high-titer inoculations that induce confluent HR across the inoculated region, the HR was scored visually on a 0 to 5 scale based on leaf tissue collapse (0 = no collapse, 5 = complete collapse) (Klement 1964). For fluorescence microscopy, the inoculated leaves were removed from plants 24 h

after inoculation and were fixed in FAA (2% formaldehyde, 5% acetic acid, 40% ethanol) for 15 min. The fixed tissues were sequentially washed with 50% and then 95% ethanol to remove chlorophyll (Yu et al. 1993). Leaf parenchyma cells were observed by fluorescence microscopy, using a fluorescein filter set (Ex 495 ± 20 nm, Em > 505 nm) (Klement 1990). Evans Blue staining was performed as previously described (Yu et al. 1998).

Bacterial growth assay in plants.

Bacterial growth within leaves was monitored by vacuum infiltration of leaves with 10 mM $MgCl_2$ with 1×10^5 CFU/ml *P. syringae* pv. *tomato* DC3000 carrying either *avrRpt2*, *avrRpm1*, *avrRPS4*, or no *avr* gene, followed by dilution-plating of homogenized leaf tissue on selective media as previously described (Yu et al. 1998).

Gene expression studies.

Plants were vacuum-infiltrated with 1×10^5 CFU per ml of *P. syringae* pv. *tomato* DC3000 carrying either *avrRpt2* or no *avr* gene. After infiltration, plants were returned to the growth chamber and above-ground plant tissue was collected 24 h after inoculation. Total RNA was extracted using Trizol (Invitrogen Corp., Carlsbad, CA, U.S.A.) according to manufacturer's instructions. *PR-1* and *PDF1.2* probes were amplified using primers 5′-GTAGGTGCTCTTGTTCTTCCC, 5′-CAC ATAATTCCCACGAGGATC, 5′-TCATGGCTAAGTTTGCT TCC, and 5′-AATACACACGATTTAGCACC, respectively. Primers for actin loading control were 5′-AGGTTCTGTTC CAG and 5′-TTAGAAGCATTTCC. RNA blot assays and other molecular biology methods were as described (Ausubel 1998).

Quantification of salicylic acid.

Both free SA and SAG were quantified from noninoculated leaf tissue as described (Uknes et al. 1993). Between 0.2 and 0.5 g of leaf tissue per sample was utilized.

Construction of *dnd2*/*nahG*$^+$ lines.

Homozygous *dnd2* plants were crossed to the characterized Col-0 homozygous *nahG*$^+$ line B15 (obtained from CIBA-Geigy/Syngenta, Research Triangle Park, NC, U.S.A.) (Lawton et al. 1995). F_1 progeny from this cross were selfed, and homozygous *dnd2*/*nahG*$^+$ F_2 lines were verified based on genetic segregation of F_3 and F_4 progeny. Plants were tested for homozygosity of *nahG*$^+$ transgene by plating on Murashige-Skoog agar medium containing 50 μg of kanamycin per ml. The *dnd2* phenotype was apparent from the size of plants grown on soil, as rosette size was either consistently large, consistently medium-small, or segregated 3:1 (large/medium-small) in *nahG*$^+$ plant lines. Additional *dnd2*/*nahG*$^+$ lines were generated by *Agrobacterium* floral dip transformation (Clough and Bent 1998) of *dnd2* plants with pCIB200 expressing *nahG* (Lawton et al. 1995).

Mapping.

Genetic mapping was carried out using DNA from F_2 individuals or F_3 families derived from crosses of Col-0 (*dnd2*/*dnd2*) × No-0. PCR-based cleaved amplified polymorphic sequence (CAPS) markers and simple sequence length polymorphism (SSLP) markers that cover the *Arabidopsis* genome were used for initial linkage analysis (Bell and Ecker 1994; Konieczny and Ausubel 1993). To narrow down the genetic interval that contained the *DND2* locus, restriction fragment length polymorphism markers g4130, m233, mi69, and g3844 were used (Lister 1993; Liu et al. 1996; Nam et al. 1989). In addition, several new PCR-based markers detecting Col-0/No-0 polymorphisms were designed during the course of *DND2* map-based cloning. New SSLP and CAPS markers (named according to BAC clone) are as follows, MXC20-AT (5′-AGCCAAACGGAGAAGTTTGT, 5′-TCAACACGAAAT TACCCCAAT), MFH8-AT (5′- AAGTGGTTTTTGGATTTG GTTT, 5′- TTGGGAGAAATGGTTTTCCTT), K19E1-1A (5′-AACATTCCCCGTTCCCTATC, 5′- CTGTCTTGCCCACCA AGTTT), MNC6-A (5′- GGAAGAAATGATTCTCTGTATTT CTCA, 5′- TGATTCATCTCTGTAATGCAAACC), MGN6-B2 (5′-TCTTGCAAAATGGGAGGTTC, 5′-AGCTCCCAGTT GCAGAAAAG) (*Bam*HI polymorphism), K19P17-H1 (5′-GC ATTGGATTTGGGAAAGAA, 5′-CATCCCACAGGGGATTT ATG) (*Mbo*I polymorphism), MDK4-A (5′- TTGACAATTTG TTAGCCTTGGA, 5′- GGAACCAGCGAAACCCTAAT), and K13P22-CTT (5′- TTCGTAAATTCGTCTGCTTGA, 5′- ACC TGTACGTTGCCCAAGTT). The *dnd2-1* mutation was verified using dCAPS primers 5′-TCCAAATGGGTTCGAGC AT and 5′-GCAATCTTGAACTGAATCC, which generates a new *Nla*III site in the PCR product (Michaels and Amasino 1998; Neff et al. 1998).

Complementation.

Three independent PCR products of *AtCNGC4* (At5g54250) were generated from Col-0 genomic DNA using primers (5′-CGTGGTGAGACCAAAAATGA, 5′- CGACAAGGATCACA TTCAACA) and the Expand Long Template PCR system (Roche Applied Science, Indianapolis, IN, U.S.A.). Primers annealed 1.3 kb upstream and 0.7 kb downstream of the *AtCNGC4* open reading frame. Independent PCR products were directly cloned using the Zero Blunt TOPO PCR cloning kit (Invitrogen) and then were digested with *Pst*I and *Bam*HI and were cloned into the binary vector pCAMBIA 3300. Transformation of *dnd2-1* plants with *AtCNGC4* constructs was performed by floral dip, as previously described (Clough and Bent 1998). T_1 seeds were sterilized and plated on selection plates containing either phosphinothricin (10 μg/liter) and carbencillin (500 μg/liter) or phosphinothricin alone. Resistant seedlings were transplanted directly into soil. Putative *dnd2-1*::*AtCNGC4* lines that exhibited wild-type size rosettes were verified using a dCAPS marker for the *dnd2-1* mutation and for the pCAMBIA 3300-derived *bar* gene. HR phenotypes of *dnd2-1*::*AtCNGC4* lines were determined by inoculating T_1 lines with (*P. syringae* pv. *glycinea* R4 pVSP61 (no *avr*) or pV288 (+*avrRpt2*) at an optical density at 600 nm value equal to 0.1.

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