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Arabidopsis thaliana cdd1 mutant uncouples the constitutive activation of salicylic acid signalling from growth defects

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SUMMARY

Arabidopsis genotypes with a hyperactive salicylic acid-mediated signalling pathway exhibit enhanced disease resistance, which is often coupled with growth and developmental defects, such as dwarfing and spontaneous necrotic lesions on the leaves, resulting in reduced biomass yield. In this article, we report a novel recessive mutant of Arabidopsis, *cdd1* (*constitutive defence without defect in growth and development*1), that exhibits enhanced disease resistance associated with constitutive salicylic acid signalling, but without any observable pleiotropic phenotype. Both *NPR1* (*NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1*)-dependent and NPR1-independent salicylic acid-regulated defence pathways are hyperactivated in *cdd1* mutant plants, conferring enhanced resistance against bacterial pathogens. However, a functional *NPR1* allele is required for the *cdd1*-conferred heightened resistance against the oomycete pathogen *Hyaloperonospora arabidopsidis*. Salicylic acid accumulates at elevated levels in *cdd1* and *cdd1 npr1* mutant plants and is necessary for *cdd1*-mediated *PR1* expression and disease resistance phenotypes. In addition, we provide data which indicate that the *cdd1* mutation negatively regulates the *npr1* mutation-induced hyperactivation of ethylene/jasmonic acid signalling.

INTRODUCTION

Plants utilize a multi-layered defence system to control diseases. This includes preformed barriers (e.g. cell wall and the cuticle) and inducible defences. Activation of inducible defences occurs on recognition of pathogen-derived factors or factors produced in response to pathogen activity (Jones and Dangl, 2006). For example, plants have evolved transmembrane pattern recognition receptors to perceive slowly evolving pathogen-associated molecular patterns (PAMPs), resulting in the downstream activation of defences that contribute to PAMP-triggered immunity (PTI). Some pathogens, however, have evolved effectors that are released into the host to suppress PTI (Gimenez-Ibanez *et al*., 2009; Gohre and Robatzek, 2008). To counteract the pathogen, resistant plants have evolved intracellular receptors encoded by *R* (*RESISTANCE*) genes to recognize pathogen-derived effectors, leading to the activation of a mechanism termed 'effector-triggered immunity' (ETI). ETI has been suggested to be an accelerated and amplified PTI response (Jones and Dangl, 2006).

Salicylic acid (SA) plays a key role in PTI- and ETI-mediated defences and has an important influence on plant defences against numerous pathogens (Tsuda *et al*., 2008, 2009). SA levels increase in pathogen-inoculated plants, resulting in the downstream activation of defence genes, including the *PR1* (*PATHOGENESIS-RELATED1*) gene, which has been used as an excellent molecular marker for the activation of SA signalling. Furthermore, the exogenous application of SA and its functional synthetic analogues 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) enhances disease resistance in a variety of plants against pathogens that primarily exhibit a biotrophic life cycle (Glazebrook, 2005). Resistance is also enhanced in plant genotypes that exhibit constitutively elevated levels of SA (Nandi *et al*., 2005; Rate *et al*., 1999; Shah *et al*., 1999, 2001; Vanacker *et al*., 2001). In contrast, the prevention of SA accumulation by the facilitation of its breakdown to catechol in transgenic *NahG* plants expressing a bacterial salicylate hydroxylase results in plants that exhibit heightened susceptibility to numerous pathogens (Delaney *et al*., 1994; Gaffney *et al*., 1993). Similarly, disease resistance is compromised in the *ics1* (*isochorismate synthase 1*) mutant, which is deficient in SA biosynthesis (Wildermuth *et al*., 2001). The *NPR1* (*NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1*) gene is a master regulator for SA signalling in Arabidopsis. Mutant *npr1* plants are more susceptible to a variety of pathogens. In contrast, plants constitutively

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overexpressing *NPR1* exhibit heightened resistance to pathogens in a variety of plant species (Cao *et al*., 1998; Chern *et al*., 2001; Lin *et al*., 2004; Makandar *et al*., 2006, 2010; Parkhi *et al*., 2010). Studies with several mutants that suppress the SA-insensitive phenotype of *npr1* plants have demonstrated that SA also activates *PR1* expression and disease resistance through an *NPR1*-independent mechanism, which is secondary to the *NPR1*-dependent SA pathway (Bowling *et al*., 1997; Clarke *et al*., 1998; Shah *et al*., 1999).

Ethylene (ET) and jasmonic acid (JA) are two other important plant hormones involved in plant defence (Bari and Jones, 2009; Pieterse *et al*., 2009; Spoel and Dong, 2008). Similar to SA, the application of ET and JA, and the constitutive activation of these signalling pathways in some plant genotypes, results in enhanced resistance primarily against pathogens that exhibit a necrotrophic life style. Co-operative and antagonistic interactions between SA and ET/JA signalling have been reported. These interactions presumably fine tune plant defences for optimal expression (Verhage *et al*., 2010).

In Arabidopsis, several mutant and transgenic plants in which either one or more of these defence signalling pathways are constitutively expressed result in heightened resistance to pathogens. However, in most of these cases, these plants with hyperactive defences also exhibit growth defects, such as dwarfing, spontaneous cell death and lesion development (Bolton, 2009; Heidel *et al*., 2004). These defects may result from hormonal imbalances and/or the allocation of resources away from growth and development to sustain the elevated level of these defence mechanisms in these plants. For the future manipulation of these signalling pathways in order to enhance plant disease resistance, it is important to identify the genetic components that de-link the growth and developmental defects from the constitutive expression of defences. In this article, we report the *cdd1* (*c*o*nstitutive* d*efence without growth* d*efect*1) mutant of Arabidopsis, identified as a suppressor of the *npr1-5* mutant phenotype, in which SA accumulation and signalling are constitutively activated without any adverse impacts on growth and development. In addition, the *cdd1* mutation also influences the cross-talk of SA and ET/JA signalling mediated by NPR1 protein.

RESULTS

NPR1-dependent and NPR1-independent defences are hyperactive in the *cdd1* mutant resulting in constitutive *PR1* expression

The *cdd1* mutant was identified in a previously described genetic screen (Shah *et al*., 1999) for mutants that constitutively expressed the SA-inducible *PR1* gene in the *npr1-5* (alias *sai1*) mutant background. As shown in Fig. 1, in comparison with the leaves of the wild-type (WT) and the *npr1-5* mutant plant, in which *PR1* gene expression was undetectable, the *PR1* gene was constitutively expressed at elevated levels in leaves of the *cdd1 npr1-5* double mutant. SA application resulted in a further increase, albeit minor, in *PR1* expression in *cdd1 npr1-5* plants (Fig. 1). However, in comparison with the SA-treated WT plants, *PR1* expression was lower in the SA-treated *cdd1 npr1-5* plants, suggesting that *cdd1* does not completely overcome the *npr1-5* defect. In support of this hypothesis, we observed that *cdd1*-mediated constitutive *PR1* expression was much higher in the *cdd1* single mutant plant, which contains the WT *NPR1* allele, than in the *cdd1 npr1-5* double mutant. The above results suggest that the *cdd1* mutation activates both NPR1-dependent and NPR1-independent mechanisms leading to *PR1* expression.

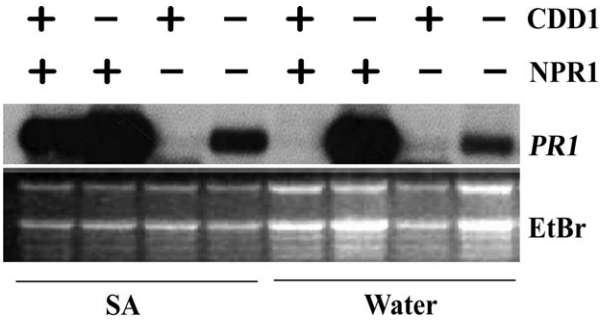

Fig. 1 *PR1* expression in *cdd1* and *cdd1 npr1-5* mutant plants. Four-week-old soil-grown plants were treated with either salicylic acid (SA) or water as control. Total RNA was harvested and probed with a radioactively labelled *PR1* probe. +, wild-type (WT); −, mutant genotype. EtBr, ethidium bromide.

cdd1 confers enhanced disease resistance

To test whether the elevated expression of *PR1* is accompanied by enhanced disease resistance, the *cdd1 npr1-5* and *cdd1* mutant plants were inoculated with a virulent strain of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (referred to as *Pst* DC3000 hereafter) and the bacterial numbers were determined 3 days post-inoculation (dpi). The WT and *npr1-5* plants provided the controls for this experiment. As shown in Fig. 2A, as expected, bacterial numbers were significantly higher in the *npr1-5* mutant relative to the WT. The presence of the *cdd1* allele resulted in a significant reduction in bacterial numbers in the *cdd1 npr1-5* double mutant compared with the *npr1-5* mutant plant. Similarly, the growth of *Pst* DC3000 carrying the *avrRpt2* avirulence gene was also restricted in the *cdd1 npr1-5* double mutant, relative to the *npr1-5* plant, confirming that the *cdd1* allele enhances resistance against virulent and avirulent pathogens in the absence of the WT *NPR1* allele (Fig. 2B). Resistance against virulent and avirulent *Pst* DC3000 was also higher in the *NPR1* allele-containing *cdd1* single mutant plant than in the WT plant (Fig. 2A, B). The *cdd1*

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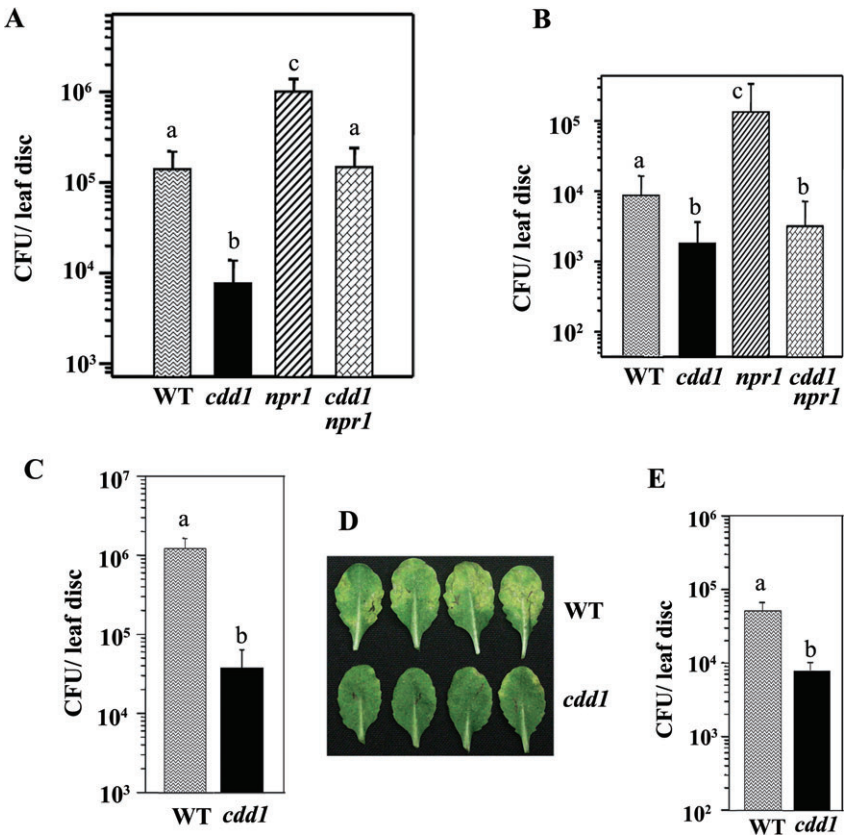

Fig. 2 Growth of pathogens in *cdd1* plants. Four-week-old soil-grown plants were infiltrated with overnight-grown bacterial cultures of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 at 10^5 colony-forming units (CFU)/mL (A), *Pst* DC3000 carrying the avirulence gene *avrRpt2* at 10^5 CFU/mL (B), *Psm* ES4326 at 10^5 CFU/mL (C,D) and *P. syringae* pv. *phaseolicola* at 10^7 CFU/mL (E), each resuspended in 10 mM $MgCl_2$. (A, C, E) Samples were harvested at 3 days post-inoculation (dpi) and CFUs/disc were determined. Each bar represents the average and standard deviation obtained from four samples, each containing five leaf discs of 5 mm in diameter. Statistical differences among samples ($P < 0.01$) are labelled with different letters. (D) Photograph of *Psm* ES4326-infected leaves taken at 3 dpi. WT, wild-type.

mutant also exhibited enhanced resistance against *P. syringae* pv. *maculicola* ES4326 (*Psm* ES4326; Fig. 2C). Disease symptom development was also lower in the *cdd1* mutant relative to the WT plant (Fig. 2D).

To determine whether *cdd1* also had an impact on resistance against nonhost pathogens, a relatively high dose [10^7 colony-forming units (CFU)/mL] of *P. syringae* pv. *phaseolicola* was infiltrated into the leaves of the *cdd1* mutant and WT plants, and the bacterial numbers were monitored at 3 dpi. As shown in Fig. 2E, the bacterial numbers were significantly lower in the *cdd1* mutant relative to the WT.

Mutant *npr1* plants are highly susceptible to the biotrophic pathogen *Hyaloperonospora arabidopsidis* (formerly called *Peronospora parasitica*) (Delaney *et al*., 1995). Previously, it has been shown that *son1*, which is a suppressor of *nim1* (*allelic to npr1*), effectively restores resistance against *H. arabidopsidis* in the *nim1 son1* double mutant (Kim and Delaney, 2002). To test whether the *cdd1* mutation behaves in a similar way in terms of suppression of susceptibility of *npr1-5* against this oomycete pathogen, 2-week-old seedlings of WT, *cdd1*, *npr1-5* and *cdd1 npr1-5* were challenged with *H. arabidopsidis*. As shown in Fig. 3A, *cdd1* mutant plants were significantly more resistant than WT plants ($P < 0.01$) and harboured three times fewer numbers of spores than WT. As expected, *npr1-5* plants were significantly more susceptible than WT. However, surprisingly, the double mutant *cdd1 npr1-5* exhibited only a modest reduction in pathogen growth and sporulation when compared with the *npr1-5* mutant (Fig. 3A). The difference between the resistance levels against *Pst* DC3000 and *H. arabidopsidis* in the double mutant compared with the *npr1* single mutant may be attributed to the difference in the growth stage at which the plants were infected. These results indicate that *cdd1*-mediated resistance against *H. arabidopsidis* requires functional NPR1.

In addition to biotrophic and hemibiotrophic pathogens, SA and NPR1 have been reported to affect resistance to necrotrophic pathogens (Ferrari *et al*., 2003). Thus, we monitored disease development following inoculation with the necrotrophic fungus *Botrytis cinerea* in *cdd1* and *cdd1 npr1-5* double mutant plants, and compared it with that in WT and *npr1-5* plants, respectively. The disease severity was comparable between the *cdd1* mutant and WT plants (Fig. 3B). We noticed an increase in susceptibility to *B. cinerea* in the *npr1-5* mutant in comparison with WT (Fig. 3B). Similar observations have been made previously by Zimmerli *et al*. (2001). However, as shown in Fig. 3B, the presence of the *cdd1* allele restored the WT level of resistance against *B. cinerea* in the *cdd1 npr1-5* double mutant, indicating that the *cdd1* allele also has an impact on defence against this necrotrophic fungus, which is evident in the absence of *NPR1*.

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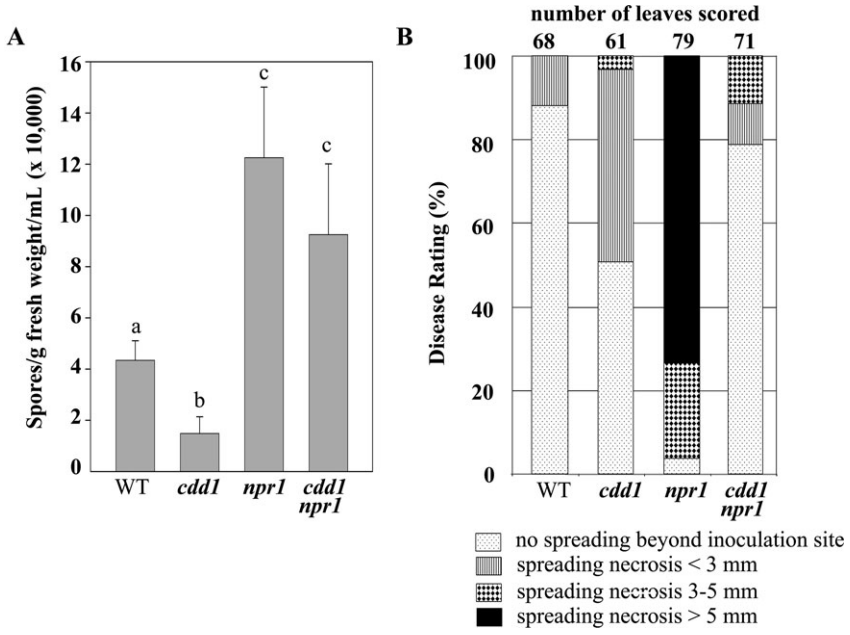

Fig. 3 Growth of *Hyaloperonospora arabidopsidis* (A) and *Botrytis cinerea* (B) in *cdd1* and *cdd1 npr1-5* plants. (A) Two-week-old soil-grown seedlings were sprayed with 10^6 spores/mL of *H. arabidopsidis* resuspended in distilled water. Spore counts were taken at 7 days post-inoculation (dpi). Each bar represents the mean and standard deviation from 55 plants. Statistical differences among samples ($P < 0.01$) are labelled with different letters. (B) Five-week-old soil-grown plants were infected with 5 μL of 7.5×10^5 spores/mL of *B. cinerea* resuspended in half-strength potato dextrose broth. Infected plants were scored at 3 dpi with a comparative scale of disease development. The number of leaves scored for each genotype is given at the top of the bars. WT, wild-type.

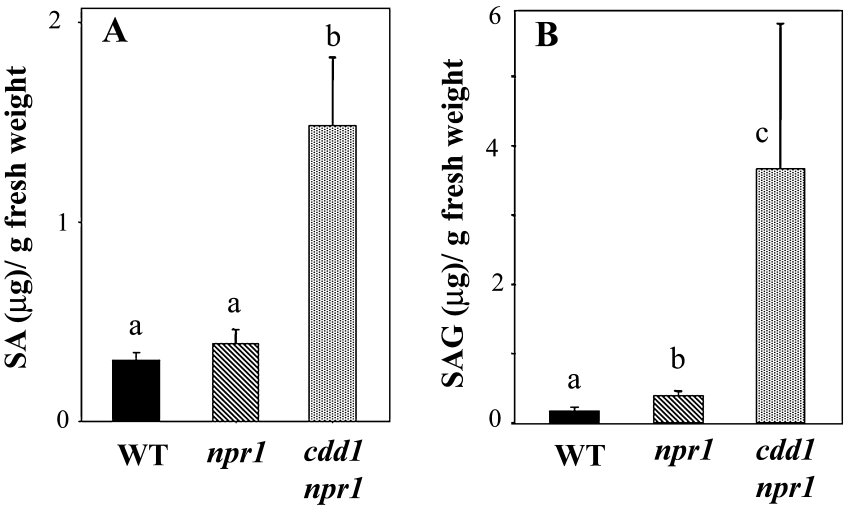

Fig. 4 Salicylic acid (SA) accumulation in wild-type (WT) and *cdd1 npr1-5* plants. Free SA (A) and bound SA (glucoside, SAG) (B) were estimated from the leaves of 4-week-old soil-grown plants. All values are the mean and standard deviations of five samples and expressed as mg/g fresh weight. Statistical differences among samples ($P < 0.05$) are labelled with different letters.

Elevated SA content is associated with *cdd1*-mediated constitutive *PR1* expression and heightened disease resistance

High-performance liquid chromatography (HPLC) analysis indicated that the basal content of SA and its glucoside (SAG) were four and 10 times higher, respectively, in the *cdd1 npr1-5* mutant relative to the WT and *npr1-5* mutant plants (Fig. 4). In addition, the expression of the *ICS1* gene, which is required for SA biosynthesis (Wildermuth *et al*., 2001), was also elevated in *cdd1* and *cdd1 npr1* mutants (Fig. S1, see Supporting Information). We therefore hypothesized that the *cdd1*-mediated constitutively high expression of *PR1* and the enhanced disease resistance phenotypes result from this elevated accumulation of SA and the corresponding hyperactivation of SA signalling. To test this hypothesis, the *cdd1* mutant was crossed with a *NahG* transgenic plant, which expresses an SA-degrading salicylate hydroxylase, and the segregating F2 population from this hemizygous plant was analysed. As *NahG* expression was dominant, 28 of 37 plants expressed the *NahG* gene (Fig. 5A) and none of the plants showed *cdd1*-mediated constitutive *PR1* expression. Of the nine plants lacking *NahG* expression, two (Fig. 5A, plants 1 and 26) showed a constitutively high level of *PR1* expression, typical of *cdd1* plants, and two other plants (Fig. 5A, plants 11 and 18) showed only modest basal expression, not associated with the *cdd1* mutation, as confirmed in the F3 population (data not shown). To further ascertain the negative correlation between the presence of the *NahG* gene and *cdd1*-mediated

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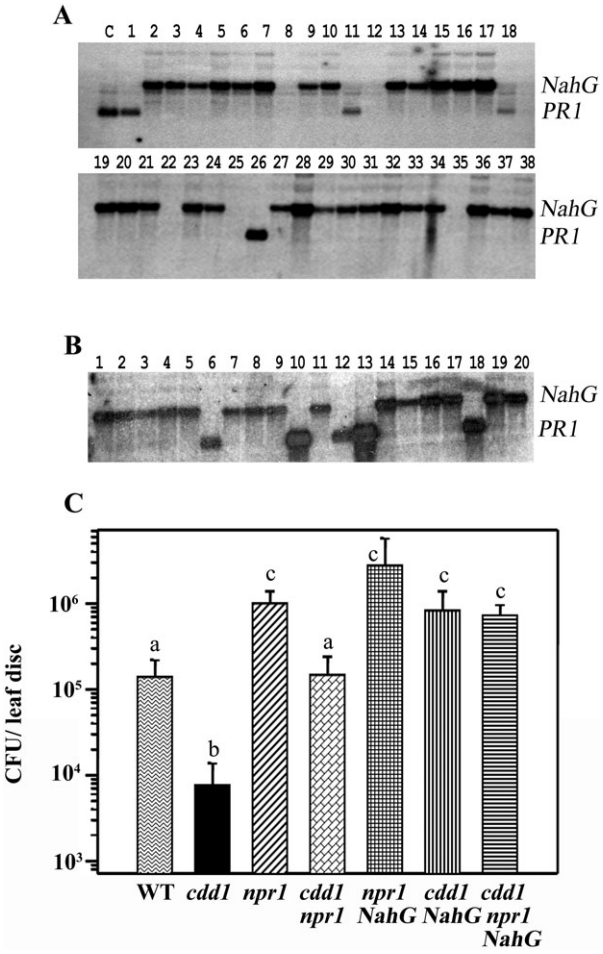

Fig. 5 Salicylic acid (SA) accumulation is required for *cdd1*-mediated *PR1* mRNA accumulation and disease resistance. (A, B) Northern blot for *PR1* and *NahG* expression in F2 and F3 populations of *cdd1* × *NahG* cross. (A) F2 plants. (B) F3 plants from F2 13. Numbers indicate the plant numbers; C, control. Probes are indicated on the right-hand side of the blot. (C) Growth of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 at 3 days post-inoculation (dpi) of 5-week-old soil-grown plants at10^5 colony-forming units (CFU)/mL. Each bar represents the average and standard deviation obtained from four samples, each containing five leaf discs of 5 mm in diameter. Statistical differences among samples ($P < 0.05$) are labelled with different letters. WT, wild-type.

constitutive *PR1* expression, several F2 plants were randomly selected and their F3 progeny were analysed to identify plants that were homozygous for the *cdd1* allele, but hemizygous for *NahG*. The F3 population of *cdd1 NahG*/+ plants showed perfect negative correlation of *PR1* and *NahG* expression (Fig. 5B), confirming that SA was required for *cdd1*-dependent *PR1* expression. The *cdd1*-mediated enhanced disease resistance was also attenuated in the *cdd1 NahG* and *cdd1 npr1 NahG* plants (Fig. 5C).

cdd1 and *cdd1 npr1-5* double mutations de-link constitutive SA signalling and defects in growth and development

Constitutive activation of SA signalling in several previously characterized mutants is accompanied by detrimental phenotypes, including dwarfing, spontaneous lesions and cell death (Bolton, 2009; Heidel *et al*., 2004). In contrast, the *cdd1* and *cdd1 npr1-5* mutants did not exhibit any obvious morphological and growth abnormalities compared with the WT plant under the conditions utilized in this study (Figs 6A, B and S2, see Supporting Information). No significant difference in biomass was observed between the *cdd1* and WT plants (Fig. 6C). The *cdd1* and *cdd1 npr1-5* mutants also did not exhibit any visible lesions or microscopic cell death phenotype, which have been reported in other constitutive SA signalling mutants (Bolton, 2009; Moeder and Yoshioka, 2008), suggesting that the *cdd1* and *cdd1 npr1-5* mutants de-link the activation of SA signalling from growth defects. Although, very rarely, we noticed a slight reduction in the size of the *cdd1* single mutant, the *cdd1 npr1-5* double mutant cultivated in parallel invariably showed a normal morphology that was indistinguishable from WT or *npr1-5* plants, despite exhibiting hyperactive SA signalling, as reported in the previous sections.

cdd1 mutation suppresses pathogen-induced JA/ET-dependent gene expression in *npr1-5* mutant plants

Arabidopsis mutants, such as *ssi1* (Nandi *et al*., 2003a; Shah *et al*., 1999), *dnd1*, *cpr6* (Jirage *et al*., 2001) and *cpr5* (Clarke *et al*., 2001; Jirage *et al*., 2001), are lesion mimics that accumulate high levels of SA and, at the same time, constitutively activate the ET/JA-inducible gene *PDF1.2* (*PLANT DEFENSIN1.2*). In contrast, mutants such as *ssi2* (Nandi *et al*., 2005) and *cpr1* (Jirage *et al*., 2001) do not show high basal expression of *PDF1.2*. The triple response of etiolated seedlings to ET after 1-aminocyclopropane-1-carboxylic acid (ACC) treatment and root growth inhibition after methyl jasmonate (MeJA) treatment in *cdd1* plants showed WT responses (Fig. S3, see Supporting Information). Moreover, we could not detect any basal expression of *PDF1.2* through Northern blot analysis in the *cdd1* and *cdd1 npr1-5* double mutant plants (data not shown). However, through real-time polymerase chain reaction (PCR), we detected a slight up-regulation of expression of *PDF1.2* and the co-regulated transcription factor gene *ORA59* (Pre *et al*., 2008) in *cdd1* and *cdd1 npr1-5* compared with WT and *npr1* plants, respectively (Fig. 7 and S4, see Supporting Information). *NPR1* has been reported to mediate cross-talk in SA-mediated suppression of ET/JA signalling (Spoel *et al*., 2003). Moreover, as discussed in the previous section, although the *cdd1* mutation had

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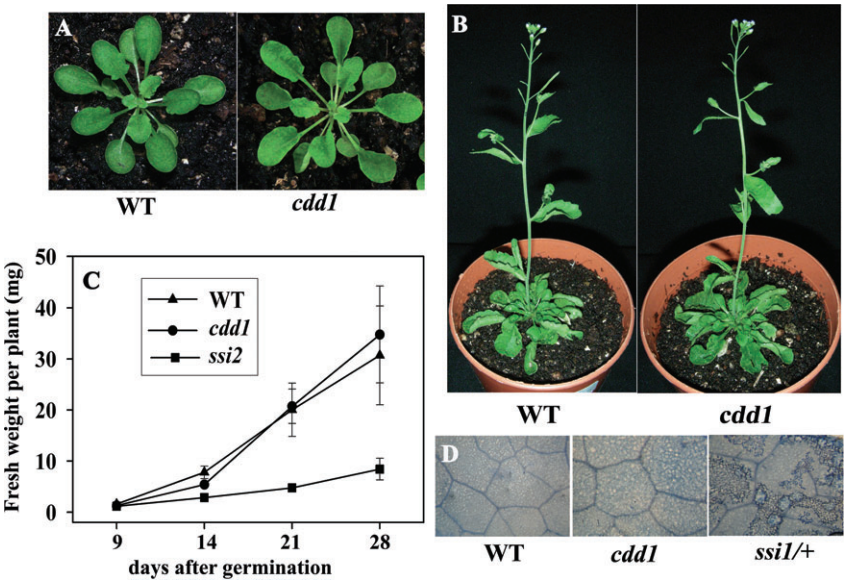

Fig. 6 Phenotype and biomass of *cdd1* mutant plants. (A) Four–week-old soil-grown plants. (B) Seven-week-old soil-grown plants. Photographs of wild-type (WT) and *cdd1* plants were taken at the same magnification. (C) Biomass comparison of WT, *cdd1* and *ssi2* plants. Each time point indicates the mean and standard deviation from 10 randomly selected plants. (D) Trypan blue staining of leaves of the indicated genotype.

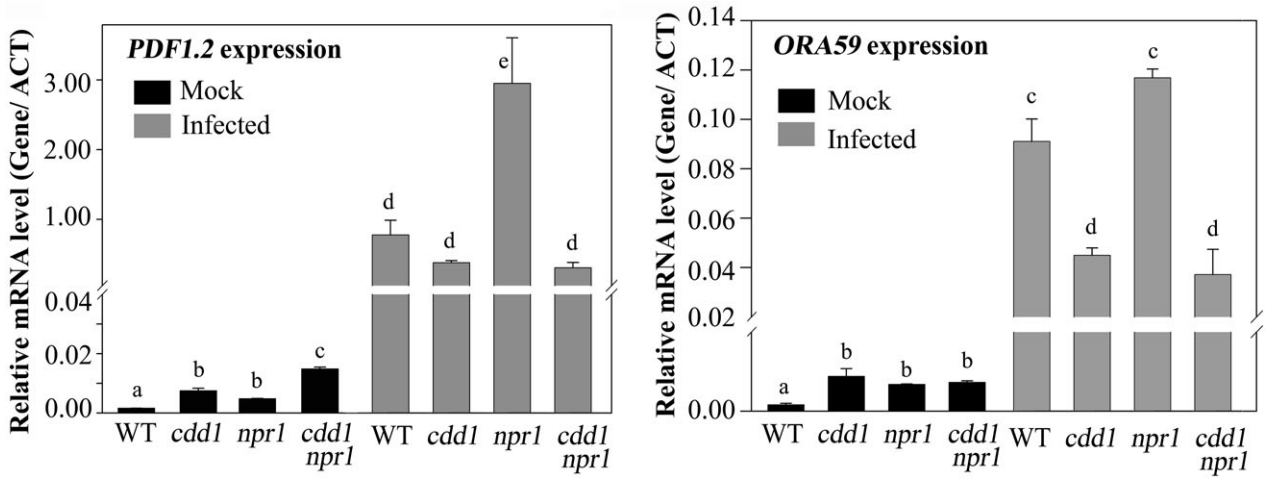

Fig. 7 The *cdd1* mutation suppresses the *npr1-5*-mediated expression of *PDF1.2* and *ORA59* after *Botrytis cinerea* infection. Five-week-old soil-grown plants were infected with 5 μL of 7.5×10^5 spores/mL of *B. cinerea* suspended in half-strength potato dextrose agar (PDA) or mock inoculated with only half-strength PDA. Samples were harvested 36 h post-inoculation and relative gene expression levels were determined by quantitative real-time polymerase chain reaction. Each bar represents the average and standard deviation of two samples. Statistical differences among samples ($P < 0.05$) are labeled with different letters over the bars. ACT, actin.

little effect on the resistance response against *B. cinerea*, it effectively suppressed the enhanced susceptibility of *npr1-5* mutant plants (Fig. 3B). Thus, we tested the *B. cinerea* infection-induced expression of *PDF1.2* and *ORA59* in the *cdd1* and *cdd1 npr1-5* double mutant plants. Consistent with disease symptom development and the role of *NPR1* in the SA-mediated suppression of ET/JA signalling, we noticed elevated *PDF1.2* and *ORA59* expression in the *npr1-5* mutant plant compared with WT (Fig. 7A, B). However, the presence of the *cdd1* allele effectively suppressed the *npr1-5*-determined high-level expression of *PDF1.2* and *ORA59* in response to *B. cinerea* infection (Fig. 7A, B). It has been reported previously that ET can bypass the requirement of NPR1 for SA-mediated suppression of JA signalling (Leon-Reyes *et al*., 2009). Thus, we estimated the level of ET production in *B. cinerea*-infected plants. ET production in *B. cinerea*-infected *cdd1 npr1* plants was at an equivalent level to that in WT and *cdd1* mutant plants and at a lower level than in *npr1* mutants (Fig. S5, see Supporting Information). Thus, *cdd1*-mediated suppression of JA signalling in *cdd1 npr1* double mutants is independent of ET accumulation in these plants.

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Genetics of the *cdd1* allele

Constitutive *PR1* expression was not observed in F1 plants derived from a cross between the *cdd1* mutant and the parental WT plant, suggesting that the *cdd1* allele is recessive to the WT *CDD1* allele. Indeed, in the F2 generation, 16 of a total of 72 plants exhibited the constitutive *PR1* expression phenotype, which is very close ($\alpha^2 = 0.296$, d.f. = 1, $N = 72$, $P = 0.59$) to the expected 1 : 3 ratio for a single-locus recessive trait, confirming the recessive nature of the *cdd1* allele.

In order to map the *cdd1* locus, the mutant plant, which is in the accession Nössen (Nö-0), was crossed with a WT plant of accession Columbia (Col-0). As expected of a recessive *cdd1* allele, the F1 hybrids did not constitutively express *PR1* at an elevated level (data not shown). The constitutive *PR1* expression phenotype segregated in the F2 progeny derived from these F1 hybrids. However, the overall extent of *PR1* expression showed a larger variation in this F2 population than that observed when *cdd1* was crossed to its parental accession Nö-0, suggesting that other loci from the accession Col-0 modify the *cdd1*-determined *PR1* expression. Only the segregants that constitutively expressed *PR1* at high levels were used to map *cdd1*. In any given mapping population, only 16%–18% of the F2 progeny showed a high level of *PR1* expression, as opposed to the 25% expected of a single recessive allele. With the help of 180 such high-*PR1*-expressing F2 plants, the *cdd1* locus was mapped to the lower arm of chromosome 5, between the LFY and G2368 markers, 21.6 cM from LFY and 7.1 cM from G2368. This region does not contain any gene that, when mutated, is reported to have phenotypes similar to that of *cdd1* (constitutive elevated SA signalling without any adverse impact on plant morphology).

DISCUSSION

Hyperactivation of SA signalling contributes to *cdd1*-mediated enhanced disease resistance

We have identified a novel Arabidopsis mutant *cdd1*, which is hyper-resistant to virulent, avirulent and nonhost pathogens. SA signalling, which was hyperactive in *cdd1*, was a major contributor to the *cdd1*-determined hyper-resistance. The *NahG*-encoded salicylate hydroxylase attenuated the *cdd1*-determined constitutive expression of *PR1* and heightened disease resistance. As the *cdd1*-determined *PR1* expression and resistance in the absence of NPR1 in the *cdd1 npr1-5* double mutant also required SA, we suggest that both NPR1-dependent and NPR1-independent SA-stimulated mechanisms are hyperactive in the *cdd1* mutant background. JA and ET signalling have been shown previously to contribute to SA- and NPR1-independent resistance in *cpr6* and *ssi1* plants, both of which also suppress *npr1*-determined enhanced disease susceptibility (Bowling *et al*., 1997; Clarke *et al*., 1998; Nandi *et al*., 2003a; Shah *et al*., 1999). Similarly, JA and ET may contribute to the SA-independent resistance in *cdd1*-containing plants. Indeed, basal expression of JA/ET-regulated *PDF1.2* and *ORA59* in *cdd1* plants was modestly higher than in WT plants. Whether these arms of the JA- and ET-regulated defence mechanisms contribute to the *cdd1*-determined resistance remains to be resolved. Moreover, we cannot rule out the possibility that the residual amount of SA in the *cdd1 NahG* and *cdd1 npr1-5 NahG* plants contributes to the remnant resistance.

cdd1 bypasses the NPR1 dependence of SA-mediated suppression of ET/JA signalling

Signalling cross-talk between plant hormones, such as SA, ET and JA, fine tunes the plant defence response (Pieterse *et al*., 2009). Both synergistic and antagonistic interactions between SA and ET/JA signalling pathways have been reported (Leon-Reyes *et al*., 2009; Nandi *et al*., 2003a). One of the prominent established interactions is the SA-induced suppression of JA signalling mediated by NPR1 (Spoel *et al*., 2003). However, the induction of ET signalling through either exogenous ET application or pathogen challenge can bypass the requirement of the NPR1 protein for SA-mediated suppression of JA signalling (Leon-Reyes *et al*., 2009). The *cdd1 npr1-5* double mutant produced WT levels of ET in both unchallenged and *B. cinerea*-challenged plants (Fig. S4); however, the *cdd1* mutation effectively bypassed the NPR1 dependence of SA-mediated suppression of the ET/JA responsive marker genes *PDF1.2* and *ORA59* (Fig. 7). These findings tempt us to postulate that the *cdd1* mutation is involved in the SA-mediated, NPR1-independent suppression of ET/JA signalling, as observed under conditions of high ET (Leon-Reyes *et al*., 2009).

The *cdd1* mutation uncouples constitutive defences from growth defects

All reported Arabidopsis mutants in which the SA signalling mechanism is hyperactivated, including the reported suppressors of *npr1* with hyperactive SA signalling, have growth and/or developmental defects. For example, the *ssi1* and *ssi2* mutants exhibit dwarfing and spontaneous cell death (Shah *et al*., 1999, 2001). The *cpr1*, *cpr5* and *cpr6* mutants are dwarfs, with *cpr5* also exhibiting cell death (Bowling *et al*., 1997; Clarke *et al*., 1998). Although the *son1* (suppressor of *nim1-1*) mutant, which was identified as a suppressor of the *npr1* allele *nim1-1*, does not exhibit a growth defect, it does not constitutively express *PR1* at elevated levels and does not contain constitutively elevated SA content (Kim and Delaney, 2002). Furthermore, the *son1*-dependent enhanced resistance phenotype was not attenuated by *NahG*, suggesting that the *son1*-determined enhanced

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resistance is caused by an SA-independent mechanism (Kim and Delaney, 2002). Constitutive plant defence and its association with the development of a lesion mimic phenotype have also been demonstrated recently in the Arabidopsis *Atsr1* mutant (Du *et al*., 2009). The *AtSR1* gene is a negative regulator of plant immunity. The mutation results in the faster activation of SA-dependent defences in response to pathogen infection when plants are cultivated at 25–27 °C. However, when cultivated at 19–21 °C, the *Atsr1* mutant plants accumulate elevated levels of SA, constitutively express *PR* genes and exhibit a short stature, indicating that the constitutive activation of defences in *Atsr1* is accompanied by a detrimental growth/developmental phenotype.

To the best of our knowledge, the *cdd1* mutant reported here is the only mutant plant of Arabidopsis known to date that uncouples constitutive activation of SA signalling and growth defects. Like all previously reported suppressors of *npr1*, the *cdd1* mutant accumulates high levels of SA, resulting in the constitutive activation of SA signalling and SA-dependent plant defences. Yet, unlike the other *npr1* suppressor mutants, *cdd1* does not display growth and developmental abnormalities (Figs 6 and S1). The recessive nature of the *cdd1* mutant allele suggests that the *CDD1* WT allele has a negative impact on SA accumulation. The expression of the SA biosynthesis gene *ICS1* increases on infection with *Erysiphe* and *P. syringae* and contributes to pathogen-induced SA accumulation in Arabidopsis (Wildermuth *et al*., 2001). Indeed, we noticed a two- to five-fold enhanced expression of the *ICS1* gene in *cdd1* and *cdd1 npr1-5* mutant plants in comparison with the corresponding WT and *npr1-5* mutant plants (Fig. S1), supporting the role of *CDD1* in the regulation of SA biosynthesis. However, it remains to be determined whether SA accumulation in *cdd1* is solely mediated through enhanced *ICS1* gene expression.

EXPERIMENTAL PROCEDURES

Plant growth conditions and pathogen infection

Arabidopsis plants and pathogens were cultivated as described previously (Nandi *et al*., 2003b). In brief, Arabidopsis seeds were germinated on Murashige and Skoog (MS) plates with 1% sucrose, and 9-day-old germinated seedlings were transferred to soil. Plants were grown at 22 °C in a growth room with a 12-h light (80 μE/m/s) and 12-h dark cycle and 65% humidity. *Pst* DC3000 and *Psm* ES4326 infections were carried out exactly as described in Nandi *et al*. (2004), except for the dose of pathogen, which is indicated in the legend to each figure. *Botrytis cinerea* infections were carried out as described previously (Nandi *et al*., 2005). *Hyaloperonospora arabidopsidis* isolate Cala2 maintenance and infection were carried out as described previously (Van der Ent *et al*., 2008). All pathogen infections were repeated at least three times with similar results.

Generation, mapping and genetics of the *cdd1* mutant

The *cdd1* mutant was identified in a screen for suppressors of *npr1-5* (Shah *et al*., 1999). Unlike the other *npr1-5* suppressors (*ssi1*, *ssi2*, *ssi4*) identified in this screen, the *cdd1 npr1-5* mutant did not exhibit any growth defects. To generate the *cdd1* single mutant, *cdd1* was crossed to the parental line 1/8E/5 (Cao *et al*., 1997), which is in the accession Nö. Northern blot analysis was conducted on the F2 progeny to identify plants that constitutively expressed *PR1*. The presence of the WT *NPR1* allele was confirmed by cleaved amplified polymorphic sequence (CAPS) marker, as described previously (Shah *et al*., 1999). To generate the *cdd1 NahG* and *cdd1 npr1 NahG* plants, the *cdd1 npr1* double mutant was crossed with a transgenic *NahG* plant in the accession Nö. The F2 progeny plants were screened by Northern blotting for expression of the *NahG* transgene and by PCR for the presence of the *NPR1* allele (Shah *et al*., 1999).

Chemical treatments

SA treatment of 4-week-old plants was performed as described previously (Shah, 1997). Water-treated plants were used as controls. For the MeJA sensitivity assay, seeds were germinated on MS medium supplemented with 10 μM MeJA. After 3 days of cold treatment, plates were placed vertically in the growth room and the root lengths were measured at 10 days post-germination (Nandi *et al*., 2003b). For ET treatment, MS plates were supplemented with the ET precursor 10 μM ACC. Surface-sterilized seeds were plated onto ACC-containing agar and the plates were wrapped in aluminium foil and kept in the cold for 3 days, followed by a 7-day incubation at 21 °C.

RNA extraction, Northern blotting and quantitative real-time PCR

Leaf tissues were ground in liquid nitrogen and RNA was extracted by guanidinium thiocyanate–phenol–chloroform as described by Chomczynski and Sachhi (1987). Radiolabelled probes were generated using random primer labelling as described previously (Shah *et al*., 1999). cDNA synthesis was performed as described by Beckers *et al*. (2009). Diluted cDNA was used as template for real-time PCR with an ABI-PRISM 7500 (Applied Biosystem, Singapore) sequence detector system. Real-time PCR experiments were repeated at least twice with two replications for each RNA sample. The following primers were used for real-time PCR: PDF1.2F, TTTGCTGCTTTCGACGCAC; PDF1.2R, CGCAAACCCCTGACCATG; Act2-F, AGTGGTCGTA CAACCGGTATTGT; Act2-R, GATGGCATGAGGAAGAGAGAAAC; ICS1-F, CTAATCTCCGCCGTCTCTGAACT; ICS1-R, TTGGAACCTG TAACCGAACGA.

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SA and SAG estimation

Leaves were harvested from 4-week-old soil-grown plants. Leaf samples (0.2 g) were extracted once with 3 mL of 90% methanol and once with 3 mL of 100% methanol. The extracts were combined and dried under nitrogen gas. Total SA and SAG determinations were carried out as described previously (Bowling *et al*., 1994)

ET estimation

Aerial rosettes of 4-week-old plants were incised from the root, weighed and carefully placed in a 35-mL glass vial containing 1 mL of distilled water. Plants were left in open vials for at least 30 min to allow the release of wound-induced ET. Subsequently, four to five leaves of each plant were inoculated with 5 μL of either *B. cinerea* at 7.5×10^5 spores/mL resuspended in half-strength potato dextrose agar (PDA) broth or half-strength PDA broth alone as mock treatment. Immediately after infection, the bottles were closed with a rubber cap and tightened with an aluminium closer. After a specific time interval of infection, 1 mL of air was taken with an airtight injection syringe and the ET content was determined by a gas chromatograph (GC955; Synspec, Groningen, the Netherlands).

Trypan blue staining

Leaves of 4-week-old soil-grown plants were floated in trypan blue staining solution (Rate *et al*., 1999) and heated in a microwave oven for 1 min, with intermittent pausing, to avoid excessive boiling. After de-staining as described by Rate *et al*. (1999), the samples were inspected under a light microscope and photographed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 *ICS1* expression in *cdd1* and *cdd1 npr1* plants. Total mRNA was isolated from 5-week-old soil-grown plants of the indicated genotype. The level of *ICS1* expression was determined by quantitative real-time polymerase chain reaction. Each bar represents the average and standard deviation of two samples. Statistical differences among samples ($P < 0.05$) are labelled with different letters over the bars. WT, wild-type.

Fig. S2 Morphological phenotypes of *cdd1*, *cdd1 npr1-5* and *ssi2* mutants. (A, C) Four-week-old soil-grown plants. (B) Seven-day-old seedlings of the indicated genotypes. WT, wild-type.

Fig. S3 Ethylene (ET) and methyl jasmonate (MeJA) responses in wild-type (WT) and *cdd1* plants. (A) Seeds were germinated in Murashige and Skoog (MS) medium alone (–) or in the presence of 10 μM 1-aminocyclopropane-1-carboxylic acid (ACC) (+) in the dark, and photographs were taken 4 days after germination. (B) Seedlings were grown in MS medium (–) or in the presence of 10 μM MeJA (+). Photographs were taken 10 days after germination.

Fig. S4 Basal levels of *PDF1.2* and *ORA59* gene expression. Leaf samples from 5-week-old soil-grown plants were harvested and the basal expression of the indicated genes was determined by real-time polymerase chain reaction. Each bar represents the average and standard deviation of two samples. Statistical differences among samples ($P < 0.05$) are labelled with different letters over the bars.

Fig. S5 Ethylene (ET) production from wild-type (WT) (W), *cdd1* (c), *npr1-5* (n) and *cdd1npr1-5* (cn) plants after *Botrytis cinerea* infection. Each bar represents the mean and standard deviation of ethylene produced from ten 4-week-old soil-grown plants. Four leaves from each plant were inoculated with 5 mL of *B. cinerea* spores having 7.5×10^5 spores/mL suspended in half-strength potato dextrose agar (PDA) or mock inoculated with

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half-strength PDA, and ethylene production was measured by gas chromatography after the indicated time period. Statistical differences among *B. cinerea*-infected samples ($P < 0.05$) are labelled with different letters over the bars.

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