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Abnormal Callose Response Phenotype and Hypersusceptibility to *Peronospora parasitica* in Defense-Compromised Arabidopsis *nim1-1* and Salicylate Hydroxylase-Expressing Plants

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To investigate the impact of induced host defenses on the virulence of a compatible *Peronospora parasitica* strain on *Arabidopsis thaliana*, we examined growth and development of this pathogen in *nim1-1* mutants and transgenic salicylate hydroxylase plants. These plants are unable to respond to or accumulate salicylic acid (SA), respectively, are defective in expression of systemic acquired resistance (SAR), and permit partial growth of some normally avirulent pathogens. We dissected the *P. parasitica* life cycle into nine stages and compared its progression through these stages in the defense-compromised hosts and in wild-type plants. NahG plants supported the greatest accumulation of pathogen biomass and conidiophore production, followed by *nim1-1* and then wild-type plants. Unlike the wild type, NahG and *nim1-1* plants showed little induction of the SAR gene PR-1 after colonization with *P. parasitica*, which is similar to our previous observations. We examined the frequency and morphology of callose deposits around parasite haustoria and found significant differences between the three hosts. NahG plants showed a lower fraction of haustoria surrounded by thick callose encasements and a much higher fraction of haustoria with callose limited to thin collars around haustorial necks compared to wild type, whereas *nim1-1* plants were intermediate between NahG and wild type. Chemical induction of SAR in plants colonized by *P. parasitica* converted the extrahaustorial callose phenotype in NahG to resemble closely the wild-type pattern, but had no effect on *nim1-1* plants. These results suggest that extrahaustorial callose deposition is influenced by the presence or lack of SA and that this response may be sensitive to the NIM1/NPR1 pathway. Additionally, the enhanced susceptibility displayed by *nim1-1* and NahG plants shows that even wild-type susceptible hosts exert defense functions that reduce disease severity and pathogen fitness.

Additional keywords: 2,6-dichloroisonicotinic acid (INA); disease resistance; downy mildew; ribosomal DNA internal transcribed spacer (ITS).

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Arabidopsis thaliana is host for a variety of pathogenic microorganisms, including bacteria, viruses, fungi, and oomycetes (Dangl 1993; Holub et al. 1995; Mauch-Mani and Slusarenko 1993). The ease of performing genetic analyses in Arabidopsis has led to rapid progress in elucidating molecular mechanisms used by plants for defense against pathogens (Delaney 1997; Glazebrook et al. 1997). *Peronospora parasitica* (Peronosporaceae family) is a naturally occurring oomycete parasite of Arabidopsis and the causal agent of downy mildew disease. Members of Peronosporaceae are obligately biotrophic plant pathogens, establish intimate relationships with a wide range of hosts, and cause significant damage to many crop species (Channon 1981). Parasitism in this group involves the development of hyphal networks within host tissues and production of haustoria that invade host cells. Haustoria penetrate plant cell walls and invaginate the host cell plasma membrane, which is accompanied by stimulated production of additional host plasmalemma (Sargent 1981). It is likely that invaded plant cells undergo changes in membrane composition and a redirection of metabolism in response to parasitism (Spencer-Phillips 1997).

A number of *P. parasitica* strains have been isolated from wild populations of Arabidopsis. These range in virulence on different Arabidopsis accessions from completely incompatible to highly virulent on specific host genotypes (Holub et al. 1994; Koch and Slusarenko 1990; Parker et al. 1993). The *P. parasitica* isolates examined that infect Arabidopsis appear to be homothallic and produce asexual and sexual propagulae within approximately 1 week after inoculation of the plant. Sexual oospores are produced within leaf tissues and released upon decomposition of dead leaves. Asexual conidia are produced from specialized sporangiophores that emerge from stomata on leaf surfaces, and the conidiospores are dislodged easily and carried to other plants by air currents. The progression of *P. parasitica* through its life cycle has been described in susceptible Arabidopsis and other cruciferous species (Kluczewski and Lucas 1982; Koch and Slusarenko 1990), and Chou (1970) provided a detailed examination of the early stages of *P. parasitica* growth in cabbage.

Plants employ a variety of pathogen-induced defenses for prevention of disease, recovery from infection, and resistance against future attack. These responses occur within several temporally distinct modes: i) rapidly, to prevent pathogen

establishment; ii) more slowly, to limit disease progression and facilitate recovery from disease; and iii) persistently, to prevent reinfection such as in the systemic acquired resistance (SAR) response (Delaney 1997). Pathogen-induced responses also differ qualitatively and include SAR (Ryals et al. 1996), induced systemic resistance (ISR) (Pieterse and Van Loon 1999), hypersensitive cell death, and production of activated oxygen species (Hammond-Kosack and Jones 1996). Collectively, these responses constitute a complex defense system analogous to the animal innate immune system, which is ancient and possibly conserved in plants (Belvin and Anderson 1996). Pathogen-induced cell wall fortifications also have been implicated in defense, which is achieved through the cross linking of cell wall proteins (Bradley et al. 1992) or by deposition of glucan polymers such as the (1-3)-β-glucan callose (Aist 1976). These diverse responses are regulated by chemical signals including salicylic acid (SA), jasmonic acid, ethylene, nitric oxide, and probably others that have yet to be described. Moreover, a specific signaling molecule may influence more than one form of defense response (Durner et al. 1998; McDowell and Dangl 2000). For example, the varied roles played by SA in defense are evident from studies of transgenic plants that express the *Pseudomonas putida nahG* gene, which encodes salicylate hydroxylase. NahG tobacco and Arabidopsis plants are unable to accumulate SA nor express SAR (Gaffney et al. 1993) and are hypersusceptible to a wide variety of pathogens, indicating that SA-dependent mechanisms also act to limit the severity of disease or aid in recovery from infection (Delaney et al. 1994). Furthermore, plants unable to accumulate SA are defective in expression of race-specific or gene-for-gene resistance against certain pathogens because some normally avirulent pathogens can grow on NahG tobacco or Arabidopsis plants (Delaney et al. 1994). Together, these observations show that SA-dependent processes contribute to the expression of several qualitatively distinct forms of disease resistance, corresponding to each temporal class of pathogen-induced defense.

To investigate the signal transduction pathways involved in SA responses, several groups have sought Arabidopsis mutants that are defective in responses mediated by SA or chemical analogs such as 2,6-dichloroisonicotinic acid (INA) (Kessmann et al. 1994). These screens yielded at least ten independent SA-insensitive mutants, all of which contain recessive mutations in the same gene called *NIM1*, *NPR1*, or *SAI1* (sai1 alleles, later renamed npr1) (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). Following infection, *nim1/npr1* mutants show normal pathogen-induced accumulation of SA but greatly reduced induction of SAR-associated genes such as PR-1. Additionally, exogenous application of SA or INA to *nim1/npr1* plants neither induces SAR genes nor activates resistance to pathogens, as would occur in wild-type plants. Therefore, the *NIM1/NPR1* gene product acts to couple accumulation of SA to the activation of defense genes and resistance.

Homozygous *nim1/npr1* plants and NahG plants are similar in that each can be considered "immune compromised," with respect to SAR induction. NahG plants, however, exhibit a more severe defense-impaired phenotype than *nim1/npr1* mutants, indicating that SA may control other resistance mechanisms beyond those dependent upon the NIM1/NPR1 pathway. It also has been speculated that the hypersusceptible phenotype of NahG plants may result in part from altered feedback regulation of the phenylpropanoid pathway brought on by SA depletion. It was suggested that this may lead to changes in the synthesis of other products of this pathway and less resistance to disease (Cameron 2000). To understand the complexity of SA-mediated resistance mechanisms will require careful examination of the biochemical and defense phenotypes of wild-type, NahG, and *nim1/npr1* plants.

Immune-compromised plants also can be important substrates on which to observe the growth and development of compatible pathogens with less interference from induced host defenses. This is illustrated by the accentuated virulence displayed by a wide range of pathogens on NahG tobacco and Arabidopsis plants compared with the wild type, showing that even susceptible hosts for phytopathogens exert defense functions that reduce pathogen fitness and constrain disease severity (Delaney et al. 1994). Furthermore, though wild-type hosts may recover from infection by compatible pathogens, *nim1* and NahG Arabidopsis plants often succumb and die, most likely as a result of a failure in activation of induced defense responses (H. Kim and T. Delaney, *unpublished data*). These observations underscore the differences between wild-type plants and those with compromised immune systems, with respect to their suitability as hosts for virulent pathogens.

To assess the roles played by SA and NIM1/NPR1-dependent processes on pathogen virulence, we examined the growth and development of a virulent *P. parasitica* isolate on *A. thaliana* wild-type and immune-compromised NahG and *nim1-1* plants. To facilitate comparisons, all host genotypes were derived from the same *A. thaliana* accession (Ws-0), which were inoculated with the virulent *P. parasitica* isolate Emwa. We dissected the pathogen's life cycle into nine stages, many of which were documented and quantified in each host genotype. We found *P. parasitica* to show the greatest fitness enhancement over wild type in NahG, followed by *nim1-1* plants, on the basis of hyphal development, pathogen rRNA accumulation, and production of conidiophores. To gain insight into the basis for enhanced pathogen growth on NahG and *nim1-1* plants, we measured accumulation of mRNA from the SAR marker gene PR-1 and characterized the abundance and morphology of callose deposits that surround haustoria. Compared with the wild type, NahG and *nim1-1* plants showed much less PR-1 accumulation, as previously observed (Delaney et al. 1994; Delaney et al. 1995). Four types of extrahaustorial callose deposits were defined, and their frequency was assessed in each host genotype. Interestingly, significant differences between host genotypes were apparent with respect to the abundance of callose deposits in the form of collars or thick encasements around haustoria. Application of INA to already infected NahG plants converted the callose profile in these plants to closely resemble the wild-type pattern, commensurate with substantial accumulation of PR-1 mRNA. In contrast, INA had no significant affect on the callose profile of *nim1-1* plants and caused only a small increase in PR-1 mRNA. Together these results suggest that extrahaustorial callose production is enhanced by treatments that activate SAR, and that this induction may involve the NIM1/NPR1 pathway.

RESULTS

Life cycle of *P. parasitica*.

To characterize the growth and development of a virulent *Peronospora* isolate (Emwa1) on wild-type Arabidopsis, we placed 2.0 μl drops of water containing approximately 50 *P. parasitica* conidiospores onto young leaves of susceptible ecotype Ws-0. Leaves were harvested every 2 h postinoculation (hpi) for the first 12 h and every 24 hpi for the next 6 days. Leaf samples were fixed and stained in lactophenol trypan blue, cleared in chloral hydrate, and observed by microscopy to visualize pathogen structures (Uknes et al. 1993). Trypan blue is commonly used to stain necrotic plant cells and fungal tissues that are living at the time of fixation. We dissected the *P. parasitica* life cycle into nine stages (Fig. 1) and recorded the approximate timing of their occurrence in wild-type, *nim1-1*, and NahG plants (Table 1).

By 2 hpi, approximately 25% of the conidiospores had germinated and many had already formed appressoria over the juncture of adjoining epidermal cells, a pattern described previously (data not shown) (Chou 1970; Koch and Slusarenko 1990). The germination frequency did not vary appreciably between host genotypes and was typical of what we normally observe for *P. parasitica* on Arabidopsis. We noticed that conidia landing close to a juncture between adjacent epidermal cells would form an appressorium directly, without a noticeable germ tube. Spores more distant from a juncture, however, would produce conspicuous germ tubes (typical is 7 to 11 μM) (Fig. 1A) that would extend until a juncture was reached. Germinated spores showed little trypan blue staining, whereas their germ tubes and appressoria stained darkly. This staining pattern may reflect the cytoplasmic contents moving out of the spore into newly formed structures in preparation for penetration (Chou 1970; Mendgen and Deising 1993).

From 4 and 8 hpi, the pathogen had not progressed beyond appressorium formation, but by 10 to 12 h, penetration of the leaf surface had occurred and one or two epidermal cells flanking the penetration site had been invaded by haustoria (Fig. 1B), similar to that described by Koch and Slusarenko (1990). In some instances, the developing hyphae had invaded the mesophyll by 12 h. At 1 day postinoculation (dpi), small colonies were established in the mesophyll layer. At this time, the infection site usually showed dark trypan blue staining caused by heavier staining of primary haustoria in the epidermal cells and in the first several mesophyll cells colonized. These haustoria usually were larger than those formed later. By day 2, a typical colony in each of the host genotypes was composed of a nonbranched hypha with approximately 70 to 90 haustoria growing within the mesophyll (Fig. 1C). Nascent haustoria were apparent near hyphal tips as small, round protuberances, whereas mature haustoria that were less proximal to the apex appeared larger and lobular (Fig. 1D and I). After day 2, intercellular hyphae continued to colonize the leaf mesophyll, forming frequent dichotomous branches and differentiating usually by one (Fig. 1D to F), but occasionally as many as three haustoria per host cell.

As early as 3 dpi, we observed regions of hyphal swelling, which stained more darkly than other hyphal sections. We attributed these swellings to production of oogonial initials as described by Dick (1994). In support of this interpretation, by day 4 mature oogonia were numerous, septate, and usually had paragynous antheridia appressed to their sides. By 5 dpi, mature, aplerotic oospores were present, which were heavily stained (Fig. 1H and I). We did not observe oospores to generate secondary vegetative hyphae, although occasionally we did notice that by 6 dpi, oospores had apiculus-like structures, suggesting that secondary somatic growth may occur in this system, as observed in other oomycetes (data not shown) (Dick 1994).

At 4 to 5 dpi, conidiophore initials were apparent as thickened hyphal sections under the epidermis near stomatal openings. Usually one, but occasionally multiple conidiophores would emerge through a single stomatal opening. By day 5, these had differentiated into mature conidiophores that were roughly the size of a leaf trichome, with branches bearing terminal conidiospores (Fig. 1G).

By 6 dpi, infected leaves contained an elaborate hyphal network that deposited haustoria into approximately three quarters of the adjacent mesophyll cells. Leaves contained numerous oospores, and their surfaces were decorated with a prominent flush of hygroscopic conidiophores that would twist in the air currents, releasing clouds of conidiospores. At this time, sexual and asexual life cycles of the pathogen were complete and, although abundant host tissue was colonized, most plant cells appeared healthy, as visualized by the lack of trypan blue staining. Leaves formed later than 1 week after infection were free of the pathogen, allowing wild-type plants to outgrow the disease.

Table 1. Stages in *Peronospora parasitica* development and their timing in wild-type, *nim1-1*, and NahG plants

Stage[a]	*Peronospora parasitica* life-cycle developmental event	Host genotype		
		Wild type	*nim1-1*	NahG
1	Conidiospore germination; appressorium formation (A[b])	2 hpi[c]	2 hpi	2 hpi
2	Formation of penetrating hypha; invasion of epidermal cells (A)	12 hpi	12 hpi	12 hpi
3	Extension of penetrating hypha into mesophyll; primary haustoria invade mesophyll cells (B)	1 dpi[c]	1 dpi	1 dpi
4	Colony well established in mesophyll; hyphae with abundant haustoria (C, D)	2 dpi	2 dpi	2 dpi
5	Extensive leaf colonization (E, F)	6 dpi	6 dpi	**5 dpi**[d]
6	Production of oogonial initials	3 dpi	3 dpi	3 dpi
7	Production of mature oogonia	3-4 dpi	**3 dpi**	3-4 dpi
8	Production of mature oospores (H, I)	5 dpi	**3–4 dpi**	5 dpi
9	Production of conidiophores (G)	5–6 dpi	**5 dpi**	**4–5 dpi**

[a] Stage numbers correspond to those in Figure 1.
[b] Letters in parentheses correspond to panels in Figure 1.
[c] Average hours (hpi) or days postinoculation (dpi) at which a specific stage was observed in most experiments performed. Where variation among experiments is common, a range is provided.
[d] Items in bold indicates differences in the timing of life-cycle stages among host genotypes.

***P. parasitica* growth on NahG and *nim1-1* plants.**

To compare growth and development of *P. parasitica* on wild-type and immune-compromised plants, we inoculated each host genotype and monitored parasite development, as described above, focusing on the timing of life-cycle events and the amount of pathogen structures formed (Table 1). Our goal was to determine which pathogen life-cycle stages were most affected by SA or NIM1/NPR1-dependent processes, phases that should show the greatest alteration in the immune-compromised plants in our study. We analyzed early pathogen growth stages up to 2 dpi, but saw no significant differences among host genotypes in spore germination or penetration efficiency, speed of colony establishment, or frequency of haustoria formation (data not shown).

By day 3, however, differences among host genotypes became evident with respect to the extent of parasite vegetative

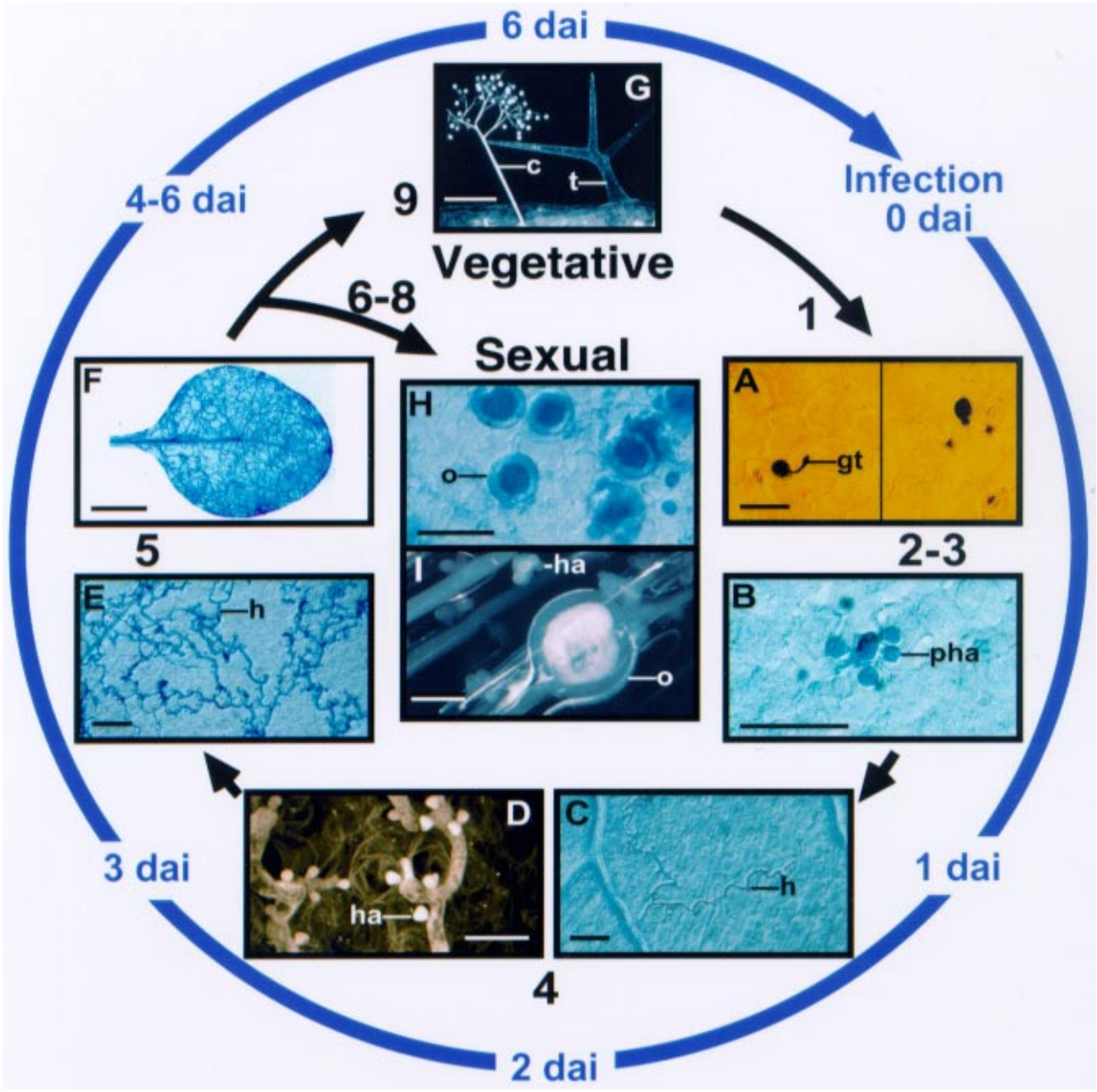


Fig. 1. Life cycle stages of *Peronospora parasitica* in wild-type Arabidopsis. Blue perimeter circle shows approximate time when each development stage appears in wild-type hosts (dai: days after inoculation). Interior black numbers correspond to the developmental stages listed in Table 1. At 0–1 dai, conidiospores germinate and form germ tubes (**A,** two fields shown; stage 1), which penetrate the host epidermis and deposit primary haustoria into plant cells at the sites of ingress (**B**; stage 2–3). Vegetative growth produces small colonies of hyphae adorned with haustoria (**C and D**; stage 4), which proliferate to form large colonies within the leaf (**E and F**; stage 5). Stages 6–8 include differentiation of antheridia and oogonia, sexual union, and formation of diploid oospores (**H and I**). At stage 9, conidiophores are formed that emerge through stomata and produce vegetative spores or conidia (**G**). c = conidiophore; gt = germ tube; h = hyphae; ha = haustorium; o = oospore; pha = primary haustorium; t = trichome (plant). Scale bars = 25 μM for **A, B, and I**; 50 μM for **D and H**; 100 μM for **C, E, and G**; 2 mm for **F**.

development and efficiency in sexual spore production (Table 1). *nim1-1* and NahG showed greater colonization than wild-type plants, with larger hyphal networks and proportionately greater numbers of haustoria (data not shown). This trend was seen in five separate replications of this experiment in which at least ten leaves from each host genotype were examined. In most experimental repetitions, *nim1-1* and NahG plants showed greater quantities of oogonia, antheridia, and mature oospores compared to wild-type plants, although the degree of variation between experiments made the significance of this observation hard to assess. For example, at 3 dpi, we usually would observe leaves of wild-type plants to show oogonial initials but only rarely did we see fully formed oogonia or oospores (NahG leaves contain initial and mature oogonia but lack mature oospores), whereas *nim1-1* showed initial and mature oogonia as well as mature oospores. By 5 dpi, *nim1-1* and NahG leaves usually showed over twice the number of oospores as found in wild-type leaves, sometimes exceeding oospore production in wild-type by fourfold (data not shown).

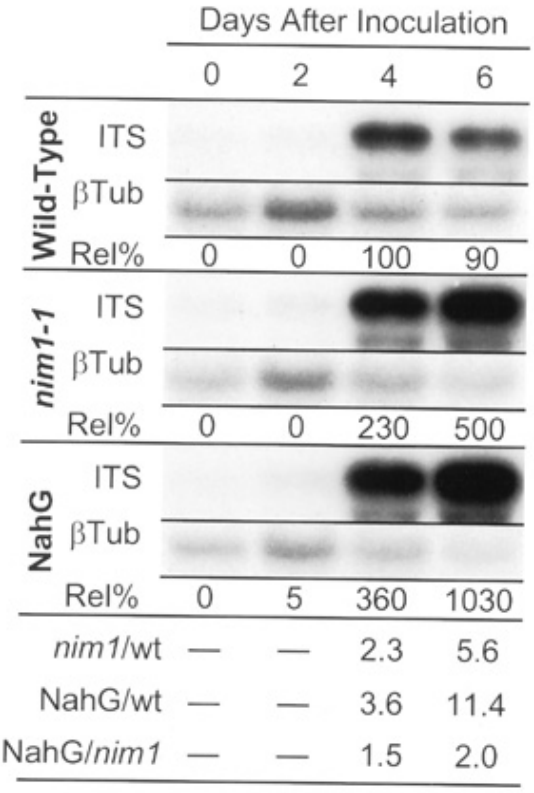


Fig. 2. NahG and *nim1-1* plants support greater pathogen growth than wild-type. *Peronospora parasitica* biomass was inferred by hybridization to a fungal 18S–28S rDNA internal transcribed spacer region (ITS) probe to RNA prepared from infected plant tissue. Wild-type, *nim1-1*, and NahG plants were inoculated with *P. parasitica*, and RNA was prepared from tissues 0, 2, 4 and 6 days later. Replicate RNA blots were hybridized to the ITS probe to quantify parasite biomass and to an Arabidopsis β-tubulin probe (βTub) to normalize for variation in the amount of RNA in the samples. Hybridization signal strength from both probes was quantified, and the normalized ITS signal expressed as the percent of the day-4 signal in wild-type plants (Rel%). At day 2, ITS levels (Rel%) were below background levels for wild-type and *nim1-1* plants. Bottom panel shows the ratios of normalized ITS values for the indicated genotypes at each time after inoculation.

At 5 dpi, *nim1-1* and NahG plants continued to show greater hyphal and haustorial colonization of mesophyll cells compared with wild type, based on the examination of stained and cleared leaves. To provide an independent measure of the quantity of pathogen biomass in each host genotype, we also examined accumulation of pathogen-produced rRNA at 0 through 6 dpi. This was performed by hybridization of total RNA from infected plants to a radioactive probe containing the 18S–28S rDNA internal transcribed spacer (ITS) region from *P. parasitica*. Variation in RNA quantity between samples was normalized by hybridization of replicate blots to an *A. thaliana* β-tubulin probe. The normalized ITS signal was expressed as a percentage of the signal produced from the ITS probe in wild-type plants at 4 dpi (Fig. 2). The ITS signal became prominent only at 4 dpi or later. NahG plants showed much greater ITS signal than wild type at days 4 and 6 (3.6- and 11.4-fold greater than wild type, respectively), whereas *nim1-1* plants showed a greater signal than wild -type but less than NahG (2.3- and 5.6-fold greater than wild type, respectively). At days 4 and 6, NahG had 1.5- and twofold greater ITS signals than *nim1-1*. The levels of ITS signal observed were consistent with visual observations of greater hyphal development in NahG and *nim1-1* plants compared with wild type (data not shown).

Although conidiophores were produced on all host genotypes occasionally as early as 4 dpi, there were notable differences in the quantity of the structures produced. NahG plants were the most prolific, showing a statistically significant increase in conidiophore number at 5 and 6 dpi compared with *nim1-1* or wild-type hosts (Fig. 3). This was most pronounced at 6 dpi when NahG leaves showed approximately 175 conidiophores per leaf, 2.7-fold more than found on wild type and 1.7-fold more than on *nim1-1* plants (Fig. 3).

Responses to *P. parasitica* infection in wild-type and immune-compromised plants.

As described above, pathogen growth was similar in each host genotype through 2 dpi. We monitored callose deposition at infection sites at 2, 12, 24, or 48 hpi and found no significant differences between host genotypes (data not shown). To determine whether the increased rate and amount of pathogen growth observed in *nim1-1* and NahG plants later than 2 dpi was linked to specific defects in the induced defenses of these plants, we examined the induction of the SAR-marker gene PR-1 (Uknes et al. 1992) and production of callose deposits around pathogen-derived haustoria. Although PR-1 induction depends upon SA accumulation and NIM1/NPR1 activity, it is not known whether callose formation has these requirements. At 5 dpi, PR-1 accumulation was strongly induced in wild-type plants, approximately 50-fold less in *nim1-1* plants, and was undetectable in NahG plants, similar to previous observations (Fig. 4) (Delaney et al. 1994; Delaney et al. 1995).

Callose deposition may serve to fortify cell walls in plants undergoing pathogen attack and is a useful marker of induced defenses in plants (Dangl et al. 1996; Hammond-Kosack and Jones 1996; Sticher et al. 1997). Callose encasements also have been suggested to play a defensive role by decreasing the efficiency of haustoria in obtaining nutrients from host cells (Allen and Friend 1983). To examine the callose response phenotype in this study, we stained wild-type, *nim1-1*, and NahG leaves 5 dpi with analine blue, which fluoresces blue

under UV light when bound to callose (Fig. 5) (Eschrich and Currier 1964). We found most of the callose fluorescence to be limited to haustoria, and was found on 48 to 68% of all haustoria examined (Figs. 5 and 6), although older hyphae closer to penetration sites occasionally were outlined in callose (not shown). We quantified the extrahaustorial callose-response phenotype of each host genotype by enumerating haustoria within an approximately 7-mm^2 leaf area in ten independent leaves from each genotype. All haustoria were assigned to one of the following classes: i) haustoria with no callose deposit (none) (Fig. 6); ii) haustoria with callose deposited in a band or collar around the base or neck of the haustorium (collar) (Figs. 5C and 6); iii) haustoria encased fully in callose (encasement) (Figs. 5E and 6); or iv) haustoria with a thick callose encasement that occupies most of the colonized plant cell volume (thick encasement) (Figs. 5A and 6). We found marked differences among host genotypes in the distribution of haustoria within the four classes (Fig. 6A to C). In wild-type leaves, extrahaustorial callose was present on 48% of haustoria examined, with collars and thick encasements found on 24 and 11% of the haustoria, respectively (Fig. 6A). In NahG leaves, however, callose deposits were found on 68% of the haustoria, mostly in the form of collars (48%), with a much lower fraction of thick encasements (4%) compared with the wild type (Fig. 6C). Extrahaustorial callose in *nim1-1* plants was found on 60% of the haustoria observed and was intermediate between wild-type and NahG plants, with respect to the frequency of collars and thick encasements that were present on 34 and 8% of the haustoria, respectively (Fig. 6B). Less variability was found for the encasement class, which comprised 13 to 18% of haustoria in each host genotype.

The largest differences in extrahaustorial callose distribution were found between wild-type and NahG plants in the fraction of haustoria showing either thick callose encasements and in haustoria exhibiting callose collars (Fig. 7). Thick encasements were most abundant in wild type and *nim1-1*, followed by NahG plants (Fig. 7A), whereas NahG plants displayed a much higher frequency of callose collars than *nim1-1* or wild-type plants (Fig. 7B). In fact, in NahG plants, 70% of the haustoria analyzed that contained callose deposits had it confined to the haustorial neck region in the form of collars; the small fraction of thick encasements observed were found predominantly on older haustoria near the colony origin. Perhaps surprisingly, callose-free haustoria (Fig. 6) were the most prevalent in wild type and least frequent in NahG plants, indicating that the complete absence of extrahaustorial callose is not correlated simply with susceptibility.

To determine whether SAR would have an affect on callose deposition, we induced this response with INA, a synthetic analog of SA that activates SAR in wild-type and NahG plants but not in *nim1-1* plants (Delaney et al. 1994; Delaney et al. 1995; Kessmann et al. 1994; Vernooij et al. 1995). *P. parasitica*-inoculated plants were allowed 2 days for the pathogen to become established, then treated with INA to induce SAR and examined 3 days later (5 dpi) to assess extrahaustorial callose formation (Fig. 5D to F). Three days after INA treatment, the fraction of haustoria with thick callose encasements increased significantly in NahG plants compared with plants not receiving INA (Figs. 6 and 7A). Furthermore, INA induced a stronger callose deposition response in NahG plants as well as restored levels of thick encasements to that of INA-

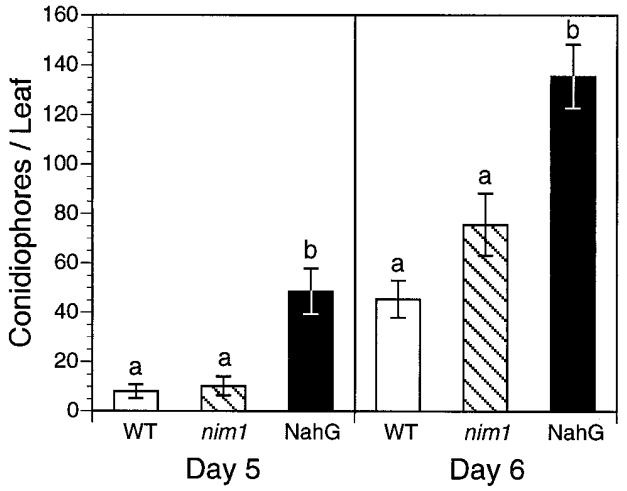


Fig. 3. Conidiophore production in wild-type, NahG, and *nim1-1* plants. To assess asexual sporulation, one leaf from 20 plants of each genotype was examined 5 and 6 days postinoculation and the number of conidiophores were counted. Bars show mean values and standard error of the means. At day 5 or 6, values that differ significantly ($P < 0.05$) are indicated by a or b.

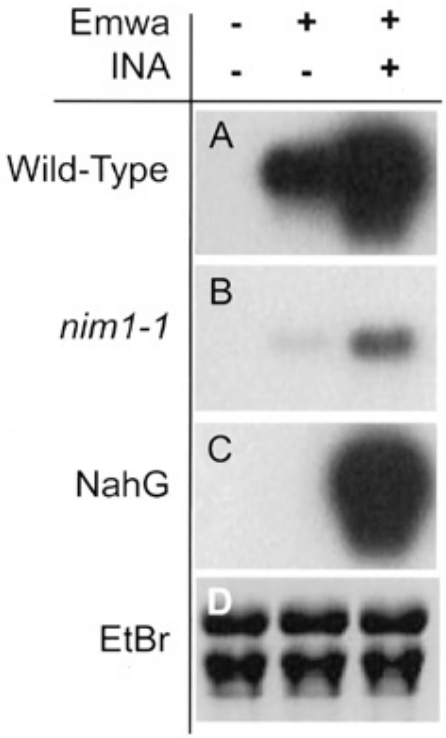


Fig. 4. PR-1 mRNA accumulation in wild-type, NahG, and *nim1-1* plants. Plants were mock inoculated (–) or inoculated (+) with *Peronospora parasitica* strain Emwa and, 5 days later, harvested for RNA (left and center lanes, respectively). To examine induction of PR-1 by 2,6-dichloroisonicotinic acid (INA) in infected plants, plants were inoculated and, 2 days later, treated with INA and harvested for RNA 5 days postinfection (right lanes). **A–C,** Autoradiograms of Northern blots containing RNA from wild-type, *nim1-1*, and NahG plants after hybridization to an Arabidopsis PR-1 cDNA probe. **D,** Ethidium bromide-stained gel of the wild-type plant RNA samples in **A**. The other RNAs were comparably loaded (not shown).

treated wild-type plants, indicating that callose induction responds to INA, a chemical inducer of SAR. Interestingly, INA treatment of *nim1-1* did not cause a significant increase in thick haustorial encasements (Fig. 7A), suggesting that a strong callose response to produce thick haustorial encasements is affected by the SAR pathway and requires the *NIM1/NPR1* gene product. To confirm that INA treatment was effective in this experiment, the INA-treated plants described above were also examined for PR-1 RNA accumulation (Fig. 4). As expected, INA treatment combined with *P. parasitica* infection caused massive accumulation of PR-1 in wild-type plants, a restoration to near wild-type PR-1 levels in NahG plants, and only a modest induction (approximately twofold) in *nim1-1* plants (Fig. 4).

We also noted less hyphal growth after INA treatment in wild-type and NahG plants as well as more trailing necrosis and callose-encased epidermal cells compared with nontreated plants (data not shown). These features are similar to that found in wild-type plants inoculated with an incompatible *P. parasitica* strain or in an SAR-induced plant inoculated with a normally virulent *P. parasitica* isolate (Hammond-Kosack and Jones 1996; Koch and Slusarenko 1990; Uknes et al. 1992), although these rapid responses normally would restrict pathogen growth at a much earlier stage than in the experiments we performed.

DISCUSSION

To examine the roles played by induced defenses on the virulence of *P. parasitica* in Arabidopsis, we compared pathogen growth in wild-type, *nim1-1*, and NahG plants while monitoring selected host responses to infection. Most phases of the parasite's life cycle were examined, including conidiospore germination, leaf invasion, vegetative colony development, and sexual and asexual reproduction. Infected plants were evaluated several times after inoculation to assess host production of callose at infection sites and around haustoria and induction of the SAR marker gene PR-1.

Through 2 dpi, no differences in pathogen performance or host response were evident in wild-type, *nim1-1*, or NahG plants with respect to conidiospore germination frequency, germ tube and appressorium formation, efficiency at epidermal penetration as early as 2 hpi, or in host production of callose at penetration sites. The similar growth of the pathogen in each host during this time suggests that SA or NIM1/NPR1 pathways are not important in determining susceptibility of Arabidopsis to *P. parasitica* soon after exposure to the pathogen.

By 3 dpi and later, however, substantial differences became apparent in pathogen fitness on each host with respect to the extent of hyphal development and accumulation of pathogen biomass as inferred by quantification of *P. parasitica* rRNA. By these measures, NahG plants supported the greatest amount of vegetative growth of the pathogen, followed by *nim1-1* and the compatible wild-type host. The parasite also displayed greater fecundity in NahG than in the wild type, which was indicated by increased conidiophore production and, in most experiments, more rapid development and production of oogonia and oospores (data not shown).

By 5 dpi, large differences in host responses to infection were apparent, with wild-type plants showing massive accumulation of PR-1 mRNA, little in *nim1-1*, and no detected response in NahG plants, which is consistent with our previous observations of PR-1 induction in these lines. The reduced induction of this defense gene in NahG and *nim1-1* plants suggests that PR-1 or other genes under similar regulation may play a role in limiting or slowing hyphal colonization in wild-type plants infected with *P. parasitica*, which is consistent with the role suggested for the tobacco ortholog PR-1a in resistance against oomycete parasites of tobacco (Alexander et al. 1993). We also observed at 5 dpi significant differences between the three hosts in their extrahaustorial callose deposit profiles. While the spacing of haustoria along hyphae appeared similar in each host (data not shown), i.e., the quantity and morphology of callose deposits around haustoria differed. Wild-type plants produced a significantly larger fraction of thick callose encasements than did *nim1-1* or NahG plants, whereas NahG plants were the most prolific producers of callose collars around haustorial necks, and wild type the least. Callose deposit profiles in *nim1-1* plants were intermediate between NahG and wild-type hosts. Curiously, the least susceptible wild-type host had the highest percentage of haustoria free from any type of callose deposit, whereas the most susceptible NahG plants had the lowest frequency of callose-free haustoria. This pattern was a result of the great abundance of callose collars in NahG plants, which were twice as frequent as what was found in wild-type plants. The abundance of callose collars and paucity of thickly encased haustoria suggest that while NahG plants are proficient in initiating callose deposits in the form of collars, they are deficient in the formation of massive deposits.

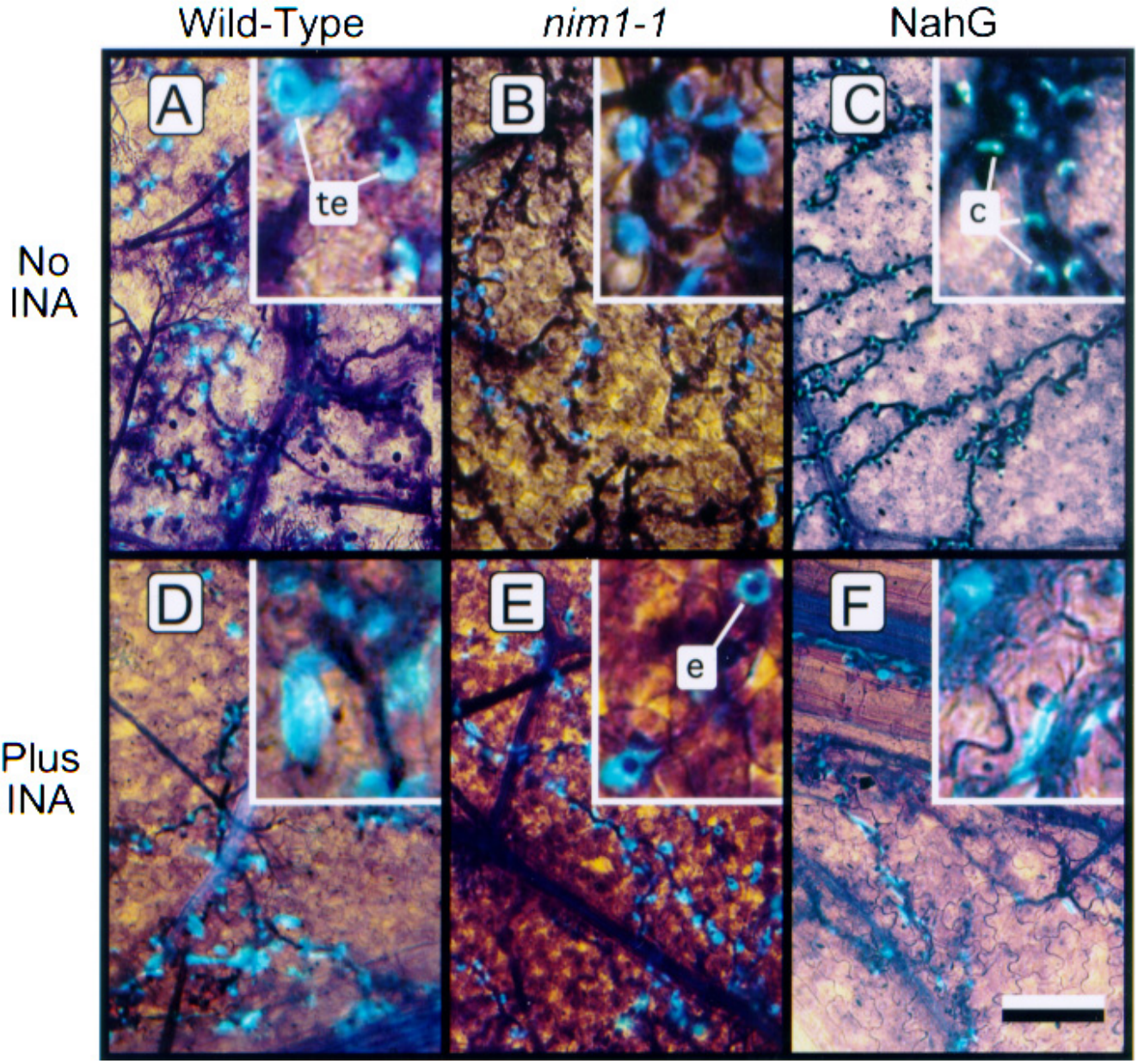
Potential role for callose in limiting pathogen growth.

Nondevelopmental callose production is associated with mechanical trauma, defense responses against fungal and oomycete pathogens, the hypersensitive response elicited by many diverse pathogens, and attempted infection by pathogens on nonhost species (Stone and Clarke 1992). Callose has been suggested to play a defense role by reinforcing the plant cell wall at attempted sites of parasite penetration or by providing a medium for the deposition of toxic compounds or toxins (Aist 1976; Kovats et al. 1991; Skou et al. 1984). Callose also may contribute to host defenses by impeding nutrient transfer from host to pathogen or possibly by delaying pathogen growth long enough for other host defenses to become active (Allen and Friend 1983).

In this study, we documented the production of extrahaustorial callose in Arabidopsis after infection by *P. parasitica*. Deposits were found in the form of collars, encasements, and thick encasements, each of which requires a greater quantity of callose accumulation than does the preceding type of deposit. The most susceptible NahG host had the smallest proportion of haustoria surrounded by thick encasements and the greatest fraction of callose collars, suggesting that thick callose deposits, but not collars, may aid in restricting pathogen growth. Alternatively, if thick encasements are a function of haustorium age, then NahG plants may have fewer thick encasements because they contain a higher proportion of young hyphae as a result of the rapid pathogen growth in these plants. This explanation, however, does not account for the higher fraction of callose collars found in NahG plants. A similar correlation between encasements and resistance was

described for potato cultivars infected with *Phytophthora infestans*: resistant tubers showed over eight times the frequency of thick callose encasements compared with tubers from a susceptible cultivar (Hachler and Hohl 1984).

A number of studies were conducted to test whether callose or papillae play a role in resistance to fungal parasites. Smart et al. (1986) concluded that callose alone was insufficient to impede penetration by *Erysiphe graminus* into oversized papillae of barley. This was demonstrated by the enzymatic removal of callose from these papillae, which was found to have little effect on their resistance to penetration, indicating that other features beside callose such as other molecules contained within the papilla are important for resistance. Later work with powdery mildew resistant *ml-o* barley, however, showed that inhibition of callose synthesis with 2-deoxy-D-glucose reduced papillae formation and led to enhanced penetration efficiency of powdery mildew fungus, suggesting that callose formation is important for *ml-o*-mediated resistance (Bayles et al. 1990). In another study, Vance and Sherwood (1976) showed that cycloheximide treatment of reed canarygrass leaf discs inhibits papillae formation and allowed penetration by spores of five genera of normally noninfecting pathogens, leading them to conclude that protein synthesis was required to produce papillae and that those structures



Fig, 5. Extrahaustorial callose deposition in wild-type, *nim1-1*, and NahG plants. To observe patterns of callose deposition, leaves were cleared in lactophenol, stained with analine blue, and visualized by fluorescence microscopy. Analine blue-stained callose fluoresces bright blue and the underlying leaf and hyphal morphology is seen by a superimposed differential interference contrast microscope image. Host genotypes are indicated at the top. **A–C,** Plants 5 days postinoculation (dpi); **D–F,** Plants were inoculated, 2 days later treated with 2,6-dichloroisonicotinic acid, and 5 dpi cleared and stained for callose. **A, C, and E,** Representative callose deposit types are illustrated. c = collars; e = encasements; te = thick encasements. Scale bar = 250 μM for the main panels; 60 μM for the insets.

performed a defensive role against fungal penetration. The conclusions of all three of these studies, however, are complicated by the potential pleiotropic affects of the treatments used.

If callose deposition is modified by inducible defense pathways, then NahG plants may be deficient in the sustained production needed to form thick deposits as a result of their defense-compromised phenotype. The reduced frequency of thick callose deposits in NahG plants is consistent with this response being supported by SA-responsive pathways, whereas the increase in frequency of thick deposits in NahG plants but not in *nim1-1* plants after INA treatment suggests that SA and NIM1/NPR1 pathways may contribute to extrahaustorial callose accumulation. Additional evidence that induced defense pathways may play a role in promoting callose deposition is seen in cucumber plants, which when expressing SAR, also show enhanced plasma membrane-associated callose synthase activity (Schmele and Kauss 1990). Sustained callose deposition in some systems has been shown to be an active host response requiring transcriptional and translational activity (Skalamera et al. 1997). Therefore, the scarcity of thick encasements in NahG plants may result from reduced expression of genes that encode callose biosynthetic enzymes or that contribute to synthesis of callose precursors such as UDP-glucose. Because callose production may be regulated by precursor supply, callose synthase gene expression, or regulation of synthase activity, defense pathways may affect production of the glucan at a number of levels. These may be direct effects such as changes in callose synthase gene expression or indirect responses to as yet unknown metabolic shifts that may accompany salicylate hydroxylase expression or mutations in the NIM1/NPR1 pathway. Resolution of the role played by callose in resistance will require more definitive tests; recent progress toward isolation of many members of the glucan synthase gene family may enable functional genomics analyses to address this issue (Burton et al. 2000; Holland et al. 2000; Richmond and Somerville 2000).

The results of this study demonstrate that NahG and *nim1-1* plants are more susceptible to *P. parasitica* than are wild-type hosts. Therefore, even susceptible wild-type plants exert defense activities against pathogens that constrain disease severity and reduce pathogen fitness. The enhanced susceptibility phenotype of NahG and *nim1-1* plants results presumably from a reduction in SA and NIM1/NPR1-dependent defenses. The heightened susceptibility of these hosts is accompanied by reduced extrahaustorial callose deposition and less PR-1 expression, suggesting that these responses may have a role in the constraint of disease severity. It is important to point out that other defense activities not examined in this study also may contribute to limiting disease. Additional studies will be required to ascertain whether callose deposition around haustoria is an important defense mechanism against oomycete or fungal pathogens or whether this process is secondary to other defense activities.

MATERIALS AND METHODS

Plant lines and pathogen strain.

A. thaliana wild-type Wassilewskija (Ws-0) seeds were obtained from the Arabidopsis Biological Resource Center at Ohio State University. The Ws-0 *nim1-1* allele was described previously (Delaney et al. 1995), and the Ws-0 NahG line was constructed as described (Delaney et al. 1994). All pathogen assays included the virulent *P. parasitica* isolate Emwa1 (Holub et al. 1994), provided by Eric Holub of Horticulture Research International, Wellesbourne, U.K. The pathogen was maintained on its susceptible host Ws-0 through weekly culture onto 2-week-old plants. Prior to inoculation, potted plants were maintained in a walk-in growth chamber at 22°C under 12-h light–12-h dark cycle at approximately 150 μE fluence provided by cool-white fluorescent lamps, with approximately 60% humidity. Plants were spray inoculated with a suspension of approximately 5 to 8 × 10^4 conidiospores per ml of water with a Preval pressurized paint sprayer (Precision Valve, Yonkers, NY, U.S.A.). The pots were placed in a horticultural flat with a wet-paper-towel floor and covered with a clear plastic dome to maintain near 100% relative humidity. The flats were placed in growth chambers (Percival Scientific, Boone, IA, U.S.A.) at a cycle of 10-h light–14-h dark (approximately 150 μE) and a day–night temperature of 18–20 and 14–16°C, respectively. After 1 week, infected plants were used as a source of inoculum by harvesting leaves bear-

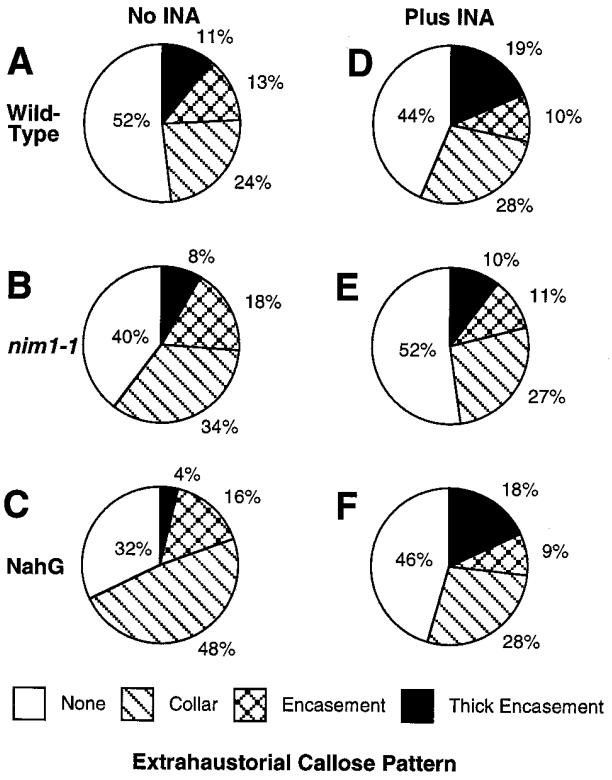


Fig. 6. Distribution of extrahaustorial callose response classes in wild-type, *nim1-1*, and NahG plants in the absence and presence of 2,6-dichloroisonicotinic acid (INA) treatment. Single leaves from 10 plants per treatment were cleared and stained with analine blue 5 days postinoculation (dpi). Haustoria within an approximately 7-mm^2 leaf area were enumerated and classified to whether they had: no callose (none), callose in the form of a collar around the haustorium base (collar), full encasements of callose around the haustorium (encasement), or thick callose deposits that nearly or completely filled the invaded cell volume (thick encasement). **A–C,** Plants 5 dpi. **D–F,** Effect of INA on callose deposition, assessed by treatment of plants at 2 dpi with INA and fixation at 5 dpi. Number of haustoria from each treatment group (10 leaves combined): 2,016, 2,509, 2,127, 1,286, 1,724, and 1,385 for **A–F,** respectively.

ing conidiophores. which were vortexed in water to dislodge conidiospores.

Pathogen inoculations and INA Treatments.

Two-week-old Ws-0, *nim1-1*, and NahG plants were spray inoculated with a conidiospore suspension, as described above. For the life-cycle experiments, the first true leaves of 2-week-old plants were inoculated with 2-µl droplets containing approximately 50 spores. Plants for all other experiments were spray inoculated with the above-mentioned paint sprayer at the spore concentration described above. INA (Kessmann et al. 1994) was sprayed as a 0.25-mg-per-ml solution of 25% INA in wettable powder in water (0.33 mM INA) with a paint sprayer, as previously described.

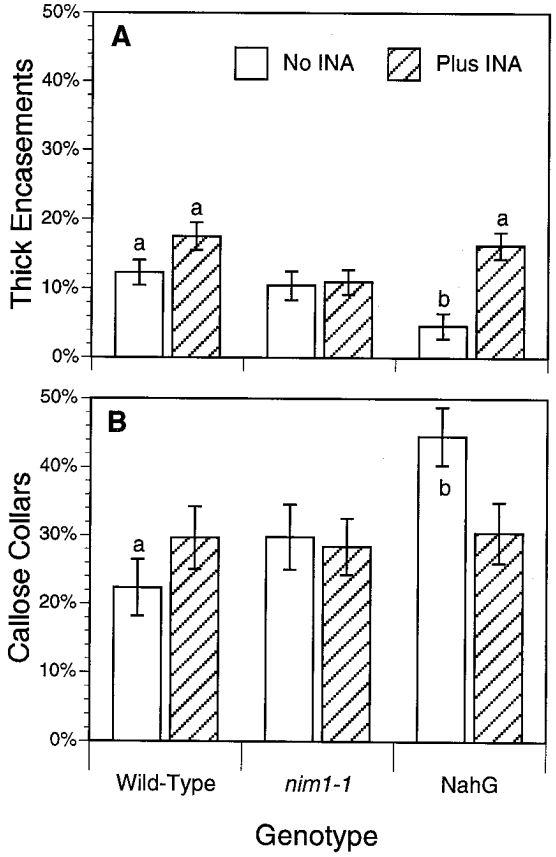


Fig. 7. Thick callose encasements and callose collars in wild-type, *nim1-1*, and NahG plants, without or with 2,6-dichloroisonicotinic acid (INA) treatment. Statistical analysis of thick encasement and collar response classes (data in Fig. 6). Mean percentages of haustoria in each callose response class are indicated with standard error of the means (n = 10 leaves per treatment). Data were analyzed with a general linear model controlling for the variable number of haustoria in the samples with a Tukey adjustment for multiple pairwise comparisons. Treatment classes that differ significantly ($P < 0.05$) are indicated by a or b. **A,** Proportion of haustoria with thick callose encasements is lower in NahG, than in wild-type, with or without INA, or in NahG plants with INA (P = 0.0396, 0.0005, 0.0014, respectively). INA-treated wild-type and *nim1-1* plants may differ if a lower threshold for significance is accepted (P = 0.13). **B,** Proportion of haustoria with callose collars is greater in NahG plants than in wild type in absence of INA (P = 0.0046). Statistically significant differences were not noted in the analysis of the callose response classes "none" or "encased" (data not shown).

Histochemical staining and microscopy.

To visualize pathogen structures, leaves were stained with lactophenol trypan blue and cleared with saturated chloral hydrate, as described (Uknes et al. 1993). After leaves had cleared (1 to 2 days), chloral hydrate was replaced with 70% glycerol for slide mounting and less noxious handling. Whole leaves were analyzed and photographed with a MZ8 stereo microscope (Leica, Wetzler, Germany) and a PM-C35 35-mm camera (Olympus, Melville, NY, U.S.A.). Detailed examination of pathogen structures was conducted with an Olympus BX60F compound microscope and differential interference contrast (DIC) optics.

Leaves were stained for callose as described (Dietrich et al. 1994), with modifications. After vacuum infiltration and boiling in lactophenol, leaves were incubated for 1 h, transferred to saturated chloral hydrate, and placed on a shaker overnight. The next day, leaves were treated to quench autofluorescence with toluidine blue-O (Smith and McCully 1978) by removing half of the chloral hydrate from each sample and adding several drops of 0.05% toluidine blue-O in 0.1 M sodium acetate (pH 4.4). After 10 min, leaves were transferred to 0.05% toluidine blue-O and incubated on a shaker for 45 min. To visualize callose, the solution was replaced with a 0.01% (wt/vol) solution of analine blue in 0.15 M K_2HPO_4. Samples were incubated in the analine blue solution on a shaker for 50 min, at which time the solution was replaced with 35% glycerol. Leaves stored in 35% glycerol in the dark remained in good condition with adequate callose fluorescence for up to 3 weeks after staining. Callose was observed by epifluorescence microscopy with an Olympus BX60F microscope and MNU filter cube (360 to 370 nM excitation filter, DM400 dichroic mirror, and BA420 long-pass filter).

Statistical analyses.

Conidiophore numbers were assessed in the three genotypes at 5 and 6 dpi. One fully expanded leaf from each of 20 plants was examined at each time and mature conidiophores were counted with the Leica stereomicroscope. Data were analyzed using one-way analyses of variance, followed by multiple pairwise comparisons with a Tukey adjustment. The analysis of callose responses was performed by examining one leaf from each of 10 plants from the six treatment groups (three genotypes, with or without INA treatment) after staining with analine blue, and inspection was performed by fluorescence microscopy and DIC, as described above. Haustoria were enumerated and classified by examining the leaf area delimited by an ocular reticle (3.20 × 2.18 mm) containing 6.98 mm^2 through the entire leaf thickness. This leaf volume contained 100 to 360 haustoria in the 60 samples examined. Data were analyzed with a general linear model, controlling for the variable number of haustoria in the samples. Data were examined for interaction between genotype and INA treatment, followed by multiple pairwise comparison with a Tukey adjustment. All statistical analyses were performed with Minitab Release 12 (Minitab, State College, PA, U.S.A.).

RNA isolation and Northern analysis.

Plants were harvested and stored at −80°C until pulverized in liquid nitrogen and extracted for RNA (Verwoerd et al. 1989). Approximately 4 µg of RNA from each sample was electrophoresed through an agarose gel, transferred to a Ny-

tran N nylon membrane (Schleicher and Schuell, Dassel, Germany), hybridized with radiolabeled probes, and washed as described (Uknes et al. 1993). The rRNA probe used contained the 18S–28S rDNA internal transcribed spacer region and 5.8S rRNA gene from *P. parasitica*. This region was amplified by polymerase chain reaction (PCR) from substrate DNA prepared by crushing conidiospores in water between glass slides with primers based on the *Saccharomyces cerevisiae* rDNA sequence (White et al. 1990) (18S primer: 5′-TCCGTAGGTGAACCTGCGG-3′; 28S reverse primer: 5′-TCCTCCGCTTATTGATATGC-3′). The Arabidopsis β-tubulin probe was generated by PCR amplification of genomic Arabidopsis DNA with primers 5′-CTTGAACGTATCGATGTCTAT-3′ and 5′-ATCCATTCCACAAAGTAG-3′ (reverse). The Arabidopsis PR-1 gene probe (Uknes et al. 1992) was provided by Novartis (Greensboro, NC, U.S.A.). Hybridization was quantified by counting radioactive emission witha PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

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