Vitamin B₁-Induced Priming Is Dependent on Hydrogen Peroxide and the *NPR1* Gene in Arabidopsis[1]

Il-Pyung Ahn*, Soonok Kim, Yong-Hwan Lee, and Seok-Cheol Suh

National Institute of Agricultural Biotechnology, Suwon 441–100, Korea (I.-P.A., S.-C.S.); and Department of Agricultural Biotechnology, Center for Fungal Genetic Resources, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151–921, Korea (S.K., Y.-H.L.)

Thiamine confers systemic acquired resistance (SAR) on susceptible plants through priming, leading to rapid counterattack against pathogen invasion and perturbation of disease progress. Priming reduces the metabolic cost required for constitutive expression of acquired resistance. To investigate the effects of priming by thiamine on defense-related responses, Arabidopsis (*Arabidopsis thaliana*) was treated with thiamine and effects of pathogen challenge on the production of active oxygen species, callose deposition, hypersensitive cell death, and pathogenesis-related 1 (*PR1*)/Phe ammonia-lyase 1 (*PAL1*) gene expression was analyzed. Thiamine did not induce cellular and molecular defense responses except for transient expression of *PR1* per se; however, subsequent *Pseudomonas syringae* pv *tomato* challenge triggered pronounced cellular defense responses and advanced activation of *PR1/PAL1* gene transcription. Thiamine treatment and subsequent pathogen invasion triggered hydrogen peroxide accumulation, callose induction, and *PR1/PAL1* transcription activation in Arabidopsis mutants insensitive to jasmonic acid (*jar1*), ethylene (*etr1*), or abscisic acid (*abi3-3*), but not in plants expressing bacterial NahG and lacking regulation of SAR (*npr1* [nonexpressor of *PR* genes 1]). Moreover, removal of hydrogen peroxide by catalase almost completely nullified cellular and molecular defense responses as well as SAR abolishing bacterial propagation within plants. Our results indicated that priming is an important cellular mechanism in SAR by thiamine and requires hydrogen peroxide and intact *NPR1*.

Plants have developed an effective immanent surveillance mechanism and pathogen invasions often induce ubiquitous plant defense responses that activate biochemical and structural changes within plant cells. A specific plant's resistance (*R*) gene product functions as a signaling receptor for the corresponding avirulence (*Avr*) gene product from the pathogen. The key differences between the compatible (susceptible) and incompatible (resistant) interactions are the early recognition of pathogen attack and the timely expression of defense responses (Yang et al., 1997; McDowell and Dangl, 2000; Lu et al., 2004; Bennett et al., 2005). One of the most noticeable features in the incompatible interaction is abrupt cell death, also termed hypersensitive response (HR), a restricted cell death defining further pathogen progress, which is initiated by the interaction between the *R* gene product and the *Avr* gene product. *R* gene-dependent resistance has been known to share several defense-related responses with systemic resistance induced by plant defense activators.

Systemic resistance is induced by exogenous application of salicylic acid (SA; Delaney, 1997), bacterial elicitors (Desikan et al., 2001), and plant defense activators, such as benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH; Friedrich et al., 1996; Gorlach et al., 1996; Bokshi et al., 2003), and β-aminobutyric acid (BABA; Zimmerli et al., 2000; Jakab et al., 2001; Ton and Mauch-Mani, 2004; Hamiduzzaman et al., 2005; Ton et al., 2005). These defense activators confer broad-spectrum resistance by stimulating common defense mechanisms; for example, oxidative burst (Iriti and Faoro, 2003), accumulation of defense-related materials (Benhamou and Belanger, 1998; Jeun et al., 2000), secondary metabolite production (Kauss et al., 1993), and pathogen-specific defense structure (Brown et al., 1998; Huckelhoven et al., 1999).

Defense activators and specific rhizobacteria confer enhanced disease protection capacity to various host species against multiple pathogens. However, some of these agents do not always trigger cellular and molecular defense responses per se (Graham and Graham, 1994; Jeun et al., 2000; Lyngkjaer and Carver, 2000; Conrath et al., 2001; Ton and Mauch-Mani, 2004; Ton et al., 2005). Subsequent pathogen challenge on plants pretreated with the above agents entailed augmented defense-related responses, such as fortified defense-related gene expression, accumulation of active oxygen species (AOS), callose deposition, and papillae formation singly or in combination. This facet brings up the rapid and efficient activation of defense systems

in immunized animals and humans infected by a pathogen (Ludewig et al., 1998; Cho et al., 2002). The enhanced capacity to express pertinent defense mechanisms is termed elicitation competency (Graham and Graham, 1994) or priming (Conrath et al., 2001, 2002). Agents inducing priming do not activate plant defense responses, but rather mobilize plants in a state of enhanced ability to suppress future pathogen attacks (Zimmerli et al., 2000; Jakab et al., 2001, 2005; Conrath et al., 2002; Pozo et al., 2004; Ton et al., 2005). In this respect, primed plants share many characteristics with resistant plants harboring *R* genes. There is ample evidence that priming is not an uncommon phenomenon in induced resistance. Low doses of plant defense activators, such as BTH, BABA, 2,6-dichloroisonicotinic acid (DCINA), SA, and methyl jasmonate are capable of priming susceptible hosts (Conrath et al., 2002; Si-Ammour et al., 2003; Faize et al., 2004). Priming is one of the most efficient types of induced resistance because the metabolic investment of the plant for the constitutive activation of the defense system is reduced or prevented. Currently, molecular research on priming has been focused on identifying plant genes through transcriptome analyses, screening of gene-trap lines, and map-based cloning.

Defense activators and subsequent pathogen infection trigger cellular defense responses involving oxidative burst and callose induction (Conrath et al., 2001; Park et al., 2002; Tanaka et al., 2003; Faize et al., 2004; Pare et al., 2005). Besides arresting pathogen proliferation in planta, AOS are involved in cell wall reinforcement (Olivain et al., 2003) and act as signaling molecules stimulating systemic resistance (Bolwell et al., 1995, 1998; Tenhaken et al., 1995; Wojtaszek, 1997; Alvarez et al., 1998; Chamnongpol et al., 1998). Callose is a fluorescent β-1,3-glucan complex and rapid deposition of callose is an indicator of defense-related responses (Devadas et al., 2002; Ton and Mauch-Mani, 2004; Flors et al., 2005). In the absence of elicitors or subsequent stresses, methyl jasmonate, SA, or DCINA alone did not induce AOS elevation and/or callose accumulation in cultured parsley (*Petroselinum crispum*) cells and cucumber (*Cucumis sativus*) hypocotyls (Kauss et al., 1999; Conrath et al., 2001). Oxidative burst and subsequent defense responses within primed plants mimics *R* gene-dependent plant defense responses in rice (*Oryza sativa*; Jia et al., 2000; Ahn et al., 2005a) and Arabidopsis (Ton et al., 2005).

Expression of a set of pathogenesis-related (*PR*) and defense-related genes has been assumed as one of the reliable molecular markers whether systemic resistance is conditioned or not (Friedrich et al., 1996; Guo et al., 1998; Reuber et al., 1998). In contrast, some agents enhanced plant resistance without direct stimulation of *PR* gene expression and successful disease protection was achieved (Midoh and Iwata, 1997; Abbasi and Graham, 2001; Graham et al., 2003). To dissect priming phenomena at the molecular level, expression profiling of defense-related genes (Kohler et al., 2002), analyses of transcriptomes altered by priming agents (Verhagen

et al., 2004; Pare et al., 2005; Wang et al., 2005), and functional analyses of priming-impaired Arabidopsis mutants have been conducted (Ton et al., 2005). More detailed investigations have revealed that expression patterns of defense genes are largely dependent on the gene that is being monitored. For example, BTH exhibits a dual role at the level of defense gene expression in suspension-cultured parsley cells (Katz et al., 1998) and intact Arabidopsis (Kohler et al., 2002).

In previous research, we presented the alternative role of thiamine as a plant defense activator (Ahn et al., 2005b). The effects of thiamine on disease resistance are prevented in Arabidopsis mutants impaired in SA accumulation. Although expression of *PR* genes was very transient, rapid and robust transcription was initiated by pathogen challenge and successful blast disease protection was achieved in rice. These phenomena indicate that activated defense status was conditioned in thiamine-treated rice plants.

The objectives of this research were to investigate the defense-associated cellular and molecular responses of plants treated with thiamine and, further, to dissect the strict correlation between defense responses and priming. To achieve these goals, the expression pattern of *PR1* and *Phe ammonia-lyase1* (*PAL1*) and the induction of cellular defense responses like AOS accumulation and callose deposition were analyzed in Arabidopsis and its several defense-defective mutants. Results here demonstrate that thiamine-induced priming is in dwelling in plants without physiological alterations and is dependent on hydrogen peroxide accumulation, SA, and *NPR1* (nonexpressor of *PR* genes 1).

RESULTS

Thiamine Protects Arabidopsis from *Pseudomonas syringae* pv *tomato* Infection

Arabidopsis disease, caused by *Pseudomonas syringae* pv *tomato* strain DC3000 (DC3000), is significantly abrogated by systemic acquired resistance (SAR) conditioned by thiamine spray (Ahn et al., 2005b). In this experiment, Arabidopsis was inoculated with DC3000 5 d after 10 mM thiamine spray to determine thiamine-induced priming. Most leaves without thiamine exhibited water-soaked symptoms at 24 to 36 h postinoculation (hpi), turned light yellow at 48 hpi, and finally wilted and died 5 d postinoculation (dpi; Fig. 1A). In contrast, Arabidopsis treated with 10 mM thiamine showed no visible symptoms by DC3000 at 24 hpi. Minute, dark-brown spots were observed at 24 to 36 hpi on the same leaves. These were similar to the symptoms of leaves infected with incompatible DC3000 (avrRpm1). No further symptoms of disease progression were seen thereafter.

Different concentrations of thiamine were applied and disease progression was assessed to determine the dose dependency of SAR. Thiamine spray did not cause any visible alterations in the plants. The 1 mM concentration had no reducing effect on the bacterial titer
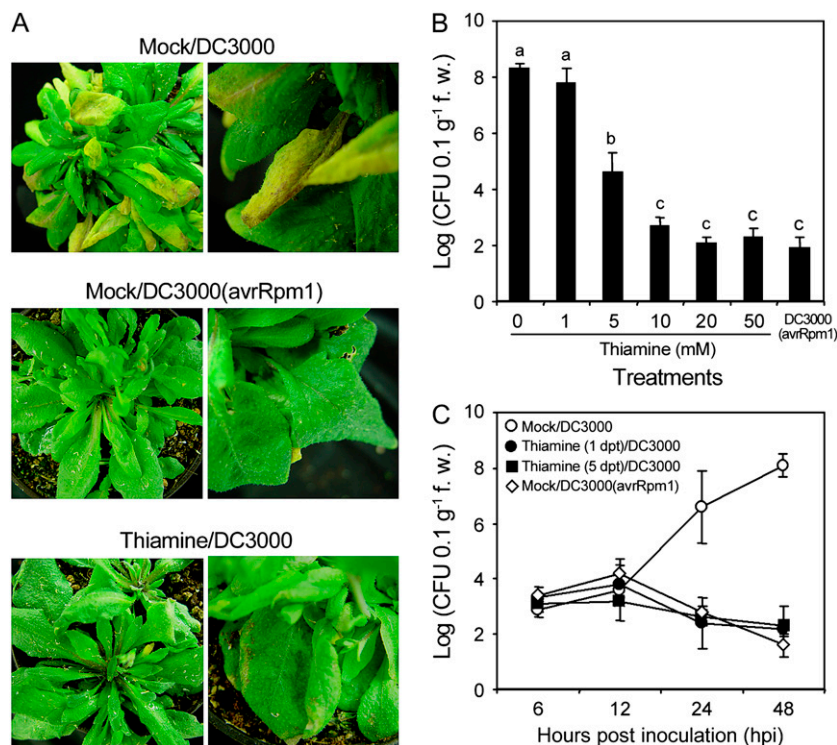
Figure 1. Effect of thiamine application on disease progression in Arabidopsis. A, Arabidopsis ecotype Col-0 plants were inoculated with virulent *P. syringae* pv *tomato* strain DC3000 (1×10^8 CFU mL^{-1} in 250 μg mL^{-1} Tween 20 [mock]) at 4 h after spraying mock or thiamine (10 mM in mock) solutions. Col-0 was also inoculated with DC3000 expressing avrRpm1 [DC3000 (avrRpm1)] as a resistance control. Samples were collected from 25 plants at 4 dpi. The necrotic lesion on Arabidopsis ecotype Col-0 caused by DC3000 was suppressed in thiamine-treated plants. B, DC3000 growth in the leaves of Arabidopsis ecotype Col-0 treated with increasing concentrations of thiamine prior to DC3000 inoculation. Samples (± 1 g fresh weight) were collected from five plants 3 dpi. Each bar represents the mean $\pm$ SE. Different letters indicate statistically significant differences between treatments (Duncan's multiple range test; $P < 0.05$). C, Inhibitory effects of thiamine on bacterial growth in Arabidopsis. Arabidopsis ecotype Col-0 was inoculated with DC3000 1 and 5 d after 10 mM thiamine treatment. Samples (± 1 g fresh weight) were collected from five plants at 6, 12, 24, and 48 h after inoculation. Each point represents the mean $\pm$ SE of bacterial count. dpt, Days posttreatment.

(Fig. 1B), but bacterial growth was reduced significantly with 5 mM and further by 10 mM. The effect on bacterial growth of higher concentrations of thiamine was not significantly different from that of 10 mM. Moreover, 10 mM thiamine inhibited bacterial growth similar to that of incompatible interaction, indicating that 10 mM is sufficient for subsequent experiments about the defense-related responses conditioned by thiamine.

In addition, Figure 1C showed that 10 mM thiamine-induced SAR was retained for more than 5 d. Leaves challenged with DC3000 1 and 5 d after 10 mM thiamine treatment showed significant reduction of pathogen growth, similar to that of the incompatible interaction. On the other hand, DC3000 propagated robustly 12 to 24 hpi in the mock-treated leaves.

Thiamine Primed Augmented Expression of *PR1* and *PAL1*

Rapid accumulation of *PR* gene transcripts has been recognized as one of the molecular indicators for the expression of plant defense responses (Friedrich et al.,

1996; van Loon, 1997; van Loon and van Strein, 1999; Kim et al., 2001). To investigate the kinetics of thiamine action, expression patterns of *PR1* and *PAL1* genes were analyzed. Transcription of both genes was observed 24 hpi in ecotype Columbia (Col-0) leaves inoculated with virulent DC3000 (Fig. 2A). On the other hand, induction of both genes peaked at 6 hpi and was retained thereafter in Col-0 leaves infected with avirulent DC3000 (avrRpm1). Thiamine (10 mM in 250 μg mL^{-1} Tween 20) treatment transiently induced *PR1* expression from 6 to 24 h after treatment (Fig. 2B). However, *PAL1* mRNA was not accumulated by thiamine treatment. Figure 2C also shows the expression patterns of *PR1* and *PAL1* in the plants treated with thiamine and challenged 1 and 5 d later with DC3000. Transcripts of *PR1* and *PAL1* were highly accumulated at 6 hpi in both treatments.

Cellular Defense-Related Responses

Cellular defense responses induced by thiamine and pathogen inoculation were analyzed. DC3000 (avrRpm1)
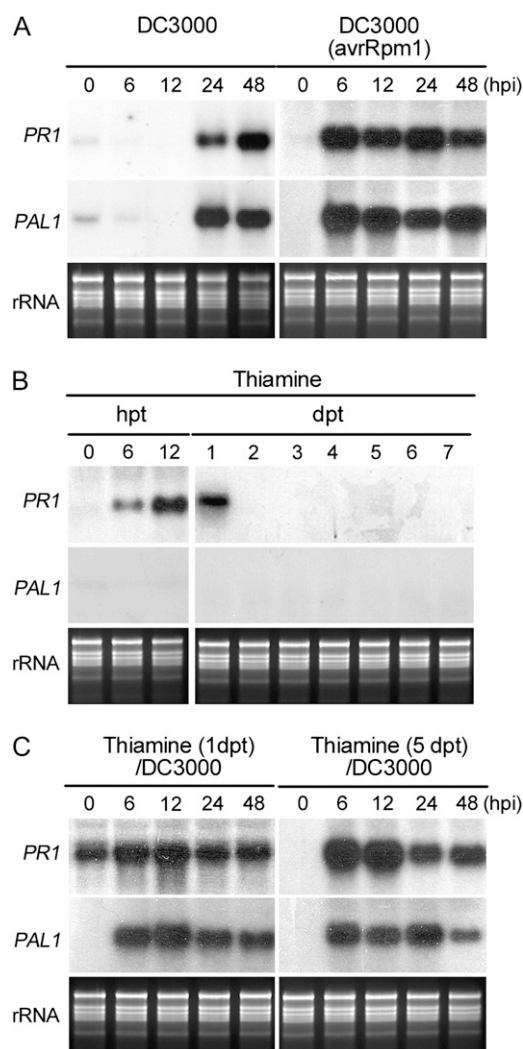
Figure 2. *PR1* and *PAL1* gene expression induced by thiamine treatment and DC3000 inoculation at varying hours posttreatment (hpt), days posttreatment (dpt), hpi, and dpi. Bacterial inoculation and thiamine treatment were performed as described in Figure 1C. A, *PR1* and *PAL1* gene expression induced by virulent DC3000 or avirulent DC3000 (avrRpm1) infection at 0, 6, 12, 24, and 48 hpi. B, Expression of *PR1* and *PAL1* genes in Arabidopsis sprayed with 10 mm thiamine at 0, 6, and 12 hpt and 1, 2, 3, 4, 5, 6, and 7 dpt. C, *PR1* and *PAL1* gene expression in Arabidopsis challenged with virulent DC3000 1 and 5 d after 10 mm thiamine treatment. Total RNA was extracted from the leaves of five Arabidopsis plants recovered 0, 6, 12, 24, and 48 hpi with DC3000.

rapidly induced HR and callose deposition within 12 and 6 hpi, respectively (Fig. 3A). On the other hand, virulent DC3000 infection did not trigger HR and callose deposition within the same time. Thiamine alone did not trigger both responses in Arabidopsis leaves, but thiamine and virulent pathogen challenge induced both responses within 6 and 12 hpi. Quantitative analyses further confirmed the results. Spectrophotometric estimation of Evans blue remained within the dead cells, indicating that virulent DC3000 infection triggered an outbreak of HR within 12 hpi in the thiamine-pretreated leaves (Fig. 3B). The amount of

cell death was comparable with that in the leaves challenged with avirulent DC3000 (avrRpm1; Fig. 3B). Callose deposition was also primed by thiamine treatment. Thiamine or DC3000 alone did not induce callose deposition, but DC3000 challenge on the thiamine-treated leaves induced rapid deposition of callose. The amount of callose accumulated within the thiamine and DC3000-treated leaves was 5 times higher than that detected in thiamine-treated or DC3000-inoculated leaves (Fig. 3C).

Oxidative burst has often been implicated in hypersensitive cell death (Levine et al., 1994); hence, the effect of thiamine on the accumulation of reactive oxygen species was analyzed. Thiamine (10 mm) treatment did not induce superoxide and hydrogen peroxide accumulation; however, DC3000 challenge provoked augmented accumulation of AOS in thiamine-treated leaves (Fig. 3, A, D, and E). Moreover, the level of hydrogen peroxide production at 6 hpi in leaves pretreated with thiamine was similar to that challenged with avirulent pathogens. Superoxide induction in primed leaves was higher than that induced by DC3000 (avrRpm1) infection.

Catalase Nullifies Priming Induced by Thiamine

Catalase was infiltrated into thiamine-treated leaves with virulent pathogens to investigate the role of hydrogen peroxide accumulation on cellular defense responses and thiamine-induced priming. Callose deposition, cell death, bacterial growth in planta, and *PR1*/*PAL1* gene expression were investigated. Thiamine-induced priming of cellular and molecular defense responses was nullified by exogenous catalase that scavenges hydrogen peroxide. Hydrogen peroxide accumulation, callose deposition, and HR in thiamine/DC3000-treated leaves were also abolished (Fig. 4A). Priming by thiamine inhibited bacterial growth in Arabidopsis; however, catalase treatment also interdicted this effect (Fig. 4B). The augmented transcription of *PR1* and *PAL1* was triggered by the virulent pathogen in thiamine-primed leaves; however, this effect was prohibited by the addition of catalase (Fig. 4C).

## Priming Is Dependent on SA and the *NPR1* Gene in Arabidopsis

Disease inhibition and *PR1* mRNA accumulation by thiamine were nullified by the expression of bacterial NahG and *NPR1* mutation (Ahn et al., 2005b). Recently, an abscisic acid (ABA)-related signaling pathway was implicated in BABA-induced priming of defense responses in Arabidopsis (Ton and Mauch-Mani, 2004). To test whether priming by thiamine acts through an ABA-dependent signaling pathway, the effects of thiamine on DC3000 proliferation within the Col-0 and *abi3-3* plants were evaluated. The level of pathogen growth in *abi3-3* plants was similar to Col-0, and thiamine-induced SAR was not affected by this mutation (Fig. 5). This result suggests that thiamine-induced

disease resistance is not related to an ABA-dependent defense-signaling pathway.

Hydrogen peroxide accumulation and callose deposition were analyzed to confirm whether thiamine-induced priming is absent in NahG and *npr1* plants. As shown in Figure 6A, thiamine spray did not affect the production and accumulation of hydrogen peroxide and callose in all plant lines tested. Fortified induction of these defense-related materials was evident

in the Col-0, *etr1* (an altered perception of ethylene mutant), *jar1* (a mutant that displays reduced sensitivity to methyl jasmonate), and *abi3-3* (a mutant insensitive to ABA) plants treated with thiamine and challenged 5 d later with DC3000. However, NahG and *npr1* plants failed to accumulate hydrogen peroxide and did not show callose deposition when challenged with DC3000. Apparently, both lines are insensitive to priming by thiamine.

Figure 3. Effects of priming by thiamine and pathogen challenge on the cellular defense responses in Arabidopsis. Arabidopsis ecotype Col-0 was sprayed with 10 mM thiamine (+) in 250 μg mL^{-1} Tween 20 (mock) or mock only (−). Five days after thiamine treatment, Arabidopsis was inoculated with virulent DC3000 (+). Mock (−) was inoculated with avirulent DC3000 (avrRpm1; +). A, Microscopic observation and quantification of hydrogen peroxide and callose deposition were performed on leaves recovered at 6 hpi. Analyses of superoxide accumulation and HR were conducted on leaves harvested 3 and 12 hpi. Blue formazan precipitate or deep-brown color indicates O_2^{-} or H_2O_2 production. The presence of fluorescence indicates callose deposition. Cell death was determined by the presence (live) or absence (dead) of luminescence. Arrowheads indicate cell death, callose deposition, and superoxide and hydrogen peroxide production. Bars = 50 μm. B, Effects of thiamine and/or DC3000 inoculation on the HR examined by staining with Evans blue. C, Effects of thiamine and/or DC3000 inoculation on callose deposition. D, Effects of thiamine and/or DC3000 inoculation on O_2^{-} accumulation. E, Effects of thiamine and/or DC3000 inoculation on hydrogen peroxide accumulation. Data presented in B to E were taken in experiments conducted three times. Each bar represents the mean ± SE. Different letters indicate statistically significant differences between treatments (Duncan's multiple range test; $P < 0.05$).
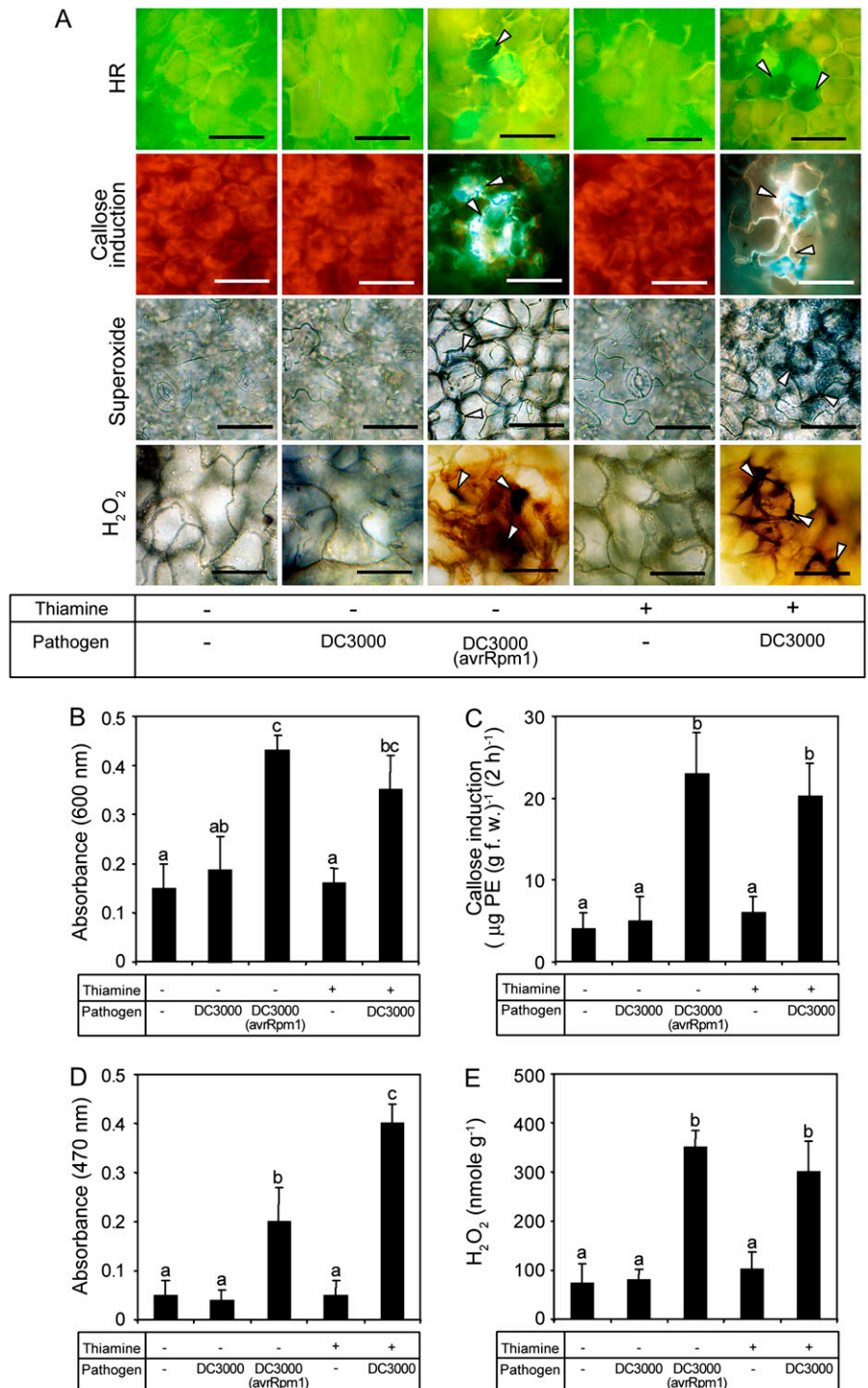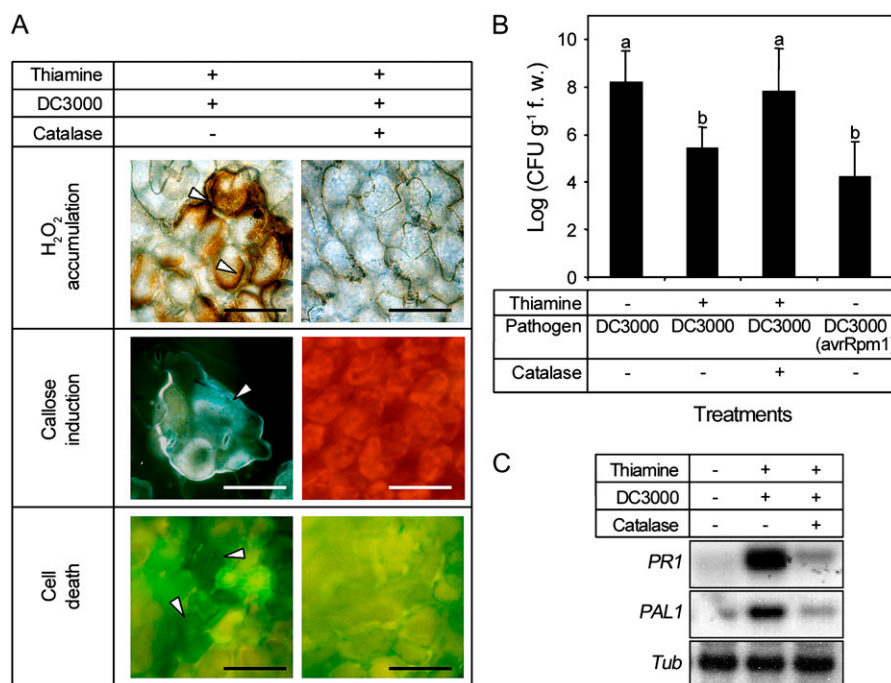
Figure 4. Effects of catalase on cellular defense responses, bacterial growth, and *PR1/PAL1* gene expression in Arabidopsis treated with thiamine and challenged with DC3000. Virulent DC3000 (5×10^6 CFU mL^{-1}) and/or 5,000 units mL^{-1} catalase (+) were infiltrated with needleless syringes into Arabidopsis (ecotype Col-0) leaves 5 d after spraying with 10 mM thiamine in 250 μg mL^{-1} Tween 20 (+) or 250 μg mL^{-1} Tween 20 only (mock; −). A, Effects of exogenous application of catalase on cellular defense responses induced by thiamine. Samples for determination of hydrogen peroxide accumulation, callose deposition, and cell death (HR) were harvested 6, 6, and 12 hpi, respectively. Arrowheads indicate each response. Bars = 50 μm. B, Titers of DC3000 in Arabidopsis Col-0 plants leaves sprayed with thiamine and/or infiltrated with catalase. DC3000 (avrRpm1) plants are infiltrated with avirulent DC3000 (avrRpm1). Each bar represents the mean ± SE. Different letters indicate statistically significant differences between treatments (Duncan's multiple range test; $P < 0.05$). C, Analysis of *PR1* and *PAL1* gene expression in the Col-0 leaves sprayed with thiamine and infiltrated with virulent DC3000 and/or catalase. Total RNA was extracted from five plants 6 h after infiltration, separated using denaturing gel electrophoresis, and transferred to nylon membrane. The blots were hybridized with Arabidopsis *PR1* and *PAL1* probes labeled with [^{32}P]dCTP. All experiments were done at least three times and similar results were obtained.

The effects of priming by thiamine at the molecular level were assessed. Expression of *PR1* and *PAL1* was assayed in thiamine- and/or DC3000-treated leaves by reverse transcription-PCR. *PR1* and *PAL1* transcripts were not accumulated in all plant leaves harvested 5 d after thiamine spray or 6 h after challenge with DC3000 (Fig. 6B). mRNA of both genes was transcribed in the Col-0 plant inoculated 6 h later with DC3000 (avrRpm1). Expression of *PR1* and *PAL1* was augmented in thiamine- and DC3000-treated Col-0, *etr1*, *jar1*, and *abi3-3* plants. In contrast, these treatments did not provoke *PR1* and *PAL1* transcriptions in the NahG and *npr1* plants.

DISCUSSION

This study further supports our previous research on the novel function of thiamine as a plant defense activator (Ahn et al., 2005b). In spite of the lack of *PR* gene transcription, rice plants treated with thiamine showed blast resistance up to 15 d. This result implies that priming might play a key role in thiamine-induced blast resistance. To further explain this phenomenon, the effects of thiamine on cellular and molecular defense responses were analyzed using Arabidopsis and *P. syringae* pv *tomato*.

Thiamine ranging from 5 to 50 mM protects Arabidopsis from bacterial infection (Fig. 1, A and B). This effect was evident in Arabidopsis challenged with DC3000 1 and 5 d after thiamine treatment (Fig. 1C). Results of the in vitro experiment also showed that thiamine did not arrest growth of bacterial pathogens (data not shown). Enhanced disease perturbation in the absence of a direct effect on the causal pathogen confirms the alternative role of thiamine as a plant defense activator. *PR1* gene expression has been used as one of the molecular markers determining whether a plant is ready to counteract against pathogen attack. Although *PR1* expression was induced by thiamine, this was very transient and disappeared 2 d after treatment. Thiamine did not affect *PAL1* transcription per se (Fig. 2B). Interestingly, transcription of both genes was fortified and advanced in Arabidopsis 5 d prior to pathogen challenge (Fig. 2C). Similar expression
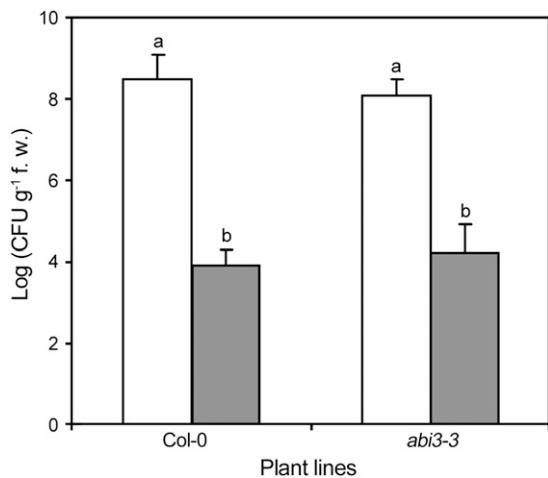
Figure 5. Effects of thiamine on pathogen growth in Col-0 and *abi3-3*. Bacterial growth is shown on Col-0 and *abi3-3* challenged with DC3000 5 d after thiamine treatment. Data were from experiments conducted independently three times. Each bar represents the mean ± SE. Different letters indicate statistically significant differences between treatments (Duncan's multiple range test; $P < 0.05$).

patterns of *PR1* and *PAL1* were evident in Arabidopsis inoculated with avirulent pathogens (Fig. 2A). Distinctive disease protection and augmented expression of defense-related genes after subsequent infection of virulent pathogens were clear molecular evidence for priming Arabidopsis by thiamine. SAR accompanied by priming was observed in Arabidopsis and grapevine (*Vitis vinifera*) treated with BABA (Hamiduzzaman et al., 2005; Ton et al., 2005) and BTH (Kohler et al., 2002). Results of this study further indicated that strong and long-lasting *PR1* transcription by chemical treatment is not crucial evidence for the induction of SAR. Disease protection in the absence of *PR1* expression was observed in wheat (*Triticum aestivum*; Gorlach et al., 1996; Stadnik and Buchenauer, 2000), barley (*Hordeum vulgare*; Jarosch et al., 2003) treated with BTH and DCA, and rice and tobacco (*Nicotiana tabacum*) treated with brassinolide (Nakashita et al., 2003).

Pathogen challenge triggered fortified AOS accumulation within leaf tissue of thiamine-treated Arabidopsis (Fig. 3). Thiamine and virulent pathogen challenge was required for potentiated AOS production. On the contrary, thiamine spray or virulent pathogen inoculation did not induce AOS burst at the same time (Fig. 3A). These results indicate that rapid AOS production should be one of the defense mechanisms of priming by thiamine. Accordingly, pronounced AOS accumulation followed by pathogen challenge is one of the typical responses of primed plants (Neuenschwander et al., 1995; Desikan et al., 1998; Huckelhoven et al., 1999; Able et al., 2000; Orozco-Cardenas et al., 2001; Pellinen et al., 2002; Shinogi et al., 2003). Thiamine did not affect callose deposition and hypersensitive cell death per se. However, pathogen challenge also provoked augmented callose induction within leaf tissues of Arabidopsis treated with thiamine. Similar results

were obtained from microscopic observation of hypersensitive cell death. Our findings indicated that primed Arabidopsis by thiamine was in a surveillance state extremely sensitive to pathogen challenge and, in addition, priming and *R* gene-dependent resistance shared several features, including AOS burst, callose deposition, hypersensitive cell death, and expression patterns of defense-related genes. Similar results were previously described (Kohler et al., 2002; Graham et al., 2003; Faize et al., 2004; Ton et al., 2005). In particular, the expression pattern of the *PAL1* gene is almost completely identical to potentiated cellular defense responses because pathogen challenge triggered strong and advanced augmentation of both responses within plants primed by thiamine application. Apparently, *PAL1* and AOS are involved in defense-related metabolism, SA accumulation (Mauch-Mani and Slusarenko, 1996; Smith-Becker et al., 1998), and callose induction (Lyngkjaer and Carver, 2000).

Hydrogen peroxide is also involved in hypersensitive cell death (Lyngkjaer and Carver, 2000; Houot et al., 2001; Kachroo et al., 2003) and acted as a signaling molecule in cellular defense responses (Alvarez et al., 1998; Hu et al., 2003; Fitzgerald et al., 2004). Catalase infiltration almost completely perturbed hydrogen peroxide accumulation by pathogen challenge in thiamine-treated Arabidopsis. This was accompanied by abolition of disease protection, callose induction, hypersensitive cell death, and *PR1/PAL1* expression (Fig. 4A). These results strongly suggest that hydrogen peroxide is required for priming by thiamine. Moreover, callose induction and hypersensitive cell death were under the control of hydrogen peroxide in priming by thiamine. Dependence of cell wall fortification on AOS was also reported in Arabidopsis (Razem and Bernards, 2003; Al-Daoude et al., 2005). Hydrogen peroxide also played a substantial role in DCINA-induced SAR (Huckelhoven et al., 1999) and could have contributed to effective papillae (comprised with callose) formation (Bestwick et al., 1997). Catalase did not inhibit pathogen proliferation (van Wees and Glazebrook, 2003); hence, results here clearly indicate that inhibition of AOS prevented priming by thiamine and resulted in SAR impairment.

No discrete hydrogen peroxide accumulation and callose induction were observed in wild-type Col-0 and mutants (Fig. 6A). Challenge of virulent pathogens at 5 d postthiamine spray strongly induced both cellular and molecular defense-related responses in wild-type and *etr1*, *jar1*, and *abi3-3* plants. In contrast, the same treatment and inoculation did not trigger both events in the NahG and *npr1* lines. In parallel, pathogen inoculation triggered fortified transcription *PR1* and *PAL1* genes in Col-0, *etr1*, *jar1*, and *abi3-3* lines and this augmented transcription was nullified by bacterial NahG expression and *NPR1* mutation (Fig. 6B). It could be concluded from our findings that priming by thiamine exerts its effects through the SA-dependent signaling pathway and might be dependent on hydrogen peroxide accumulation. Recently, priming effects
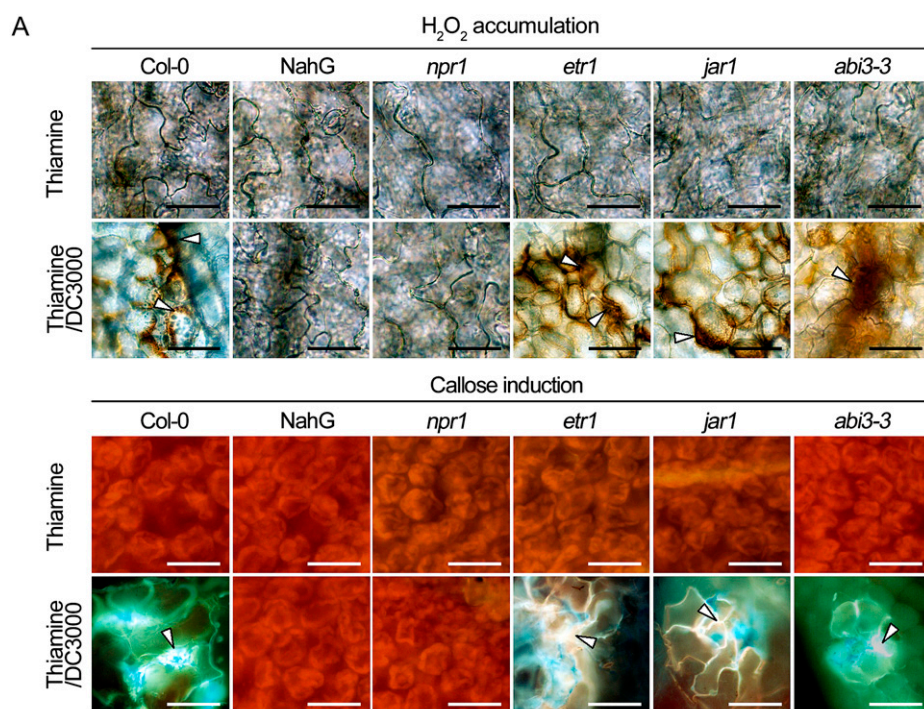
Figure 6. Effects of priming by thiamine on the accumulation of hydrogen peroxide, callose deposition, and *PR1/PAL1* transcription in Arabidopsis Col-0 and its mutants. DC3000 was inoculated 5 d after thiamine spray and samples were recovered 6 hpi. A, Hydrogen peroxide accumulation and callose deposition in Arabidopsis Col-0, NahG, *npr1*, *etr1*, *jar1*, and *abi3-3*. B, Transcription of *PR1* and *PAL1* in Arabidopsis Col-0, NahG, *npr1*, *etr1*, *jar1*, and *abi3-3*. Data were from Arabidopsis sprayed with 10 mM thiamine and 250 μg mL^{-1} Tween 20 (+) or 250 μg mL^{-1} Tween 20 only (mock; −). Samples were harvested 6 hpi. In addition, leaves of Col-0 were harvested 1 d after thiamine spray (T [1 dpt]) and avirulent DC3000 (avrRpm1) inoculation.
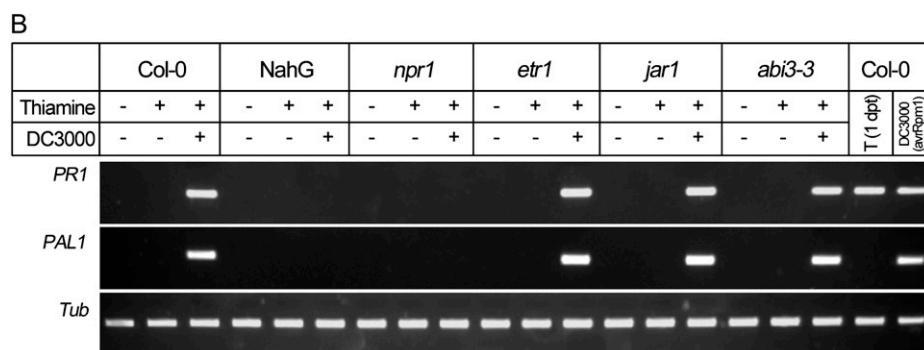
by BABA were investigated in tobacco (Siegrist et al., 2000) and Arabidopsis (Ton and Mauch-Mani, 2004). Interestingly, the mode of action of priming by BABA in tobacco and Arabidopsis was distinct. Protection of tobacco mosaic virus infection by BABA-induced priming was nullified by the expression of bacterial NahG, whereas abolition of *Alternaria brassicicola* and *Plectosphaerella cucumerina* infections was not influenced by SA accumulation, but was dependent on ABA. This indicated that acting sites of thiamine and BABA are different in the Arabidopsis defense-signaling cascades and imply that the mode of action of a priming agent is highly influenced by the kind of hosts and pathogens.

The results further demonstrated that priming and its associated cellular and molecular defense mechanisms were induced by thiamine. Thiamine altered the plant into a highly competent state for a long time in the absence of detectable variations. Subsequent pathogen challenge triggered augmented molecular and cellular defense-related responses in thiamine-applied

Arabidopsis. Besides its physiological and genetic importance, priming by thiamine could be one of the most economical and effective resistances because expression of defense-related mechanisms in the absence of pathogen requires the plant's metabolic investment necessary for growth or other fitness-related processes (Purrington, 2000; Heil, 2001; van Hulten et al., 2006). Along with conventional antibiotics, previously developed plant defense activators, biocontrol organisms, and improved seed varieties, thiamine could provide novel disease control strategies that satisfy environmental regulations.

MATERIALS AND METHODS

Plant Materials, Chemical Treatment, and Pathogen Challenge

Seeds of the Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0, the Col-0 expressing bacterial *NahG* gene, and mutants (*npr1*, *etr1*, *jar1*, and *abi3-3*) from this line were obtained from The Arabidopsis Information Resource. Arabidopsis

was grown in a growth chamber at 22°C, 65% to 70% relative humidity, and 16 h of illumination daily. Four-week-old Arabidopsis was used for chemical treatment and pathogen inoculation.

Thiamine (10 mM, unless otherwise indicated) in 250 μg mL^{-1} Tween 20 (mock) was hand sprayed onto Arabidopsis until all the plants were covered with fine droplets. The DC3000 and DC3000 (avrRpm1) strains of *Pseudomonas syringae* pv *tomato*, each containing pVSP61 and pVSP61 carrying avrRpm1, were cultivated on King's medium B with 50 μg mL^{-1} kanamycin for 48 h at 28°C. To inoculate Arabidopsis with DC3000 and DC3000 (avrRpm1), bacterial cells were retrieved from the medium with 0.85% NaCl and mock, and the concentration was adjusted to 1×10^8 or 5×10^6 colony-forming units (CFU) mL^{-1}. At least 25 plants of Arabidopsis ecotype Col-0 were inoculated per treatment. Bacterial suspension (1×10^8 CFU mL^{-1}) was sprayed until all leaves were covered with fine droplets 1 or 5 d after thiamine or mock treatment. To investigate the effect of catalase on priming by thiamine, bacterial suspension (5×10^6 CFU mL^{-1}) and catalase (5,000 units mL^{-1}) were also infiltrated into the parenchyma tissue of rosette leaves with a 1-mL needleless plastic syringe. The inoculated plants were kept in a dew chamber for 16 h at 25°C and 100% relative humidity and then transferred to a growth chamber with a 16-h light/8-h dark regime at 25°C and 80% relative humidity. Bacterial growth was assessed by determining the CFU of 1 g (fresh weight) of leaves of five Arabidopsis plants through plating appropriate dilutions on King's B medium containing 50 μg mL^{-1} kanamycin.

Histochemistry: Superoxide, Hydrogen Peroxide, and Callose

To investigate the effect of thiamine on oxidative burst and callose deposition, more than 10 plants applied with 10 mM thiamine or mock were challenged 5 d later with virulent DC3000. Histochemical detection of superoxide and hydrogen peroxide were performed as described previously (Wohlgemuth et al., 2002). To observe superoxide production, leaves were harvested at 3 hpi and infiltrated immediately with 10 mM sodium azide (NaN$_3$; Sigma) and 0.1% (w/v) nitroblue tetrazolium solution. To determine the accumulation of hydrogen peroxide, leaves were recovered at 6 hpi and stained with 0.1% (w/v) diaminobenzidine (Sigma). Then leaves were cleared with 96% (v/v) ethanol, preserved in 50% (v/v) ethanol, and observed under a light microscope. Superoxide and hydrogen peroxide were indicated as blue formazan formation and red-brown precipitate under the light microscope. To determine callose deposition, leaves were recovered at 6 hpi, fixed with lactophenol, and stained with 0.1% (w/v) aniline blue. Fluorescence of callose was detected with an epifluorescence microscope (E800; Nikon) using a V-2A filter (Reuber et al., 1998). More than 15 leaves from five randomly selected plants were observed in each experiment. These experiments were done at least three times.

Quantitative Determination of Superoxide Radical, Hydrogen Peroxide, and Callose

Superoxide and hydrogen peroxide were extracted from thiamine-treated and/or pathogen-inoculated Arabidopsis leaves and quantified as described (Neuenschwander et al., 1995; Ukeda et al., 1997; Willekens et al., 1997; Frahry and Schopfer, 2001) with some modifications. Arabidopsis leaves were harvested, pulverized with Geno/Grinder (SPEX CertiPrep), extracted with perchloric acid, and debris was removed by centrifugation at 5,000g, 4°C for 15 min. The recovered supernatants were purified using AG 1-X8 resin (Bio-Rad Laboratories). Superoxide level within purified plant extract was determined spectrophotometrically in the presence of 500 μM Na, 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) bezensulfonic acid hydrate (Sigma), and NADH. Hydrogen peroxide levels were determined using Autolumat LB953 luminometer (EG & G Derthold) as described (Baker et al., 2002). Each reaction mixture contained 0.72 units of peroxidase and 77.6 mM luminol. Extraction and measurement of callose from Arabidopsis leaves were done as described (Kohler and Conrath, 2000). Arabidopsis leaves were harvested and pulverized after complete removal of chlorophyll with ethanol. Callose was extracted from the remaining tissues through boiling in dimethyl sulfoxide and the debris was removed by centrifugation at 5,000g, 4°C for 15 min. After centrifugation, 1 M NaOH and loading mixture containing aniline blue were added to the supernatant. Total fluorescence at 479 nm was determined and fluorescence of callose was calculated by subtracting the autofluorescence in the parallel assay performed without aniline blue. Callose within 1 g fresh weight was quantified based on comparison with the epifluorescence of known amounts of the commercial β-1,3-glucan pachyman

(Calbiochem). Samples were harvested from 20 plants in each experiment and these experiments were repeated more than three times.

Detection and Quantification of Cell Death

The effects of thiamine and DC3000 inoculation on cell death were determined. Rosette leaves were recovered 5 d after 10 mM thiamine spray, inoculated with DC3000, and harvested 12 h later. Dead cells or tissues were detected by dye-staining methods. Recovered Arabidopsis leaves were stained with 100 μg mL^{-1} fluorescein diacetate (Sigma). After 30 min, leaf tissues were observed under a fluorescence microscope using an excitation filter at 450 nm. To quantify dead cells, leaf discs (0.5 mm in diameter) were stained for 30 min with 0.25% Evans blue (Sigma) and washed to remove excess stain (Mino et al., 2002). Dye bound to dead cells was extracted with 1 mL of 50% methanol supplemented with 1% SDS for 1 h at 50°C. Absorbance at 600 nm was estimated with 10-fold dilution of the above dye extract.

RNA Isolation and Expression Analyses

Total RNA was extracted using the lithium chloride precipitation method (Davis and Ausubel, 1989). Hybridization analysis was performed as described (Ahn et al., 2005b). Analyses of *PR1* and *PAL1* gene expression were performed using reverse transcription-PCR as described (Pieterse et al., 1998). Leaves of Col-0, NahG, *npr1*, *etr1*, *jar1*, and *abi3-3* were recovered from five plants of each treatment and total RNA was prepared. First-strand cDNA was synthesized from 50 ng total RNA using a Reverse-iT first-strand synthesis kit and anchored oligo(dT) as indicated by the manufacturer's instructions (AB Gene). Independent PCR using equal aliquots (0.5 μL) of cDNA samples was performed using the gene-specific primers described (Vieira Dos Santos et al., 2003). The *tubulin* gene was amplified as a quantitative control (Lee et al., 2000). The experiments were repeated at least twice and similar results were obtained.

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