Vitamin B\textsubscript{1}-Induced Priming Is Dependent on Hydrogen Peroxide and the NPR1 Gene in Arabidopsis\textsuperscript{1}

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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) gene expression analysis 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-carboxylic acid S-methyl ester (BTH; Friedrich et al., 1996; Gorlach et al., 1996; Bokshi et al., 2003), and \(\beta\)-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...
Primed plants share many characteristics with intact Arabidopsis (Kohler et al., 2002). 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
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)
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 \textit{PR1}/\textit{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 interrupted this effect (Fig. 4B). The augmented transcription of \textit{PR1} and \textit{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 \textit{NPR1} Gene in Arabidopsis

Disease inhibition and \textit{PR1} mRNA accumulation by thiamine were nullified by the expression of bacterial NahG and \textit{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 \textit{abi3}-3 plants were evaluated. The level of pathogen growth in \textit{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 (+) or 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 O₂⁻ or H₂O₂ 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 in O₂⁻ 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).
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). 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 [32P]dCTP. All experiments were done at least three times and similar results were obtained.

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 [32P]dCTP. All experiments were done at least three times and similar results were obtained.

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
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 PRI transcription by chemical treatment is not crucial evidence for the induction of SAR. Disease protection in the absence of PRI 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 PALI 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, PALI 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 PRI/PALI 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 PRI and PALI 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 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).
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

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**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.
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 μM, 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 pVS61 and pVS61 carrying avrRpm1, were infiltrated on Kingdom medium B with 50 μg mL⁻¹ kanamycin for 24 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 x 10⁸ or 5 x 10⁷ colony-forming units (CFU) mL⁻¹. At least 25 plants of Arabidopsis ecotype Col-0 were inoculated per treatment. Bacterial suspension (1 x 10⁷ 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 x 10⁷ 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 counting the CFU of 1-g (fresh weight) leaves of *Arabidopsis* at 24 hpi. These experiments were done at least three times.

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 μM 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 μM sodium azide (Na₃N₃, Sigma) and 0.1% (w/v) nitroblue tetrathion 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 (EB800, 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; Ueda et al., 1997; Willekens et al., 1997; Frhary and Schopier, 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 I-X8 resin (Bio-Rad Laboratories). Superoxide level within purified plant extract was determined spectrophotometrically in the presence of 500 μM Na, 3-[(4-phenoilamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (Sigma), and NADH. Hydrogen peroxide levels were determined using Autolumat LB953 lumimeter (EG & G Derthold) as described (Baker et al., 2002). Each reaction mixture contained 0.72 units of peroxidase and 77.6 μM 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 ml 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 μM 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% w/v 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 PKI and PALI gene expression were performed using reverse transcription-PCR as described (Piepere et al., 1998). Leaves of Col-0, NahG, npr1, etr1, jar1, and ahb3-3 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 *tabulius* 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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