Vitamin B_1 Functions as an Activator of Plant Disease Resistance[1]

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Vitamin B_1 (thiamine) is an essential nutrient for humans. Vitamin B_1 deficiency causes beriberi, which disturbs the central nervous and circulatory systems. In countries in which rice (*Oryza sativa*) is a major food, thiamine deficiency is prevalent because polishing of rice removes most of the thiamine in the grain. We demonstrate here that thiamine, in addition to its nutritional value, induces systemic acquired resistance (SAR) in plants. Thiamine-treated rice, Arabidopsis (*Arabidopsis thaliana*), and vegetable crop plants showed resistance to fungal, bacterial, and viral infections. Thiamine treatment induces the transient expression of pathogenesis-related (PR) genes in rice and other plants. In addition, thiamine treatment potentiates stronger and more rapid PR gene expression and the up-regulation of protein kinase C activity. The effects of thiamine on disease resistance and defense-related gene expression mobilize systemically throughout the plant and last for more than 15 d after treatment. Treatment of Arabidopsis ecotype Columbia-0 plants with thiamine resulted in the activation of *PR-1* but not *PDF1.2*. Furthermore, thiamine prevented bacterial infection in Arabidopsis mutants insensitive to jasmonic acid or ethylene but not in mutants impaired in the SAR transduction pathway. These results clearly demonstrate that thiamine induces SAR in plants through the salicylic acid and Ca^{2+}-related signaling pathways. The findings provide a novel paradigm for developing alternative strategies for the control of plant diseases.

Plants, like animals, are continually exposed to pathogen attack and have developed an innate surveillance mechanism that enables them to rapidly ward off attempted invasions by pathogens. The key differences between the compatible (susceptible) and incompatible (resistant) interactions are the timely recognition of pathogen attack and the rapid, appropriate expression of defense responses (Yang et al., 1997; McDowell and Dangl, 2000; Kim et al., 2001a; Umemura et al., 2003; Lu et al., 2004; Bennett et al., 2005). In incompatible interactions, the plant's resistance (*R*) gene product acts as a signaling receptor for the pathogen's avirulence (*Avr*) gene product in the presence of resistance-regulating factors such as RAR1 and SGT1, leading to a form of cell death termed hypersensitive response (HR; Flor, 1971; Shen et al., 2003; Allen et al., 2004; Belkhadir et al., 2004; Bieri et al., 2004; Bohnert et al., 2004; Zhang et al., 2004; Rowland et al., 2005). HR-mediated cell death is triggered sequentially through an increase in the intracellular cytosolic Ca^{2+} concentration by an influx of external Ca^{2+} and the secretion of Ca^{2+} from the calcium stores into the cytoplasm (Bowler and Fluhr, 2000; Grant et al., 2000; Chung et al., 2004), a burst of reactive oxygen species (Levine et al., 1994; Sandermann, 2000; Tanaka et al., 2003; de Jong et al., 2004; Tsukamoto et al., 2005), changes in the extracellular pH and membrane potentials (Bolwell et al., 1995), and variations in protein phosphorylation patterns (Dietrich et al., 1990; Peck et al., 2001; de Jong et al., 2004). Finally, key mediators such as salicylic acid (SA) accumulate and resistance is induced systemically (Gaffney et al., 1993; Durrant and Dong, 2004; Pieterse and Van Loon, 2004).

HR eliminates infected host cells that support continuous plant-pathogen interactions. The plant begins to express a subset of pathogenesis-related (PR) genes locally at the point of infection, and induced resistance develops systemically with increases in the concentrations of key mediators (Mittler et al., 1997; McDowell and Dangl, 2000; Sasaki et al., 2004). The rapid and timely elevation of PR gene transcripts has been recognized as one of the most important events in and indicators of the resistant-incompatible interaction (Bent, 1996; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Tanaka et al., 2003; de Jong et al., 2004; Kim et al., 2004). Although different subsets of the defense signaling cascade are expressed in each pathosystem during pathogen recognition and infection, most defense mechanisms are common to the different systems. Oxidative bursts and ion fluxes have been observed in numerous incompatible plant-pathogen interactions, and secondary signaling messengers are up-regulated systemically prior to the expansion of

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local lesions (Bolwell et al., 1995; Grant et al., 2000; Martinez et al., 2000; Kachroo et al., 2003). Furthermore, treatment with SA or its commercial derivative benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) inhibits disease progress in many compatible plant-microbe interactions (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996; Wurms et al., 1999; Kohler et al., 2002; Bokshi et al., 2003; Achuo et al., 2004) and prevents the infection of plant roots with root-parasitic weeds (Sauerborn et al., 2002). These findings support the use of chemicals that induce plant resistance for the control of plant diseases.

Systemic acquired resistance (SAR) is enhanced resistance against many but not all fungal, bacterial, and viral pathogens, and is generally triggered by pathogen-induced localized cell death, HR, which occurs as local lesions and can spread over the entire plant. SAR induces long-lasting, efficient resistance against a broad spectrum of pathogens (McIntyre et al., 1981; Ryals et al., 1994; Achuo et al., 2004). SAR could serve as a basis for novel disease control strategies involving genetically engineered plants with enhanced disease resistance and agrochemicals that induce the mimicry of incompatible interactions. To accomplish this goal, mutant lines, including the constitutive expression of PR genes (*cpr*; Bowling et al., 1994; Clarke et al., 2000) and constitutive immunity (*cim*) mutants of Arabidopsis (*Arabidopsis thaliana*; Maleck et al., 2002), and several chemicals, including BTH, dichloroisonicotinic acid (DCINA; Schweizer et al., 1997; Colson-Hanks and Deverall, 2000), and probenazole (Midoh and Iwata, 1996; Yoshioka et al., 2001), have been characterized and developed. Chemical plant defense activators have several advantages over disease control methods that depend on traditional fungicides or bactericides and breeding for resistance. Importantly, strategies that exploit SAR are generally environmentally safe and do not affect the appearance of chemical-tolerant strains or induce the breakdown of resistance; their effects last for long periods and the application range is relatively large.

In recent years, the importance of vitamins as nutrients and as disease control agents has been emphasized. Genetically engineered rice (*Oryza sativa*) with increased endosperm provitamin A content has been developed to reduce deficiency of this nutrient (Beyer et al., 2002). A novel function of riboflavin (vitamin B_2) in disease resistance has also been described (Dong and Beer, 2000). Treatment with riboflavin protects tobacco (*Nicotiana tabacum*) and Arabidopsis plants from fungal and bacterial infections without inhibiting pathogen growth.

Thiamine is a B-complex vitamin that is produced in plants and microbes, including brewer's yeast (*Saccharomyces cerevisiae*; Burrows et al., 2000) and *Salmonella typhimurium* (Beck and Downs, 1999). Thiamine deficiency causes beriberi, Wernicke-Korsakoff syndrome, Alzheimer's syndrome, and alcoholic ketoacidosis (Mimori et al., 1996). Thiamine occurs in animals, plants, and microbes as free thiamine and the phosphorylated forms thiamine monophosphate (TMP), thiamine pyrophosphate (TPP), and thiamine triphosphate. These forms act as coenzymes in numerous physiological processes, including glycolysis, the pentose phosphate pathway, and the synthesis of nucleic acids and the niacin-containing coenzyme NADPH.

In this study, we present a novel role for thiamine as a plant defense activator that induces SAR. Thiamine activates SAR-related genes in rice, tobacco, tomato (*Lycopersicon esculentum*), cucumber (*Cucumis sativus*), and Arabidopsis and prevents several diseases caused by semibiotrophic and biotrophic pathogens. The effects of thiamine on disease resistance are prevented in Arabidopsis mutants impaired in SA accumulation as well as by treatment with the calcium channel blocker $LaCl_3$, demonstrating that thiamine induces SAR in plants through the SA- and Ca^{2+}-related signaling pathways.

RESULTS

Thiamine Induces Disease Resistance

To evaluate the plant defense activation activity of thiamine, thiamine-treated rice plants (cv Hwacheong) were inoculated with the compatible blast fungus *Magnaporthe grisea* strain KJ201 (Fig. 1A). Control plants not treated with thiamine developed typical diamond-shaped lesions, and massive conidia formed at the center of each lesion at 10 d after inoculation. By contrast, disease protection was evident in plants treated with 50 mM thiamine 4 h prior to inoculation with *M. grisea*. Microscopic observations revealed that fungal growth was restricted to areas within the infection sites, and rapid cell death was observed at the site of attempted penetration of host cells (data not shown). These responses are typical for HR in the rice cultivar Hwacheong inoculated with the avirulent *M. grisea* strain KJ401, an incompatible interaction (Kim et al., 2001b). These observations indicate that thiamine stimulates the rice defense system by converting a compatible interaction into an incompatible interaction.

Thiamine treatment of the rice cultivar Nakdong also induced resistance to the compatible bacterial leaf blight pathogen *Xanthomonas oryzae* pv *oryzae* strain KXO21 (Fig. 1B). In control rice plants, typical blight symptoms clearly appeared at 48 h after inoculation and began to progress along the vascular systems. On leaves that had been treated with 50 mM thiamine, the inoculated, clipped sites rapidly changed to a dark brown color within 36 h after inoculation, and no disease progress was observed thereafter.

In addition to rice plants, we tested the effects of thiamine in cucumber, tobacco, and Arabidopsis against fungal, bacterial, and viral infections. Thiamine protected susceptible tobacco plants (cv Samsun NN) against infection by *Pepper mild mottle virus* (*PMMoV*; Fig. 1C). Typical symptoms of systemic *PMMoV* infection appeared in the leaves of untreated

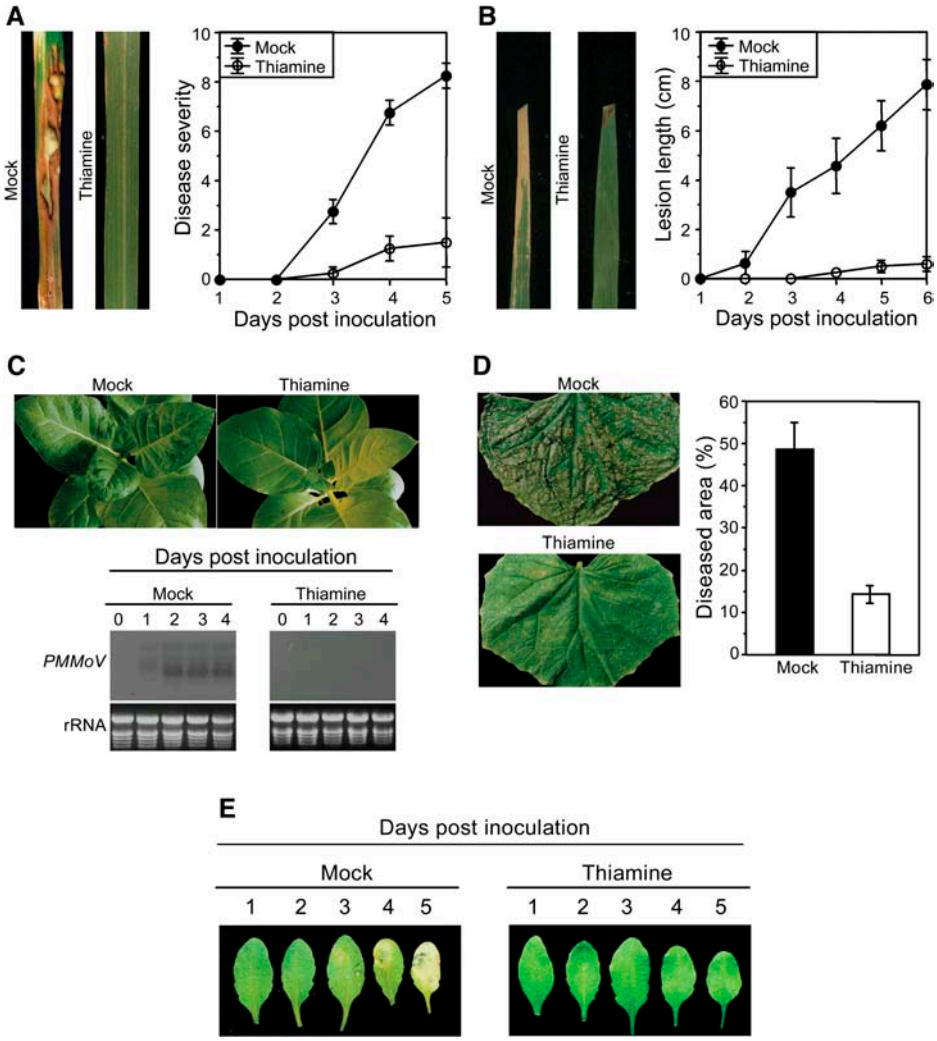

Figure 1. Effects of thiamine application on disease progress in rice, tobacco, cucumber, and Arabidopsis. Plants were inoculated with each pathogen at 4 h after spraying of mock (control, 250 μg mL^{-1} Tween 80) or thiamine (thiamine, 50 mM in 250 μg mL^{-1} Tween 80) solutions. A, The necrotic lesions normally caused on the rice cultivar Hwacheong by the rice blast disease pathogen *M. grisea* strain KJ201 (5×10^5 conidia mL^{-1}) are suppressed in thiamine-treated leaves. The leaves were photographed at 7 d post inoculation. The graph shows the protection against blast disease provided by the pretreatment with thiamine. The disease severities were evaluated daily as described in "Materials and Methods." Each point represents the mean ± SE of 10 plants. B, The yellow lesions normally caused on the rice cultivar Nakdong by the rice bacterial leaf blight pathogen *X. oryzae* pv *oryzae* strain KXO21 (1×10^8 CFU mL^{-1}) are suppressed in thiamine-treated rice plants. The leaves were photographed at 10 d post inoculation. The graph shows the reduced lesion lengths observed after pretreatment with thiamine. Each curve represents the mean ± SE of 10 plants. C, Thiamine acts as an antiviral compound against *PMMoV* infection in the genetically susceptible tobacco cultivar Samsun NN. The second true leaf of mock- or thiamine-treated plants was inoculated with *PMMoV* and photographed at 10 d post inoculation. Three inoculated leaves were harvested at 0, 1, 2, 3, and 4 d post inoculation for RNA extraction. Northern-blot hybridization analyses were conducted using an RT-PCR product of *PMMoV* labeled with [^{32}P]dCTP as a probe. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel. D, The necrotic lesions normally caused on the cucumber cultivar Sunmi-Baekdadaki by the cucumber anthracnose pathogen *C. lagenarium* are suppressed in thiamine-treated cucumber plants. The graph shows the levels of disease protection observed in thiamine-treated plants and untreated controls. The percentage of the symptomatic area was evaluated at 7 d after inoculation and photographed as described in "Materials and Methods." Each bar represents the mean ± SE of five plants. E, The necrotic lesions normally caused on Arabidopsis ecotype Col-0 by the Arabidopsis pathogen *Pst* DC 3000 are suppressed in thiamine-treated plants. Representative samples were collected from 10 plants at 1, 2, 3, 4, and 5 d after spraying with a bacterial solution of 1×10^8 CFU mL^{-1}.

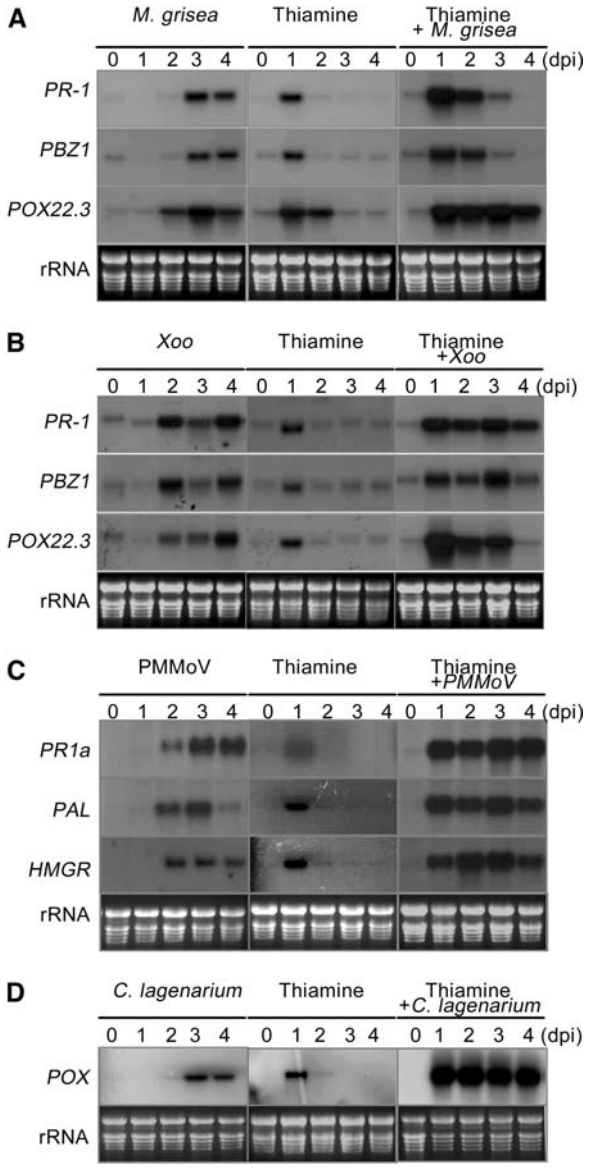

Figure 2. Defense-related gene expression induced by thiamine treatment and pathogen inoculation. Pathogen inoculation and thiamine treatment were performed as described in Figure 1. dpi, Days post inoculation. A, Rice (cv Hwacheong) defense-related gene expression induced by spraying with a thiamine solution and/or infection by *M. grisea* strain KJ201. Total RNA was extracted from the leaves of five rice plants at 0, 1, 2, 3, and 4 d after inoculation with *M. grisea*. B, Rice (cv Nakdong) defense-related gene expression induced by spraying with a thiamine solution and/or infection with *X. oryzae* pv *oryzae* strain KXO21 (*Xoo*). Total RNA was extracted from the leaves of five rice plants at 0, 1, 2, 3, and 4 d post inoculation with *Xoo*. C, Defense-related gene expression induced in tobacco by spraying with a thiamine solution and/or infection with *PMMoV*. Total RNA was extracted from the second true leaves of five tobacco plants at 0, 1, 2, 3, and 4 d following inoculation with *PMMoV*. D, Expression of the cucumber acidic peroxidase gene (*POX*) induced by spraying with a thiamine solution and/or infection with *C. lagenarium*. Total RNA was extracted from the second and third true leaves of five cucumber plants at 0, 1, 2, 3, and 4 d following inoculation with *C. lagenarium*. Total RNA was extracted, separated by denaturing gel electrophoresis, and transferred to nylon membrane. The blots were hybridized with probes labeled with [^{32}P]dCTP. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel.

control plants, but no clear symptoms or visible disease progress were observed in thiamine-treated tobacco plants. Replication of *PMMoV* was almost completely inhibited in thiamine-treated leaves. Furthermore, thiamine protected cucumber plants against anthracnose (*Colletotrichum lagenarium*; Fig. 1D) and powdery mildew (*Sphaerotheca fuliginea*) infection (data not shown). Thiamine also protected the Arabidopsis ecotype Columbia-0 (Col-0) against infection with the virulent *Pseudomonas syringae* pv *tomato* strain DC 3000 (*Pst* DC 3000; Fig. 1E). These data strongly suggest that thiamine protects not only rice but also cucumber, tobacco, and Arabidopsis against a broad spectrum of fungal, bacterial, and viral pathogens.

The effects of thiamine on the growth of *M. grisea* and *X. oryzae* pv *oryzae* were determined in vitro by growing the pathogens in media supplemented with thiamine to concentrations ranging from 0 to 50 mM. Both pathogens grew well at all of the thiamine concentrations tested, indicated by the similar colony diameters of the fungal cultures on agar plates and the similar numbers of colony-forming units (CFU) of the bacterial suspension cultures, respectively (data not shown).

Kinetics of Thiamine-Induced Resistance

To understand the mechanisms involved in thiamine-induced resistance in rice, we first analyzed the expression patterns of three rice PR genes (Chitoor et al., 1997; Kim et al., 2001b): *PR-1* (a gene encoding PR protein 1), *PBZ1* (a gene encoding intracellular *PR-10*, probenazole inducible), and *POX22.3* (a gene encoding peroxidase, *PR-11*). Inoculation of the rice cultivar Hwacheong with the virulent *M. grisea* strain KJ201 and of the cultivar Nakdong with the virulent *X. oryzae* pv *oryzae* strain KXO21 induced the expression of these genes at 72 and 48 h after inoculation, respectively (Fig. 2, A and B). However, thiamine treatment without pathogen inoculation induced the expression of these genes at 24 h after treatment, which is more rapid than that which occurs after pathogen inoculation. Much higher expression was observed after pathogen inoculation following thiamine treatment. These expression patterns are similar to those induced in rice plants inoculated with the avirulent *M. grisea* pathogenic strain KJ401, an incompatible combination (Kim et al., 2001b).

To determine whether thiamine affects the accumulation of defense-related mRNAs in other plants, we investigated the expression patterns of *PR-1a*, *PAL* (a gene encoding Phe ammonia lyase), and *HMGR* (a gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase) in tobacco and *POX* (a gene encoding acidic peroxidase; Narusaka et al., 1999) in cucumber (Fig. 2,

C and D). At 48 h after inoculation of tobacco cultivar Samsun NN with the virulent *PMMoV*, the expression of these genes was induced. Thiamine had triggered the accumulation of these transcripts by 24 h after treatment, which is more rapid than that observed after pathogen inoculation. As noted above, increased expression was observed after pathogen inoculation following thiamine treatment. These expression patterns of defense-related genes are similar to those in resistant tobacco cv Xanthi-nc inoculated with *PMMoV* (an incompatible combination; Ahn et al., 2002). Similar mRNA accumulation patterns were also induced in thiamine-treated cucumber plants challenged with the virulent anthracnose pathogen. The rapid and strong induction of defense-related genes by a virulent pathogen suggests that thiamine treatment mimics some aspects of genetic resistance through the conversion of the susceptible phase into the resistant phase.

The Specificity of Thiamine Effects

The specificity of defense-related gene expression and the resistance induced by thiamine were further investigated by treating plants with the thiamine derivatives TMP and TPP. Both chemicals induced defense-related gene expression in rice and protected the plants against rice blast disease and bacterial leaf blight in the same manner as thiamine but at an even lower concentration, 1 mM (Fig. 3A). In addition, these thiamine derivatives induced rapid and strong *PR-1* gene expression in rice plants challenged with both pathogens (Fig. 3B).

The Duration of Thiamine Effects

The expression of defense-related genes induced by thiamine had abated by 3 d after thiamine treatment. Therefore, it was important to understand the duration of the resistance induced following thiamine treatment. To address this question, rice plants were inoculated with the blast pathogen at various times after thiamine treatment. As shown in Figure 4A, the disease protection by thiamine lasted up to 15 d after the treatment. Minute dark brown lesions were frequently observed to be induced as a result of abrupt cell death around the infection site on leaves of the cultivar Hwacheong inoculated with the virulent strain KJ201. Defense-related gene expression was undetectable at 3 d after treatment, but was induced within 24 h of challenge with the blast pathogen, indicating that thiamine-potentiated rice plants display activated defense-related gene expression for up to 15 d (Fig. 4B). This potentiation might have a practical application, since constitutive expression of defense genes results in physiological disorders in other plants (Ahn et al., 2002).

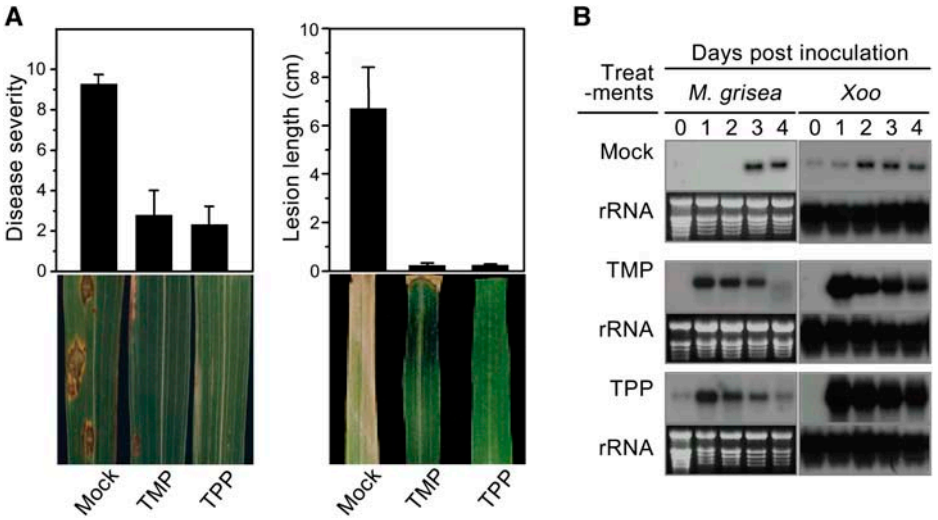

Figure 3. Specificity of the activation of plant defenses by thiamine. Rice plants were sprayed with solutions of TMP (1 mM TMP with 250 μg mL^{-1} Tween 80) or TPP (1 mM TPP with 250 μg mL^{-1} Tween 80). The rice cultivars Hwacheong and Nakdong were inoculated with the *M. grisea* strain KJ201 or the *X. oryzae* pv *oryzae* strain KXO21 at 4 h after TMP and TPP treatments, respectively. A, Effects of TMP and TPP on the progress of rice blast disease and bacterial leaf blight disease. The progress of rice blast disease and lesion lengths of plants with bacterial leaf blight disease were evaluated at 7 d after inoculation and photographed as described in ''Materials and Methods.'' Each bar represents the mean ± SE of 10 plants. B, Rice *PR-1* gene expression induced by infection with *M. grisea* or *X. oryzae* pv *oryzae* (*Xoo*, mock) in the presence or absence of 1 mM TMP or 1 mM TPP. Total RNA was extracted from rice leaves harvested from five rice plants, separated using denaturing gel electrophoresis, and transferred to nylon membrane. The blots were hybridized with a rice *PR-1* probe labeled with [^{32}P]dCTP. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel or hybridization with a radioactive 18S rRNA probe after removal of the *PR-1* probe.

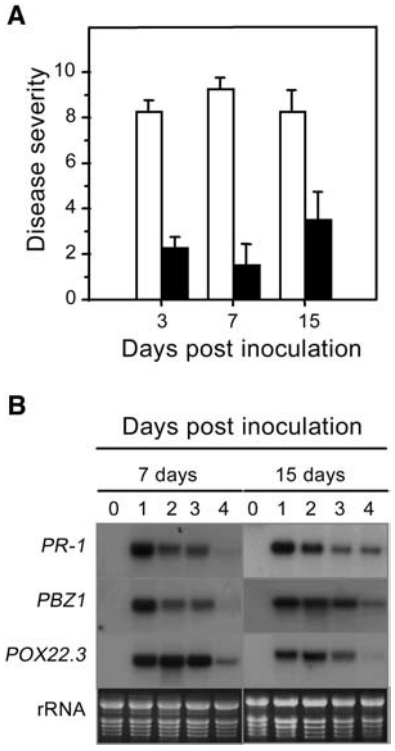

Figure 4. Thiamine suppresses rice blast disease for up to 15 d following treatment through the induction of resistance responses. The rice cultivar Hwacheong was inoculated with the rice blast pathogen *M. grisea* strain KJ201 at 3, 7, and 15 d after thiamine treatment. The disease severity was evaluated daily as described in ''Materials and Methods.'' A, Rice blast disease progress in mock-treated and thiamine-treated rice leaves following *M. grisea* infection at 3, 7, and 15 d after treatment. Each bar represents the mean value from 10 plants. White bars, Mock-treated rice leaves; black bars, thiamine-treated rice leaves. B, Expression patterns of defense-related genes in thiamine-treated rice leaves inoculated with *M. grisea* at 7 and 15 d after thiamine treatment. Total RNA was extracted from the leaves of three plants at 0, 1, 2, 3, and 4 d after fungal inoculation, separated by denaturing gel electrophoresis, and transferred to nylon membrane. The blots were hybridized with a rice *PR-1* probe labeled with [^{32}P]dCTP. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel.

Mode of Thiamine Action

To further investigate the mode of action of thiamine, we tested the induction of *PR-1* and *PDF1.2* by thiamine in Arabidopsis Col-0 and several mutants (Fig. 5A). *PR-1* expression was induced in wild-type Col-0; *etr1*, an altered perception of ethylene mutant; and *jar1*, a mutant that displays reduced sensitivity to methyl jasmonate. However, no *PR-1* expression was detected in *nahG*, an Arabidopsis line expressing the bacterial NahG, or *npr1*, a mutant that does not accumulate *PR-1* in response to SA. No *PDF1.2* transcript was detected in any of the Arabidopsis plants treated with thiamine, indicating that the expression of this gene is independent of jasmonic acid and ethylene signaling (data not shown). To further analyze the mode of action of thiamine in the resistance of Arabidopsis against *Pst* DC 3000, the ecotype Col-0 and the above mutants were treated with thiamine 4 h prior to bacterial inoculation. The *nahG* and *npr1* lines were not protected, but *etr1* and *jar1* were protected at levels similar to that in the wild-type Col-0 (Fig. 5B). These results strongly suggest that the defense-related gene expression induced by thiamine is dependent on the SA pathway. However, the role of the SA-dependent signaling pathway in the rice defense system remains to be elucidated because rice has high endogenous levels of SA (Silverman et al., 1995).

Thiamine Exerts Its Effects Systemically through the Ca^{2+}-Dependent Signaling Pathway

To determine whether the effects of thiamine on disease resistance could be transferred from the site of treatment to other parts of the plant, thiamine was sprayed on rosette leaves of the Arabidopsis ecotype Col-0 or on both rosette and cauline leaves, and the leaves were harvested at 24 h after treatment. As

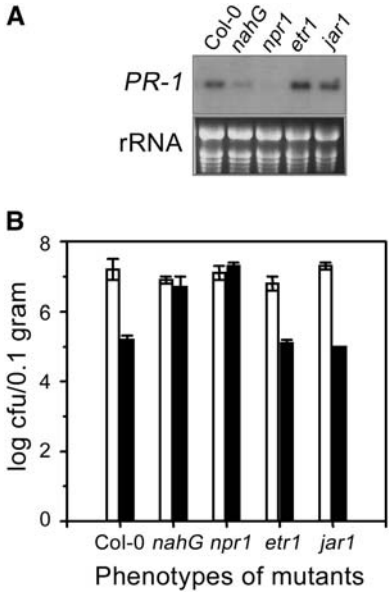

Figure 5. Analysis of *PR-1* gene expression and quantification of resistance to *Pst* DC 3000 infection following thiamine treatment of Arabidopsis Col-0, *nahG*, *npr1*, *etr1*, and *jar1* plants. A, Effect of thiamine on the accumulation of *PR-1* transcripts in Arabidopsis. Total RNA was extracted from the leaves of five plants at 24 h after thiamine treatment, separated by denaturing gel electrophoresis, and transferred to nylon membrane. The blots were hybridized with an Arabidopsis *PR-1* probe labeled with [^{32}P]dCTP. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel. B, Numbers of *Pst* DC 3000 in leaves of the Arabidopsis ecotype Col-0 and signaling mutants of the same ecotype treated with thiamine 4 h prior to bacterial inoculation. Samples were collected from five wild-type and five mutant plants at 3 d after inoculation, and all experiments were conducted three times independently, with three replicates. White bars, Mock-treated Arabidopsis leaves; black bars, thiamine-treated Arabidopsis leaves. The data represent the mean ± SE of three pools of five plants. The experiments were repeated at least five times, with similar results each time.

shown in Figure 6A, *PR-1* gene expression was induced in both types of leaves, indicating that the effect of thiamine mobilizes to other parts of the plant.

Among the earliest cellular events in plant-pathogen interactions, ion fluxes across the membrane, such as of Ca^{2+}, play important roles in the development of HR (Blume et al., 2000; Romeis et al., 2001). To determine whether calcium signaling is involved in thiamine-induced resistance, we tested the effect of thiamine on protein kinase C (PKC) activity in rice plants. As shown in Figure 6B, inoculation with *M. grisea* up-regulated PKC activity in rice plants, but thiamine treatment did not. Pretreatment of plants with thiamine prior to inoculation with *M. grisea* resulted in a significantly greater increase in PKC activity. This result is consistent with the patterns of induction of defense-related gene expression by thiamine and pathogen inoculation. The involvement of calcium in the defense-related gene expression induced by thiamine was also confirmed by treatment of plants with the calcium channel blocker $LaCl_3$ (Govrin and Levine, 2000). Infiltration of Arabidopsis ecotype Col-0 plants with $LaCl_3$ prevented the accumulation of the *PR-1* gene transcript that is induced by thiamine treatment (Fig. 6C).

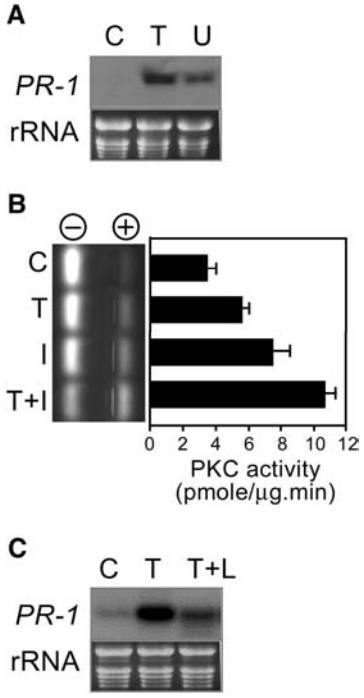

Figure 6. Accumulation of the *PR-1* transcript in Arabidopsis ecotype Col-0 and induction of PKC activity in rice triggered by thiamine treatment and/or pathogen inoculation. A, Systemic expression of *PR-1* induced by thiamine treatment of the Arabidopsis ecotype Col-0. Thiamine was sprayed only on the rosette (T), and the upper cauline leaves were left untreated (U). Total RNA was extracted from rosette and cauline leaves of five plants harvested at 24 h after thiamine treatment. Total RNA was also extracted from thiamine-untreated leaves (C). The blots were hybridized with an Arabidopsis *PR-1* gene probe labeled with [^{32}P]dCTP. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel. B, Thiamine treatment increases PKC activity in rice following infection with *M. grisea*. Plants of the rice cultivar Hwacheong that had been treated with thiamine or mock treated were inoculated with *M. grisea* at 4 h after thiamine treatment. Abbreviations: C, Treated with 250 μg mL^{-1} Tween 80 only; T, treated with 50 mM thiamine; I, infected with *M. grisea*; T + I, infected with *M. grisea* after thiamine treatment. Total protein was extracted from the leaves of five plants harvested at 24 h after the thiamine treatment. C, Infiltration of Arabidopsis plants with $LaCl_3$, a calcium channel blocker, resulted in the suppression of *PR-1* gene induction by thiamine. Abbreviations: C, Infiltrated with distilled water at 4 h after spraying with 250 μg mL^{-1} Tween 80; T, infiltrated with distilled water at 4 h after spraying with 50 mM thiamine; T + L, leaves infiltrated with 1 mM $LaCl_3$ at 4 h after spraying with 50 mM thiamine. Total RNA was extracted from the leaves of five plants at 24 h after thiamine treatment, separated using denaturing gel electrophoresis, and transferred to nylon membrane. The blots were hybridized with an Arabidopsis *PR-1* probe labeled with [^{32}P]dCTP. Equal sample loading was confirmed by ethidium-bromide staining of the rRNA in the gel.

DISCUSSION

Thiamine Induces SAR Responses

Our results demonstrate that thiamine endows rice, tobacco, and cucumber with resistance to fungal, bacterial, and viral infections. The disease-inhibiting activities of thiamine were evident in repeated inoculation experiments. Several mechanisms that mediate the disease protection induced by certain chemicals have been described, including the direct inhibition of pathogen growth, blocking of the disease cycle (Fabritius et al., 1997; Thompson et al., 2000; Vicentini et al., 2002), and the induction of plant resistance to pathogen infection (Dong and Beer, 2000; Zimmerli et al., 2000; Kachroo et al., 2003; Nakashita et al., 2003). Given the disease-progress-inhibiting activities of thiamine against fungal, bacterial, and viral pathogens, it would be unusual if this compound acted as a specific antibiotic. Media containing thiamine did not inhibit the growth of *M. grisea* or *X. oryzae* pv *oryzae* on plates (data not shown). These results imply that thiamine induces resistance in plants to infection by various pathogens. Broad-spectrum effects and the absence of direct effects on the pathogen are distinctive characteristics of other plant defense activators, including BTH (Lawton et al., 1996), DCINA (Delaney, 1997), probenazole (Midoh and Iwata, 1996), probenazole derivatives (Yoshioka et al., 2001), and brassinolide (Nakashita et al., 2003). In addition, thiamine did not result in phytotoxicity at any of the tested concentrations. These results show that thiamine satisfies the requisites for an activator of plant SAR, as previously suggested (Friedrich et al., 1996).

The resistance-inducing effects of TMP and TPP on rice plants further confirm the above explanations. Both chemicals contain the thiamine structure and conclusively protect host plants from infection by the rice blast fungus and rice bacterial leaf blight. These results indicate that thiamine itself should act as a plant defense activator.

Thiamine Triggers Augmented Defense Responses

Thiamine affected defense-related gene expression in the tested plant species. In the compatible interaction, transcripts of the tested defense-related or SAR-related genes began to accumulate at a relatively late point in time after pathogen infection. The transcripts of all of the tested defense-related genes accumulated within 24 h after thiamine treatment, but the high transcript levels did not persist. Thiamine treatment itself triggers transient defense-related gene expression. Rhizobacteria (Zhang et al., 1998; Ahn et al., 2002) and some chemicals, including β-aminobutyric acid (Zimmerli et al., 2000; Ton and Mauch-Mani, 2004), which activate plant resistance, also induce the transient expression of defense-related genes, although the resulting expression patterns are not identical. By contrast, treatment of intact Arabidopsis, tobacco, wheat (*Triticum aestivum*), or potato (*Solanum tuberosum*) plants with BTH or DCINA resulted in strong expression of defense-related genes within 12 to 24 h of the treatments, and the induced expression lasted for more than 20 d, even in the absence of pathogen inoculation (Friedrich et al., 1996). However, following pathogen infection, SAR-related genes were rapidly and strongly expressed in thiamine-treated plants, mirroring the expression patterns that occur during the interaction between resistant host plants and avirulent pathogens. According to the terminology for a phenotypically similar phenomenon in mammalian monocytes (Hayes and Zoon, 1993), thiamine triggers the ''priming'' of the plants. Similar priming effects in intact plants have been reported after treatment with SAR-inducing rhizobacteria (Zhang et al., 1998) and some chemicals, including β-aminobutyric acid (Zimmerli et al., 2000; Siegrist et al., 2002) and acibenzolar *S*-methyl (Narusaka et al., 1999).

These priming effects were observed to persist for a long period. To investigate the priming period, the intervals between thiamine treatment and pathogen inoculation were expanded up to 15 d. As expected, *PR-1* transcripts were not detected at 4, 7, or 15 d after thiamine treatment. However, following pathogen infection, *PR-1* transcripts rapidly accumulated to high levels and disease protection was evident. This result indicates that thiamine is a candidate for an effective plant defense activator.

Thiamine-Induced Defense Signaling Is Dependent on SA and Calcium

Thiamine treatment resulted in the inhibition of disease development through the activation of plant defense systems and SAR. We examined the mechanisms induced by thiamine by investigating SAR-related (*PR-1*) and defensin gene (*PDF1.2*) expression and by assessing the disease-inhibiting effects of thiamine in Arabidopsis mutants that fail to metabolize SA, jasmonic acid, or ethylene. We also assessed the effect of calcium channel blockers on the induction of the SAR-related gene by thiamine and analyzed PKC activity in thiamine-treated and/or *M. grisea*-inoculated rice.

Thiamine-treated wild-type Arabidopsis ecotype Col-0 showed high *PR-1* gene expression. By contrast, no defensin expression was observed, whether pathogen had been inoculated or not. Thiamine did not trigger *PR* gene expression in the *nahG* and *npr1* lines. The in planta bacterial population was clearly reduced in thiamine-treated Col-0, *etr1*, and *jar1* plants, but this inhibitory effect was not evident in *nahG* or *npr1*. These results clearly suggest that thiamine exerts its effects through the SA-dependent signaling pathway. Similar dependencies on SA were also observed in β-aminobutyric-acid-treated tobacco (Siegrist et al., 2002) and Arabidopsis (Zimmerli et al., 2000).

In addition, the thiamine-induced accumulation of SAR-related transcripts was prevented by $LaCl_3$, a blocker of plasma-membrane-localized calcium channels. Previous reports have revealed prominent differences in the cytosolic concentrations of calcium ion in the incompatible resistant interaction and the compatible susceptible interaction. This is consistent with results in plant cell cultures treated with fungal elicitors from virulent and avirulent strains (Gelli et al., 1997) and in numerous intact plants, including wheat (Takezawa, 1999).

The activation of plant resistance by thiamine suggests a regulatory role for thiamine in defense and signal transduction. To characterize the mechanisms that underlie these phenotypic changes, we studied the effect of thiamine on Ca^{2+}-dependent protein kinase by measuring PKC activity. Morello et al. (1993) suggested that a certain rice PKC(s) shares biochemical characteristics with animal PKC proteins. Among the earliest cellular events in incompatible host-pathogen recognition, fluxes of appropriate ions, including Ca^{2+}, across the plasma membrane result in a set of oxidative bursts that produce reactive oxygen species (Harding et al., 1997; Reddy et al., 2003). These events are followed by HR and result in the blocking of continuous interactions between pathogens and hosts. Although thiamine alone did not up-regulate PKC activity, thiamine-treated rice infected with the rice blast pathogen showed a 2-fold increase in PKC activity, as compared to mock-treated rice at 24 h after infection. Therefore, the site of thiamine action is upstream of the mobilization of Ca^{2+}, and the resistance induced by thiamine treatment mimics the single-plant-gene-mediated HR-dependent resistance that occurs during incompatible plant-microbe interactions. This is concomitant with the above SAR-related gene expression induced by thiamine and/or pathogen inoculation.

Taken together, our results demonstrate a novel biological function for thiamine. Thiamine confers disease resistance through the priming of several plant defense responses, leading to a restriction of pathogen growth in planta and suppressed propagation of the inoculum. The maintenance of the resistance mimic

status for a long period indicates that thiamine is a good candidate as a plant defense activation agent. Along with conventional antibiotics, previously developed plant defense activators, biocontrol organisms, and improved seed varieties, thiamine should provide novel disease control strategies that satisfy environmental regulations. Although the precise signaling pathways involved in the induction of SAR by thiamine remain unknown, our findings demonstrate that thiamine exerts its effects via the SA- and calcium-dependent signaling pathways. These findings add to our understanding of the novel signaling pathways in SAR that are mediated by thiamine.

MATERIALS AND METHODS

Plant Materials and Chemical Treatments

The rice (*Oryza sativa*) cultivars Hwacheong and Nakdong were grown in a greenhouse, as described (Kim et al., 2001b). The tobacco (*Nicotiana tabacum*) cultivar Samsun NN and the cucumber (*Cucumis sativus*) cultivar Sunmi Baekdadaki were grown in a greenhouse at 25°C to 30°C under natural light. Seeds of the Arabidopsis (*Arabidopsis thaliana* ecotype Col-0) and mutants (*npr1*, *etr1*, and *jar1*) in this line were obtained from The Arabidopsis Information Resource (TAIR). Transgenic Col-0 containing the *nahG* gene was kindly provided by Dr. X. Dong (Duke University, Durham, NC). Arabidopsis plants were grown in a growth chamber at 22°C and 65% to 70% relative humidity, with 16 h of illumination daily. Four- to 5-week-old rice, cucumber, and Arabidopsis plants and 2-month-old tobacco plants were used for chemical treatments. The plants were sprayed with 250 μg mL^{-1} Tween 80 (mock) or 50 mM thiamine, 1 mM TMP, or 1 mM TPP (Sigma-Aldrich, St. Louis) supplemented with 250 μg mL^{-1} Tween 80 at 4 h prior to pathogen inoculation, unless stated otherwise. The calcium ion inhibitor $LaCl_3$, which blocks plasma membrane calcium channels, was used in an aqueous solution. Four hours after spraying of Arabidopsis Col-0 plants with 50 mM thiamine, 1 mM $LaCl_3$ was infiltrated into the leaves using a needleless syringe.

Pathogen Maintenance and Inoculation

The effects of thiamine on disease progress were examined to evaluate its disease inhibitory activity. *Magnaporthe grisea* strain KJ201 and *Xanthomonas oryzae* pv *oryzae* strain KXO21, the causal agents of rice blast and bacterial leaf blight, respectively, were propagated and inoculated onto leaves of the rice cultivars Hwacheong and Nakdong, as described (Kim et al., 2001b). The disease severities and the lesion lengths were assessed according to the rating scale of the International Rice Research Institute (1988). *PMMoV* was maintained and inoculated on tobacco leaves as described by Ahn et al. (2002), and in planta propagation of *PMMoV* was measured by northern-blot hybridization analysis using a reverse transcription (RT)-PCR product of the viral RNA as the probe. *Colletotrichum lagenarium*, the causal pathogen of cucumber anthracnose, was propagated on green bean agar (Goode, 1958) and inoculated on cucumber plants as described by Raupach and Kloepper (1998). Seven days after pathogen challenge, the second and third leaves of each plant were assessed for anthracnose disease, the percent of the leaf area that was diseased was recorded, and the leaf was photographed. *Pst* DC 3000 was cultivated on the King's medium B containing 50 μg mL^{-1} rifampicin for 2 d at 28°C. To inoculate Arabidopsis with *Pst* DC 3000, bacterial cells were retrieved from medium containing 10 mM $MgCl_2$ and 250 μg mL^{-1} Tween 80, and the concentration was adjusted to 10^7 CFU mL^{-1}. At least 20 plants of the Arabidopsis ecotype Col-0 or mutants in this line were inoculated by spraying with the bacterial suspension until all of the leaves were covered with fine droplets. 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 16:8-h light:dark regime at 25°C and 80% relative humidity. The disease severity was assessed at 3 d after inoculation by determining the CFU within 0.1 g (fresh weight) of Arabidopsis leaves from five plants through plating appropriate dilutions on King's B medium containing 50 μg mL^{-1} rifampicin.

Effect of Thiamine on Pathogen Growth

Mycelial blocks (0.6 cm in diameter) of *M. grisea* strain KJ201 were cultured on potato dextrose agar supplemented with 0, 5, 10, 20, or 50 mM thiamine at 25°C for 7 d, after which the diameters of the fungal colonies were measured. *X. oryzae* pv *oryzae* strain KXO21 was cultured in 50 mL of nutrient broth containing equal concentrations of thiamine on a shaker at 150 rpm and 28°C for 48 h. The cultures were started by adding 500 μL of sterile distilled water or bacterial inoculum (4.8×10^5 CFU). The populations of bacteria in the suspension cultures were estimated by counting the CFU after appropriate dilution on peptone-Suc agar. Five replicates were performed for each pathogen and thiamine concentration.

Determination of the Duration of the Control Period

To estimate the length of the control effect by thiamine, the rice cultivar Hwacheong was inoculated with conidial suspensions of *M. grisea* strain KJ201 at 4 h, 3 d, 7 d, and 15 d after spraying with thiamine, and the disease progress was evaluated as described above.

Systemic Translocation of Thiamine-Mediated Defense Signals

To investigate the systemic translocation of defense responses induced by thiamine treatment, rosettes sprayed with 50 mM thiamine and mock-treated upper cauline leaves were harvested from the same plant at 24 h after treatment in the presence or absence of *Pst* DC 3000 inoculation. The stems and cauline leaves were completely covered with plastic wrap while the rosette leaves were sprayed with thiamine, and the plastic was not removed until the chemical droplets had dried completely. The expression of the *PR-1* gene in the rosette and cauline leaves was assayed using northern-blot hybridization analysis.

RNA Extraction and Northern-Blot Hybridization Analysis

Total RNA was extracted from inoculated and/or thiamine-treated plants and control plants using the lithium chloride precipitation method (Davis and Ausubel, 1989). For hybridization analysis, 15 μg of total RNA were separated electrophoretically in denaturing formaldehyde-agarose gels (8% formaldehyde, 0.5× MOPS, 1.5% agarose) and blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary transfer. RNA gel blots were hybridized and washed as described (Kim et al., 2001b) and exposed to x-ray film (Agfa-Gevaert N.V., ISO 9001, Mechelen, Belgium). DNA probes were labeled with [^{32}P]dCTP using random primer labeling (Boehringer Mannheim, Tutzing, Germany). The tobacco *PR-1a*, *PAL*, and *HMGR* genes and the cucumber acidic peroxidase (*POX*) gene were kindly provided by Dr. Doil Choi at the Korea Research Institute of Bioscience and Biotechnology and Dr. Hiroshi Ishii at the National Institute of Agro-Environmental Sciences, Japan.

Protein Kinase Assay

Inoculated and/or thiamine-treated rice (cv Hwacheong) leaves were harvested, macerated in liquid N_2, and resuspended in 100 μL of protein extraction buffer (50 mM potassium phosphate, pH 7.6, 10 mM β-mercaptoethanol, 4 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride). The mixture was centrifuged at 13,000 rpm for 40 min at 4°C. The protein concentrations in the supernatants were quantified using the Bradford method (Bradford, 1976). PKC activities were analyzed using a nonradioactive assay system, according to the manufacturer's instructions (Promega, Madison, WI). One unit of kinase is defined as the number of pmoles of phosphate transferred per minute to a substrate.

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