Riboflavin-induced Priming for Pathogen Defense in Arabidopsis thaliana

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Abstract

Riboflavin (vitamin B₂) participates in a variety of redox processes that affect plant defense responses. Previously we have shown that riboflavin induces pathogen resistance in the absence of hypersensitive cell death (HCD) in plants. Herein, we report that riboflavin induces priming of defense responses in Arabidopsis thaliana toward infection by virulent Pseudomonas syringae pv. tomato DC3000 (Pst). Induced resistance was mechanistically connected with the expression of defense response genes and cellular defense events, including H₂O₂ burst, HCD, and callose deposition in the plant. Riboflavin treatment and inoculation of plants with Pst were neither active but both synergized to induce defense responses. The priming process needed NPR1 (essential regulator of systemic acquired resistance) and maintenance of H₂O₂ burst but was independent of salicylic acid, jasmonic acid, ethylene, and abscisic acid. Our results suggest that the role of riboflavin in priming defenses is subject to a signaling process distinct from the known pathways of hormone signal transduction.

Key words: callose deposition; defense responses; hypersensitive cell death; hydrogen peroxide; priming; riboflavin.

Riboflavin (vitamin B₂) biosynthetic and functional pathways affect plant growth, development, and defense responses by multiple mechanisms. Riboflavin is involved in anti-oxidation (Upreti et al. 1991; Packer et al. 1996) and peroxidation (Zubay 1998), which both affect the production of reactive oxygen species (ROS) during oxidative burst. H₂O₂ is an important form of ROS and is essential to the induction of hypersensitive cell death (HCD) and pathogen defense (Levine et al. 1994; Lamb and Dixon 1997; Alvarez et al. 1998). Levels of riboflavin in plants are believed to be important to these processes (Dong and Beer 2000). Exogenous application of riboflavin promotes plant growth (Peng et al. 2002) and induces resistance to fungal, bacterial, and viral pathogens (Aver'yanov et al. 2000; Dong and Beer 2000; Taheri and Hofte 2006). Plant resistance can also be induced when riboflavin is applied as an ingredient of spraying mixture (Wang and Tzeng 1998).

Some mechanistic connections have been established between stimulation of plants by riboflavin treatment and several known signaling pathways. The ethylene signaling pathway is activated to facilitate plant growth (Peng et al. 2002). NPR1 (nonexpressor of PR genes1), essential regulator of systemic acquired resistance (SAR) (Cao et al. 1994; Cao et al. 1997; Dong 1998), and undefined protein kinases are recruited (Dong and Beer 2000) during the induction of pathogen resistance by riboflavin. Salicylic acid (SA), which usually mediates the SAR pathway (Delaney et al. 1994; Ryals et al. 1996), is not required (Dong and Beer 2000). Pathogen defenses are often associated with HCD, which, however, is not necessary to defense responses induced by riboflavin (Dong and Beer 2000). Moreover, riboflavin does not directly affect pathogens, but instead, it induces defensive responses in plants (Aver’yanov et al. 2000; Dong and Beer 2000; Taheri and Hofte 2006). It is supposed that riboflavin-induced defense has functional linkages with particular signaling pathways in plants (Dong and Beer 2000). A possible intermediate tache may be priming of...
plant defensive mechanisms towards infection by pathogens (Conrath et al. 2002, 2006; Kohler et al. 2002).

In many plants, defense priming can be induced by biotic and abiotic factors to increase capacity of resistance to pathogens (Conrath et al. 2002, 2006; Beckers and Conrath 2007). Inducers of defense priming tested thus far include natural and synthetic compounds, such as β-aminobutyric acid (Zimmerli et al. 2000; Ton and Mauch-Mani 2004; Hamiduzzaman et al. 2005; Ton et al. 2005), vitamin B1 (Ahn et al. 2007) and SA or its structural analogs (Ryals et al. 1996; Sticher et al. 1997; Dempsey et al. 1999; Kohler et al. 2002). In general, molecular mechanisms that underlie the priming process is instrumental in improving plants’ ability to perceive stress stimuli more rapidly, thereby coping with different forms of stress more efficiently (Conrath et al. 2002). Under priming conditions, specific molecular and cellular defense responses are activated quicker and greater than those during regular infection (Ton et al. 2005; Ahn et al. 2007). An important response is the expression of defense response genes, like those encoding pathogenesis-related protein (PR) and phenylalanine ammonia-lyase (PAL) (Friedrich et al. 1996; Guo et al. 1998; Reuber et al. 1998). H2O2 burst (Levine et al. 1994; Lamb and Dixon 1997; Alvarez et al. 1998), HCD (Peng et al. 2003; Ahn et al. 2007), and callose deposition (Kohler et al. 2002; Ton and Mauch-Mani 2004; Hamiduzzaman et al. 2005) are often induced coordinately during the priming process. Hence, priming is an important mechanism of induced resistance in plants (Pieterse et al. 2006; Beckers and Conrath 2007). It is unclear whether these primed events contribute to plant resistance induced by riboflavin.

This study is aimed at the mechanistic connection between riboflavin-induced priming and pathogen resistance in Arabidopsis. We describe that riboflavin induces priming of molecular and cellular defense responses to cope with infection by the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Pst). We also show evidence that riboflavin-induced priming is dependent on H2O2 burst and NPR1 but is not affected by several known pathways of hormone signal transduction.

Results

Riboflavin and Pst synergize to induce priming of cellular and molecular defense responses

Previously we have shown that riboflavin induces plant resistance to Pst (Dong and Beer 2000) but omitted the mode of riboflavin action. To study whether riboflavin plays a role in priming of defense responses, we tested H2O2 burst, callose deposition, and HCD in plants pretreated with riboflavin or water and then maintained either no inoculation, or were inoculated with Pst at 5 d post-treatment (dpt). H2O2 burst, callose deposition, and HCD were determined at 6, 12, and 12 h postinoculation (hpi), respectively, when the responses become evident based on previous studies (Peng et al. 2003; Ahn et al. 2007). The three responses were induced only when riboflavin treatment and subsequent inoculation with Pst were applied to plants (Figure 1A). H2O2 burst, callose deposition, and HCD were not evident in plants treated with only riboflavin and in water-treated plants with and without Pst inoculation. In contrast, conspicuous H2O2 burst, callose deposition, and HCD were observed in plants pretreated with riboflavin and inoculated with Pst. In these plants, the extents of H2O2 burst and HCD were significantly greater (ANOVA tests, P < 0.01) than those in plants treated with only riboflavin and those in water-treated plants with and without inoculation (Figure 1B).

PR and PAL gene transcripts are molecular indicators for the activation of plant defensive pathways (Friedrich et al. 1996). Our previous study showed that PR-1 and PR-2 were expressed in Arabidopsis plants treated with riboflavin (Dong and Beer 2000). Here, it was found that the expression of PR-1, PR-2, and PAL1 in leaves of plants observed 6 hpi is a part of defense priming (Figure 1C). Transcription of the three genes was not evident in water-treated plants inoculated with Pst or those that were not inoculated. Conversely, profuse amounts of the three transcripts were observed in riboflavin-pretreated and Pst-inoculated plants. In these plants, transcript levels were much greater compared with those in plants treated with riboflavin but not inoculated. Clearly, riboflavin treatment primes Arabidopsis plants for augmented induction of defense responses when challenged by the pathogen.

Catalase nullifies priming induced by riboflavin

We used catalase in a pharmacological study of the role of H2O2 in riboflavin-induced priming of plant defense responses. Catalase acts to scavenge H2O2 but does not inhibit pathogen proliferation (van Wees and Glazebrook 2003). We confirmed that catalase applied alone to plants did not induce any evident responses, whereas, riboflavin-induced priming for defense responses was arrested by the enzyme present in Pst inoculum (Figure 2). When catalase was present in Pst suspension, leaf symptoms became severe (Figure 2A, top) and bacterial numbers increased (Figure 2B) relative to those in plants treated with only riboflavin. The enzymes also caused great compromises on combinative effects of riboflavin and Pst in eliciting H2O2 burst, callose deposition, and HCD (Figure 2A, lower three photo panels). Induced expression of the defense response genes were also depressed by the application of catalase (Figure 2C). Therefore, riboflavin treatment requires H2O2 burst as an essential signal to prime the defense responses.

Riboflavin-induced priming is dependent on NPR1 but independent of the basal defense signals

To reveal possible crosstalk between riboflavin-primed defenses and basal defense pathways of SA, jasmonic acid (JA)
Riboflavin Signaling in Pathogen Defense

Figure 1. Combinative effects of riboflavin and *Pseudomonas syringae* pv. *tomato* (Pst) on defense priming in *Arabidopsis*.
The defense responses were tested in accordance with treatments noted in the lower caption panels. *Arabidopsis* plants were sprayed with a riboflavin solution (Rib+) or water (Rib−). Plants were incubated 5 d post-treatment (dpt) with Pst (Pst+) or remained free from the pathogen (Pst−).

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and ethylene signal transduction (Dong 1998; Dangl and Jones 2001). *Arabidopsis* mutants that have defects in basal defenses were compared with wild-type (WT) plants in the induction of defense priming responses. H$_2$O$_2$ burst differed in the plant genotypes (Figure 3A). NahG (a transgenic plant that does not accumulate SA), *etr1-1* (a mutant insensitive to ethylene), and *jar1-1* (a mutant insensitive to JA) were similar to WT. In these plants, H$_2$O$_2$ burst was induced evidently subsequent to riboflavin treatment followed by Pst inoculation in comparison with only riboflavin treatment. In contrast, the induction of H$_2$O$_2$ burst was eliminated in *npr1-1*, which carries a deficient *NPR1* gene (Cao et al. 1994; Cao et al. 1997). In assays with the same set of plants, the induction of HCD and callose deposition was impaired in *npr1-1* but not in the other three genotypes compared with WT (Figure 3B). Despite pretreatment with water or riboflavin, leaf symptoms were more severe (Figure 3C) and *in planta* Pst cell numbers were greater (Figure 3D) in *npr1-1* plants than in other genotypes. These genotypes, in contrast to *npr1-1*, had fewer symptoms and Pst cell numbers, which are functions of riboflavin versus water. Differences in bacterial population were significant (ANOVA tests, $P < 0.01$) between *npr1-1* and other genotypes following treatment with riboflavin and inoculation with Pst (Figure 3D). Thus, a functional *NPR1* is required for riboflavin to induce priming of defense responses.

**ABA signaling does not affect riboflavin-induced priming of pathogen defense**

To gain more information about the relationship between riboflavin-primed defense and hormone signaling pathways, we tested the effects of ABA signaling on the function of riboflavin. In *Arabidopsis*, transcription factor ABI4 acts to repress ABA-dependent gene expression during oxidative stress (Staneloni et al. 2007), which is affected by riboflavin (Zubay 1998). We chose to assay the *Arabidopsis* ABA-insensitive mutant *abi4-1* since it dominates other *abi* mutants in blocking pathogen resistance in plants (Pourtau et al. 2004; Ton and Mauch-Mani 2004; Kaliff et al. 2007). We found that *abi4-1* did not affect riboflavin-induced priming of *Arabidopsis* defense responses.
Figure 2. Inhibitory effects of catalase on riboflavin-induced priming of Arabidopsis defense responses.

Plant treatments (inoculation) are shown at the bottoms.

(A) Leaf symptoms and the defensive responses. Leaf symptoms were observed 3 d postinoculation (dpi). H$_2$O$_2$ burst, callose deposition, and cell death were determined at 6, 12 and 12 h postinoculation (hpi), respectively. Scale bars, 300 μm.

(B) In planta Pst population. Logarithmic numbers of bacteria recovered from leaves of plants 3 dpi were quantified versus fresh weight (FW) of leaves and are shown as mean ± SD (n = 3 repeats).

(C) Expression of defense response genes in untreated leaves of plants tested 6 hpi.

toward infection by Pst. As shown in Figure 3A, cellular defense responses evaluated were all induced in abi4-1, similar to WT when riboflavin was applied before inoculation with Pst. Leaf symptoms were alleviated (Figure 3B) and in planta bacterial population (Figure 3C) was decreased at close extents in abi4-1 and WT plants pretreated with riboflavin. Consistent with the dispensability of ABI1 and ABI2 in pathogen defense elicited by a biotic elicitor (Dong et al. 2005), neither abi1-1 nor abi2-1 were involved in the riboflavin action (data not shown). Overall, these results suggest that ABA signaling is not engaged in riboflavin-induced priming.

Discussion

We have studied defense priming by riboflavin in Arabidopsis. The molecular basis for priming was characterized. Riboflavin induces plant resistance to Pst, while Pst grows well in the presence of riboflavin (Dong and Beer 2000). Enhanced disease perturbation without a direct effect on the causal pathogen confirms the alternative role of riboflavin as an inducer of plant defense priming. Systemic expression of defense response genes as an indicator of activated SAR (Uknes et al. 1992, 1993) was observed in the leaves of riboflavin-treated plants (Dong and Beer 2000; the present study). The gene expression in riboflavin-treated plants was augmented subsequent to plant inoculation with Pst. Augmented resistance provides clear evidence that riboflavin treatment primes Arabidopsis defense.

We have established a mechanistic connection between riboflavin-primed defenses with plant cellular responses. Neither riboflavin treatment nor inoculation of plants with Pst were active, but both synergized to trigger H$_2$O$_2$ burst, HCD, and callose deposition (Figure 1A,B). HCD and callose deposition frequently occurred as a consequence of ROS burst (Levine et al. 1994) and the three cytological events are recognized
as typical responses to pathogen infection in primed plants. Our findings indicate that riboflavin-primed Arabidopsis in a surveillance state was extremely sensitive to pathogen infection. Riboflavin and pathogen challenge were required for H$_2$O$_2$ production (Figure 1A), and rapid H$_2$O$_2$ production is thus considered as one of the mechanisms of defense priming by riboflavin. Catalase scavenges H$_2$O$_2$ but did not inhibit pathogen proliferation (van Wees and Glazebrook 2003). Catalase present in Pst inoculum eliminated a significant portion of H$_2$O$_2$ accumulation as combinative effects of Pst and riboflavin. The inhibitory role of catalase accompanied abolition of disease resistance, callose deposition, HCD, and expression of defense response genes (Figure 2A–C). These observations suggest that H$_2$O$_2$ is critical for riboflavin-induced defense priming.

Defense priming by riboflavin seems distinct from several known pathways of hormone signal transduction but is dependent on NPR1 (Figures 3, 4). Primed defense responses in cytology were absent in npr1-1 (Figure 4A), which carries a deficient NPR1 (Cao et al. 1994; Delaney et al. 1995; Cao et al. 1997). The NPR1-regulated SAR pathway needs SA signaling (Ryals et al. 1996; Dong 1998), which, however, did not affect priming (Figure 4). The roles of signaling by JA and ethylene were ruled out similarly (Figure 4). ABA signaling is also not likely to play a role in the priming process based on the behavior.

**Figure 4.** Comparison of the specific genotypes of Arabidopsis in riboflavin-induced defense priming.

The plant mutants NahG and npr1 (defect at salicylic acid (SA) signaling), etr1 (defective in ethylene perception), and jar1 (insensitive to jasmonic acid (JA)) were compared with wild type (WT) for the defensive responses. Experiments were similar as in Figure 1. Scale bars, 300 μm in (A) and (B). In (C), histograms represent means ± SD bars (n = 3 repeats).

(A) Assays of H$_2$O$_2$ burst.
(B) Assays of cell death and callose deposition.
(C) Differences in leaf symptoms.
(D) Pst population in leaves.
of abi4-1 (Figure 3). However, abi4-1 dominates over other abi mutants to block plant defenses towards infection by pathogens and environmental cues (Pourtau et al. 2004; Ton and Mauch-Mani 2004; Jakab et al. 2005; Kaliff et al. 2007). These results support the notion that modes of priming are largely influenced by the types of stimuli (Ahn et al. 2007).

In this study, we have demonstrated that priming and its associated cellular and molecular defense mechanisms were induced by riboflavin. Figure 5 depicts a working model of the process. Schematic linkages between the events are mechanistic. The physiological connection remains to be studied. Protein kinases have been implicated in riboflavin-induced plant resistance to pathogens (Dong and Beer 2000) and whether they donate to priming also requires further study. Moreover, whether riboflavin-mediated redox is related to redox status for NPR1 activation and whether riboflavin signaling uses the scheme similar to that used by NPR1 to regulate PR gene transcription (Mou et al. 2003) are of great interest.

Figure 5. Model of plant defense priming by riboflavin.

Only the components that are relevant to this study are shown. Arrows point to event fluids. Bars indicate inhibition. The two question marks indicate unknown factors that are supposed to act at those places.

Materials and Methods

Plants and pathogens

The Arabidopsis genotypes tested were the ecotype Columbia (Col-0), npr1-1, jar1-1, etr1-1 and abi4-1 mutants (seed stock nos.: CS1092, CS3726, CS8072, CS237, CS8104 at http://www.arabidopsis.org) as well as transgenic plant NahG. NahG seeds were a gift from Dr Zuhua He. Seeds were chilled at 4°C for 5 d and sown in 60-mL pots containing a mixture of sand and potting soil. Plants were incubated in chambers with a 12:12 h light : dark cycle (light 200 μE/m² per s at 24°C) (night, 20°C) for 40 d before use. Pseudomonas syringae pv. tomato DC3000 (Pst) without any foreign avirulence genes was lyophilized, stored at −80°C, and cultured on Luria Bertani Agar medium (Gerhardt et al. 1981) before inoculation.

Plant treatment and resistance scoring

Plants were sprayed with water (CK) or 0.5 mmol/L riboflavin (EMD Biosciences, Darmstadt, Germany) in the presence of the surfactant Silwet L-77 (0.03%). Plants were inoculated 5 dpt by spraying plant tops with a Pst suspension (1 × 10⁸ cfu/mL). For use of catalase (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), a Pst suspension (5 × 10⁶ cfu/mL) with and without catalase (5 000 units/mL) was infiltrated into leaf intercellular spaces of the top two expanded leaves of a plant. The method was chosen to allow efficient function of catalase in intercellular leaf spaces. Symptoms caused by Pst on leaves were observed and bacterial population in leaves were determined 3 dpi using a previous method (Dong et al. 1999). Induced resistance in riboflavin-treated plants was defined based on decreases in symptom severity and in planta bacterial numbers relative to those in CK plants.

Gene expression analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using the kit RT-PCR Beads as per the manufacturer’s protocol (Amersham Pharmacia Biotech, Piscataway, NJ, USA). RNA was isolated from leaves as described (Clark 1997; Dong and Beer 2000). The EF1α gene that is highly conserved and constitutively expressed in eukaryotes (Berberich et al. 1995; Gallie et al. 1998) was used as a loading control. Each gene was amplified for 25–30 cycles. PR-1 and PR-2 gene specific primers were the same as used in a previous study (Peng et al. 2003). Primers specific for the PAL1 gene were as follows: 5′-GGAGTGGACGCTATGTTTG-3′ and 5′-CATTGACGCCATTCCAGATC-3′. Sequences of RT-PCR products were confirmed by sequencing and comparison using the Blast program (Liu et al. 2006). RT-PCR products were resolved by electrophoresis with agarose gel and then captured with Bio-Rad Molecular Imager Gel Doc XR System (BIO-RAD Laboratories-Segrates, Milan, Italy) after staining with ethidium bromide.

Studies of cellular defense responses

The concentration of H₂O₂ in leaves was determined at 6 hpi with Pst 5 d after water or riboflavin treatment by monitoring the A₄₁₅ of the titanium-peroxide complex (Jiang and Zhang 2001). H₂O₂ accumulation was visualized by microscopic observations on detached leaves stained with 3,3′-diaminobenzidine tetrachloride (DAB) (Amresco, Solon, OH, USA) as previously described (Rea et al. 2004). Leaves were immersed with
1 mg/mL solution of DAB (pH 3.8) for 8 h under light at 25 °C and decolorized by immersion of leaves in ethanol (96%) for 8–12 h.

Hypersensitive cell death that had occurred in the form of micro-HR was observed by microscopy of leaves stained with trypan blue (Peng et al. 2003). Levels of cell death were determined based on Evan’s blue uptake by dead cells (Andl et al. 2001). Micro-HR was monitored at 12 hpi with Pst by microscopic observation of the treated leaves after staining with trypan blue, a protocol that can visualize cell death that has occurred at low frequency and is undetectable by eye (Peng et al. 2003). Levels of cell death that had occurred in leaf tissues were estimated at 12 hpi by Evan’s blue uptake assay (Andl et al. 2001). In the assay, leaf discs were incubated for 20 min with 0.05% Evan’s blue and washed extensively with highly pure water to remove unbound dye. Dye bound to dead cells was solubilized by incubating for 45 min at 50 °C in an aqueous solution of 50% methanol and 1% sodium dodecyl sulfate (SDS). The absorbance of the solubilized dye was determined by spectrophotometry at 600 nm.

Callose deposition in plants was visualized at 12 hpi with Pst by staining with aniline blue (Reuber et al. 1998). The top two leaves were infiltrated with 5 mL of a solution made of phenol, glycerol, lactic acid, water, and 95% ethanol (1:1:1:1:2, v/v). Leaves in solution were incubated in a 65 °C bath until they were judged clear and then stained with aniline blue. The staining reaction was held in the dark for 4 h for leaves. Samples were observed by microscopy under ultraviolet light.

Data treatment

All experiments were carried out in three replicates with similar results; each replicate contained 10 plants unless otherwise noted. Quantitative data were subjected to ANOVA tests ($P < 0.05$ and $P < 0.01$) to evaluate significance in differences between CK and other individuals of treatments.

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References


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