Riboflavin Induces Disease Resistance in Plants by Activating a Novel Signal Transduction Pathway

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ABSTRACT

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The role of riboflavin as an elicitor of systemic resistance and an activator of a novel signaling process in plants was demonstrated. Following treatment with riboflavin, *Arabidopsis thaliana* developed systemic resistance to *Peronospora parasitica* and *Pseudomonas syringae* pv. *Tomato*, and tobacco developed systemic resistance to *Tobacco mosaic virus* (TMV) and *Alternaria alternata*. Riboflavin, at concentrations necessary for resistance induction, did not cause cell death in plants or directly affect growth of the culturable pathogens. Riboflavin induced expression

of pathogenesis-related (PR) genes in the plants, suggesting its ability to trigger a signal transduction pathway that leads to systemic resistance. Both the protein kinase inhibitor K252a and mutation in the *NIM1/NPR1* gene which controls transcription of defense genes, impaired responsiveness to riboflavin. In contrast, riboflavin induced resistance and PR gene expression in NahG plants, which fail to accumulate salicylic acid (SA). Thus, riboflavin-induced resistance requires protein kinase signaling mechanisms and a functional *NIM1/NPR1* gene, but not accumulation of SA. Riboflavin is an elicitor of systemic resistance, and it triggers resistance signal transduction in a distinct manner.

Additional keywords: protein kinase cascade, systemic acquired resistance.

Two forms of induced resistance in plants, systemic acquired resistance (SAR), and induced systemic resistance (ISR), have been demonstrated. SAR refers to a distinct signal transduction pathway (19,37,66,71,72) that is mediated by salicylic acid (SA) and activates defense genes, such as pathogenesis-related (PR) genes (2,6,26,48,52,56,79,82). Transgenic *Arabidopsis* and tobacco plants (NahG) expressing the bacterial *nahG* gene, which encodes salicylate hydroxylase that degrades SA, are incapable of accumulating sufficient SA to induce SAR when challenged with pathogens (21,29). For many SAR-inducing compounds identified, SA is one of several plant-derived substances that elicit SAR (39,41,66). Several synthesized analogs of SA, such as 2,6-dichloroisonicotinic acid (INA), mimic SA in resistance induction (28,33,47,55,78). INA was an inducer of SAR in mutant screens, permitting discovery of a regulatory gene in SAR signal transduction known as *NIM1* (20,68), *NPR1* (9-11), or *SAI1* (69). *NIM1/NPR1* encodes a protein that controls expression of various sets of defense genes (8,10,16,40,65,88). *Arabidopsis nim1* mutants fail to develop SAR following infection by pathogens and treatment with SAR inducers (20,68). ISR, on the other hand, refers to resistance developed in plants following colonization by biocontrol rhizobacteria (36,51,75,76). ISR is mediated by jasmonic acid (JA) and ethylene and is not associated with expression of the set of SAR-associated genes (54,60,63,64). Although the pathways are different, both SAR and ISR require *NIM1/NPR1* for induction (26,65).

Responses mediated by SA, JA, and ethylene suggest that these plant-derived substances have important physiological roles and great potential as elicitors and mediators of resistance signal transduction. All three compounds induce unique types of resis-

tance when topically applied, and they affect a variety of processes in plants, including environmental acclimatization and defense against pathogens and insect pests (21,41,42,79,80,84,85). However, disease resistance may develop through alternative pathways that use different components. For example, oxidative burst (46) is an immediate response to inoculation with avirulent pathogens or treatment with resistance elicitors and leads to hypersensitive response (HR), a programmed cell death associated with disease resistance in plants (3,18,38,72,74,77). The reaction involves complex redox processes that require participation by specific signal molecules, such as H₂O₂ (12,46), and nitric oxide (22,50), and antioxidants, such as tocopherols and riboflavin (62,74,84).

Riboflavin is a vitamin produced by plants and microbes that acts as a coenzyme in many physiological processes in animals, plants, and microbes (30,34,83,90). Riboflavin is involved in anti-oxidation (62,74) and peroxidation (90); both processes affect the production of reactive oxygen intermediates (ROIs) in oxidative burst and consequent HR (3,18,22,38). Foliar application of riboflavin effectively controls several diseases of tobacco (25), and it reduces powdery mildew of strawberry plants in combination with methionine, metal ions, and surfactant (81). Thus, riboflavin may function as a resistance elicitor or a mediator of resistance signal transduction.

We report here evidence that riboflavin is an activator of a novel signaling pathway leading to systemic resistance. Riboflavin activates PR genes in *Arabidopsis* and tobacco and induces systemic resistance to four pathogens, including three model systems routinely used to study resistance (17,19,66), suggesting that riboflavin initiates resistance signal transduction. The response to riboflavin is negated by the protein kinase inhibitor K252a and by mutation of the *NIM1/NPR1* gene, but is unaffected in NahG plants, demonstrating that riboflavin-triggered signaling requires protein kinases and regulation by NIM1/NPR1, but does not require accumulation of SA. These findings should stimulate studies of novel roles for riboflavin and novel mechanisms of resistance signal transduction in plants.

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MATERIALS AND METHODS

Plant growth and treatment. Tobacco cvs. NC89, Xanthi (NN), and Xanthi (NN) containing the *nahG* gene from *Pseudomonas putida* (21,29) were grown in the greenhouse. *Arabidopsis thaliana* accession Columbia (Col-O), transgenic Col-O containing *nahG*, accession Ws-O, and Ws-O *nim1-1* (hereafter *nim1*) mutants were grown in a chamber at 18 to 20°C with 14 h of illumination. Six- to seven-week-old tobacco and two-week-old *Arabidopsis* plants were examined routinely for resistance induction. Plants were sprayed with water or 0.5 mM riboflavin (Sigma-Aldrich Chemical Co., St. Louis, MO), unless stated otherwise. The SAR-inducing chemical 2,6-dichloroisonicotinic acid (INA, 0.125 mg/ml) (55,78) and the HR-elicitor harpin (15 µg/ml) produced by *Erwinia amylovora* (23) were used as positive controls for PR gene expression and microscopic cell death. INA was provided as 25% wettable powder by K. Lawton, Novartis Crop Protection Inc. (Research Triangle Park, NC). Harpin was prepared and applied according to previous methods (23).

Pathogen maintenance and inoculation. *Tobacco mosaic virus* (TMV) was maintained in an aqueous solution (18 mg/ml) at 4°C. A diluted solution of 1 µg/ml was used to inoculate leaves of tobacco (Xanthi) by rubbing the leaf lamina with a finger dipped in inoculum mixed with diatomaceous earth as an abrasive. *Alternaria alternata* was propagated on potato dextrose agar (PDA) plates at 27°C and maintained on slants at 4°C. A spore suspension of 5×10^6 spores per ml was used to inoculate tobacco leaves by placing 25-µl drops on six sites on the upper leaf surfaces of tobacco (NC89). Maintenance, propagation, and inoculation of *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato* DC3000 were done as described previously (29,41,71). Col-O and NahG transgenic *Arabidopsis* were inoculated with the *Peronospora parasitica* isolate Noco2, and ecotype Ws-O and *nim1* mutant plants were inoculated with *Peronospora parasitica* isolate Emw1. All plant genotypes were inoculated with *P. syringae* pv. *tomato* DC3000 (31,43). Inoculated plants were incubated as described, except those inoculated with the bacterium, which were kept at room temperature (20 to 23°C).

Responses of plants. *Arabidopsis* and tobacco plants were sprayed with riboflavin at 0, 0.1, 0.5, 2.5, 5.0, 7.5, and 10.0 mM, respectively. Plants were assessed for possible phytotoxicity caused by riboflavin. In addition, leaves of *Arabidopsis* were detached 6 days after treatment, stained with trypan blue in lactophenol, cleared with chloral hydrate solution, and examined under an Olympus BX60 microscope (Olympus Corporation, NY) for microscopic cell death, according to previous methods (24,73).

Effects of riboflavin on pathogen growth. *A. alternata* was cultured on plates of PDA medium containing 0, 0.1, 0.5, 2.5, 5.0, 7.5, and 10.0 mM riboflavin at 25°C for 7 days; diameters of the fungal colonies were measured. *P. syringae* was cultured in Luria-Bertani medium containing equal concentrations of riboflavin on a shaker at 200 rpm and 25°C for 20 h. Cultures were started uniformly by adding 10 µl of sterile water or 10 µl of the seed culture suspension ($\approx 5 \times 10^8$ CFU/liter). Relative populations of bacteria in the suspension cultures were estimated by measuring absorbance at 620 nm (31). Five replicates were done for each pathogen.

Evaluation of pathogen infection and disease resistance. Infection by TMV and *A. alternata* in tobacco was assessed 7 and 10 days after inoculation, respectively. The number of lesions per leaf was counted, and the diameters of 10 to 20 lesions per leaf were measured. Infection of *Arabidopsis* by *Peronospora parasitica* was judged based on growth of conidiospores on the leaves 5 to 7 days after inoculation; the percentage of infected plants was recorded, and the amount of conidiospores per leaf was estimated by counting conidiospores in leaf washes with a hemacytometer. Oomycete growth in leaves was examined after staining with lactophenol-trypan blue (24,73). Infection by *P. syringae* in *Arabidopsis* was observed 5 to 7 days after inoculation; the bacterial

populations in leaf tissues were determined by recovering bacteria from leaves of inoculated plants and dilution-plating according to previous methods (21).

To determine if resistance develops systemically, riboflavin or water was sprayed on the three lower leaves or infiltrated in intercellular leaf spaces of tobacco, and 5 days later two untreated upper leaves were inoculated with TMV or *A. alternata*. In *Arabidopsis*, riboflavin or water was infiltrated into intercellular leaf spaces of two lower leaves, followed 5 days later by inoculation of whole plants with *Peronospora parasitica*.

Each test was repeated three to five times, and each replicate contained 30 to 50 *Arabidopsis* seedlings or 25 to 50 viral or fungal lesions on tobacco leaves, unless specified otherwise. *F* tests were done to determine any significant differences between treatments with riboflavin and water (*F* test two-sample for variances, Data Analyses Tools, Microsoft Excel Software).

Northern blot analyses. mRNA corresponding to *PR-1* and *PR-2* in *Arabidopsis* and *PR-1a* in tobacco was assessed in treated plants by northern blot hybridization. Plants for RNA analyses were treated with caution to prevent spraying the apices and the youngest three leaves. Total RNA was isolated from the untreated, youngest leaves with the RNeasy plant mini kit (Qiagen, Chatsworth, CA). RNA was size-fractionated by agarose gel electrophoresis and transferred to Immobilon transfer membrane (Millipore Corp., Bedford, MA), as described previously (15,82). Replicate blots of RNA from *Arabidopsis* and tobacco were hybridized to 32 P[dCTP]-labeled *Arabidopsis PR-1* and *PR-2* probes and tobacco acidic *PR-1a* probe, respectively. Hybridized membranes were washed at high stringency (13). For *Arabidopsis* and tobacco, 4 and 8 µg of total RNA per lane was used, respectively. Uniform loading was verified by staining with ethidium bromide.

Determination of effects of K252a. The protein kinase inhibitor K252a (Calbiochem, Novabiochem International Inc., San Diego, CA) was applied by spraying at 25 nm on a single leaf of tobacco or whole plants of *Arabidopsis* 30 min after treatment with riboflavin or water. The development of resistance and expression of PR genes in the treated plants was assessed as above. To determine if K252a alone affects infection and PR gene expression, plants of *Arabidopsis* treated only with K252a were tested for infection and PR gene expression.

RESULTS

Riboflavin-induced resistance in *Arabidopsis*. Reduction in infection by the downy mildew pathogen, *Peronospora parasitica*, was observed in *Arabidopsis* after treatment with riboflavin, as compared with water treatment. Differences in degree of infection between treatments were determined (Fig. 1A). In plants treated with water, *Peronospora parasitica* caused heavy infection in both accessions (Col-O and Ws-O) within 5 days, evidenced by heavy growth of hyphae and oospores in leaf tissues, as shown by microscopy of trypan blue-stained leaves. By day 7, massive numbers of conidiospores covered leaf surfaces. In riboflavin-treated plants, the pathogen did not produce visible masses of conidiospores on leaf surfaces but was restricted to leaf margins and a few small interior islands, as indicated by staining with trypan blue. Based on conidiospores per leaf, water-treated plants supported production of a large number of spores, while riboflavin-treated plants had only $\approx 0.1\%$ of the number on water-treated plants during the same period (Fig. 2A). In addition, the number of infected plants treated with riboflavin was significantly lower than those treated with water 12 days after inoculation (Fig. 3A). Therefore, riboflavin-induced resistance to the oomycete pathogen was demonstrated qualitatively based on the obvious reduction in infection and quantitatively by reduction in conidiospores produced per leaf and percentage of infected plants.

The occurrence of chlorosis and necrosis in *Arabidopsis* inoculated with *P. syringae* pv. *tomato* DC3000 was reduced by

treatment with riboflavin (Fig. 1B). The degree of infection was also evaluated based on the amount of bacteria recovered from leaf tissues daily for 5 days following inoculation. At each time, the number of bacteria recovered from control plants was significantly higher than that recovered from plants treated with riboflavin (Fig. 2B). Thus, riboflavin effectively induced resistance to the bacterial pathogen.

Riboflavin-induced resistance in tobacco. Tobacco plants treated with riboflavin developed resistance to *A. alternata* and TMV, based on reduced numbers and smaller diameters of lesions that developed following inoculation with the fungus or virus (Fig. 1C, Table 1). The degree of reduction in lesion numbers and diameters following riboflavin application was different for the two pathogens. In plants inoculated with TMV, riboflavin treatment significantly reduced the number of lesions. Lesion diameter was also reduced in riboflavin-treated plants, but the reduction was not always statistically significant (Table 1). In plants inoc-

ulated with *A. alternata*, reductions in both numbers and diameters of brown spot lesions were similar. Seven days after inoculation, brown spot lesions in water-treated plants were generally ≈ 10 mm in diameter, while lesions in riboflavin-treated plants were only ≈ 3 mm in diameter (Fig. 2C). Based on the reduction in lesion diameter, riboflavin-induced resistance was more effective against *A. alternata* than TMV (Fig. 2C and D).

Kinetics of riboflavin-induced resistance. Resistance in *Arabidopsis* to *Peronospora parasitica* and *P. syringae* and resistance in tobacco to TMV and *A. alternata* developed systemically throughout the plants, based on the reduction in the degree of infection on the untreated upper plant parts following treatment of lower leaves with riboflavin (Fig. 2, Table 1).

According to the time course of pathogen growth in the plant tissues, resistance in riboflavin-treated *Arabidopsis* became apparent after 3 days, reached its highest level at 5 days, and declined quickly 9 days after treatment (Fig. 3A and B). The time

Fig. 1. Infection of *Arabidopsis thaliana* by *Peronospora parasitica* and *Pseudomonas syringae* and infection of tobacco by *Tobacco mosaic virus* (TMV) and *Alternaria alternata*. Plants were treated with water (W) or riboflavin (R) at 0.5 mM and 5 days later were inoculated with either pathogen. Infection was observed 7 days after inoculation. **A.** Representative *Arabidopsis* leaves infected by *P. parasitica*. Leaves were photographed directly (left panels) or stained with trypan blue and observed under a microscope to visualize the in planta growth of the oomycete (right panels). Pathogen hyphae and oospores, infected sites of leaves stained blue, and uninfected leaf tissues (except veins) appear pale. **B.** *P. syringae* in *Arabidopsis* plants. **C.** Infection of tobacco by TMV (left two leaves) and *A. alternata* (right two leaves). Brown spots caused by *A. alternata* were covered with spores.

course of development of resistance to TMV and *A. alternata* in tobacco plants was similar. Resistance became apparent after 5 days and reached its highest level at 10 to 12 days after treatment (Fig. 3B). However, the duration of resistance to both pathogens differed. Brown spot symptoms were reduced for 40 days post-treatment, while diameters and numbers of TMV lesions were no longer reduced after 20 days, based on periodical inoculation at intervals up to 40 days posttreatment (Fig. 3B; data not shown).

Effects of riboflavin on plants and pathogens. The dose effect of riboflavin showed that concentrations of 0.5 to 1 mM were

effective and sufficient for resistance induction; higher concentrations did not increase the effect (Fig. 4). To determine the possibility of phytotoxicity, plants were treated with 0.1 to 10 mM riboflavin. Treated leaves did not exhibit any necrosis or other abnormalities when examined macroscopically or microscopically (Fig. 5). On the other hand, the effect of riboflavin on the growth of *A. alternata* and *P. syringae* was determined in vitro by growth of the pathogens in media supplemented with riboflavin ranging from 0 to 10 mM. Both pathogens grew well at all concentrations, indicated by the similar colony diameters of the fungal cultures on

Fig. 2. Development of infection in *Arabidopsis* and tobacco as affected by treatment with riboflavin. Leaves were infiltrated with water or riboflavin (Rib) at 0.5 mM and were inoculated 5 days after treatment, either (locally) on the same leaves or (systemically) on untreated upper leaves. Bars indicate standard errors. **A**, *Peronospora parasitica* in *Arabidopsis*. Curves reflect five replicates, each involving five leaves. **B**, *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis*. Each datum point represents the mean of three replicates, each containing three leaves. **C**, *Alternaria alternata* in tobacco. Lesion diameter is the mean of 50 lesions measured at each time. **D**, *Tobacco mosaic virus* in tobacco. Lesion diameter is the mean of 48 lesions measured at each time point.

TABLE 1. *Tobacco mosaic virus* infection in wild-type and NahG Xanthi tobacco plants treated with water and riboflavin

Treatment	Leaf ^y	Wild-type ^z		NahG ^z	
		No. of lesions per leaf	Lesion diameter (mm)	No. of lesions per leaf	Lesion diameter (mm)
Water	Treated	185 ± 8 (A)	6.6 ± 0.5 (A)	290 ± 15 (A)	7.5 ± 0.5 (A)
	Upper	170 ± 10 (A)	5.3 ± 0.3 (A)	255 ± 25 (A)	7.8 ± 0.8 (A)
Riboflavin	Treated	91 ± 16 (B)	5.0 ± 0.5 (A)	115 ± 30 (B)	5.0 ± 0.7 (B)
	Upper	77 ± 14 (B)	3.1 ± 0.2 (B)	140 ± 20 (B)	5.2 ± 1.0 (B)

^y Water or riboflavin was sprayed on lower leaves (treated) of plants. Seven days later, the upper two leaves were inoculated.

^z Lesions were examined 10 days after inoculation. Numbers followed by the same letter within columns do not differ significantly at $P \leq 0.05$.

agar plates and the similar absorbance values of the bacterial suspension cultures, respectively (Table 2).

Activation of PR genes. *Arabidopsis* and tobacco plants treated with riboflavin or water were tested for expression of PR genes by northern blot hybridization. Accumulation of transcripts of *PR-1* and *PR-2* genes were detected in the untreated upper leaves of the two accessions of *Arabidopsis*, in which the lower leaves had been treated with riboflavin 2 days prior to RNA extraction. Level of PR gene expression was estimated based on visual intensity of bands from northern blot hybridizations (Fig. 6A and B). Expression of both *PR-1* and *PR-2* increased with time after treatment, but their patterns of expression over the course of the experiment were slightly different. Transcription of *PR-1* was evident at 2 days and reached its highest level 4 days after treatment with riboflavin. Expression of *PR-2* increased slightly from 2 to 6 days (Fig. 6A) in tobacco. Similar, expression of *PR-1a* was detected in the untreated upper leaves of plants on which the lower leaves had been treated with riboflavin (Fig. 6C). In *Arabidopsis* and tobacco, treatment with INA, an elicitor of SAR, induced levels of PR gene expression similar to treatment with riboflavin, while treatment with water did not induce detectable expression of PR genes (Fig. 6).

Fig. 3. Effect of time between treatment with water or riboflavin and inoculation on development of infection by **A**, *Peronospora parasitica* in *Arabidopsis*, and **B**, *Alternaria alternata* in tobacco. Infection was examined 7 days after inoculation. Data at each time represents means (plus/minus standard errors) from 90 to 150 *Arabidopsis* seedlings or 75 to 150 lesions.

Impairment by K252a of resistance and PR gene expression. In tobacco, application of protein kinase inhibitor K252a after treatment with riboflavin greatly reduced riboflavin-induced resistance to TMV and *A. alternata* (Fig. 7A). Plants treated with water and inoculated with the virus or fungus developed large brown spots or viral lesions. K252a alone slightly increased

Fig. 4. Effects of concentration of riboflavin on the development of infection by **A**, *Peronospora parasitica* in *Arabidopsis*, **B**, *Alternaria alternata* in tobacco, and **C**, Tobacco mosaic virus in tobacco. Plants were sprayed with water or riboflavin and inoculated 5 days after treatment. Infection was evaluated 7 days (**A** and **C**) and 10 days (**B**) after inoculation. Each datum point represents the mean (plus/minus standard errors) of three replicates, involving **A**, 30 to 50 *Arabidopsis* seedlings; **B**, 30 to 50 lesions; and **C**, 50 lesions.

Fig. 5. Macroscopic and microscopic appearance of leaves of plants treated with riboflavin. Riboflavin was sprayed at several concentrations and appears as yellow residues at 5 and 10 mM. **A**, *Arabidopsis* leaves photographed 6 days after treatment (upper panel); leaves were detached and photographed after staining with trypan blue (lower panels, 5X). Trypan blue stains necrotic cells of the CK leaf, which had been treated with harpin that induces micro-HR (3,24). **B**, Tobacco leaves photographed 7 days after treatment.

susceptibility of tobacco to both pathogens, based on diameters of spots and lesions. Riboflavin reduced fungal and viral necrosis diameters by ≈ 60 and $\approx 40\%$, respectively. However, addition of K252a to riboflavin-treated plants reduced the effect of riboflavin by $\approx 80\%$, based on lesion diameters.

The role of riboflavin in resistance induction in *Arabidopsis* similarly was affected by K252a (Fig. 7B). K252a alone slightly increased infection by *Peronospora parasitica* in Col-O but not in Ws-O. Riboflavin reduced infection by ≈ 45 and $\approx 60\%$ in Ws-O and Col-O, respectively. Subsequent application of K252a to riboflavin-treated plants evidently obviated the effects of riboflavin.

Northern blot hybridization indicated that riboflavin-activated PR gene expression also was counteracted by K252a (Fig. 6A and C). Thus, the protein kinase inhibitor concomitantly inhibits the role of riboflavin in inducing resistance and activating PR gene expression.

Induction of resistance and PR gene expression in NahG plants. To determine if accumulation of SA is necessary for induction of resistance and PR gene expression by riboflavin, NahG *Arabidopsis* and tobacco were treated and examined as were non-transgenic plants. Induction of both resistance and PR gene expression occurred in NahG plants (Figs. 1A and B, and 6B and C). In riboflavin-treated NahG *Arabidopsis*, growth of *Peronospora parasitica* was greatly reduced (Fig. 1A), and fewer plants were infected (Fig. 8); *P. syringae* did not cause obvious symptoms, similar to what occurred in the wild-type (Fig. 1B). In NahG tobacco, the numbers and diameters of lesions caused by TMV were significantly decreased by treatment with riboflavin (Table 1). *Arabidopsis* PR-1 and PR-2 genes and tobacco PR-1a gene were expressed in NahG plants following treatment with riboflavin (Fig. 6B and C). Therefore, riboflavin is able to induce resistance and activate defense genes in the absence of SA accumulation.

Compromise of resistance and PR gene expression in nim1 Arabidopsis. To determine if a functional NIM1/NPR1 gene is required for the action induced by riboflavin, *Arabidopsis* nim1 mutants were examined for resistance development and PR gene expression following application of riboflavin. No PR gene expression was detected in nim1 mutant plants (Fig. 6B). The mutant also failed to develop resistance after treatment with riboflavin (Fig. 8). Both the wild-type and nim1 mutant supported growth of a number of conidiospores on leaf surfaces when treated with water (control), but nim1 plants were more sensitive to the pathogen (Fig. 8). Resistance developed in wild-type plants but did not develop in nim1 plants after treatment with riboflavin. A similar percentage of infection was observed in nim1 plants, whether treated with water or riboflavin. These data suggest a requirement for regulation by the NIM1/NPR1 gene in the induction of resistance by riboflavin.

DISCUSSION

The terminology used for different types of resistance in plants (to infection by pathogens) has become quite confusing. Subtle

TABLE 2. Effect of riboflavin on growth in vitro of *Alternaria alternata* and *Pseudomonas syringae* pv. *tomato* DC3000^y

Riboflavin concentration (mM)	<i>A. alternata</i> colony diameter (cm) ^z	Relative growth of DC3000 (OD ₆₂₀) ^z
0	2.4 ± 0.2	2.03 ± 0.03
0.1	2.3 ± 0.2	2.23 ± 0.03
0.5	2.4 ± 0.1	2.23 ± 0.04
1.0	2.3 ± 0.3	2.22 ± 0.07
2.5	2.4 ± 0.1	1.88 ± 0.04
5.0	2.5 ± 0.1	1.79 ± 0.04
7.5	2.3 ± 0.3	1.75 ± 0.08
10.0	2.3 ± 0.2	1.68 ± 0.04

^y Optical density (OD) at 620 nm.
^z Each number represents the mean of five replicates.

changes in modifiers or word order in combination with “induced” and “resistance” have been proposed to indicate resistances induced by specific biological, chemical, or physical factors. Often these subtleties are lost or hard to express in conversation or translation to languages other than English. Therefore, we propose that the term “induced resistance” be used generally to encompass resistant plant phenotypes that result from any treatments that cause plants to develop resistance, which is not constitutive. The inducer of the resistance can precede the “induced resistance” to clearly define the process. For example, SA-induced resistance (52,66), Plant growth promoting rhizobacteria-induced resistance (51), harpin-induced resistance (23,24), and, based on the present report, riboflavin-induced resistance. More specific terms, such as “the SAR pathway” (66) and “the ISR pathway” (65,75), are used to refer to a specific signal transduction process leading to the phenotype of induced resistance to a specific array of pathogens.

The studies reported here indicate that treating plants with riboflavin results in pathogen resistance, which differs from previously described types of resistance; the process requires plant metabolism time between treatment and the development of resistance. Thus, clearly it is a form of induced resistance. Comparison of riboflavin-induced resistance with several previously described types indicated that riboflavin induces resistance specifically by a novel mechanism involving a pathway that differs from those studied previously.

Fig. 6. Northern blot hybridization showing riboflavin-induced pathogenesis-related gene expression as affected by plant genotype and application of K252a. Plants were treated with W = water; I = positive control 2,6-dichloroisonicotinic acid at 0.125 mg/ml; K = protein kinase inhibitor K252a at 25 mM; R = riboflavin at 0.5 mM; or +K = riboflavin followed by K252a. RNA was extracted from the untreated upper leaves at days indicated after treatment. Uniform loading was verified by staining with ethidium bromide (EtBr). **A**, Transcripts of PR-1 and PR-2 from *Arabidopsis* (ecotype Col-O). **B**, Expression of PR-1 in *Arabidopsis* NahG transgenic but not in *nim1* mutant plants 5 days after treatment. **C**, Expression of PR-1a in tobacco (Xanthi) and NahG plants 5 days after treatment.

Basis of the hypothesis that riboflavin induces resistance through activating plant defense mechanism. The important role of riboflavin (vitamin B₂) as an indispensable vitamin for healthy animals and as an effective therapeutic treatment for many physiological diseases in animals and humans (34,59,83) inspired external application of the vitamin to plants. Although plants can synthesize riboflavin themselves, levels of riboflavin vary greatly in organs and during stages of development (70,90). Riboflavin is an antioxidant (62,74), and other antioxidants induce disease re-

sistance in plants (61). These features are consistent with the concept that riboflavin might play a role in plant disease resistance.

In this study, we first determined the development and kinetics of resistance in *Arabidopsis* and tobacco following application of riboflavin. We ruled out the possibility that riboflavin might directly inhibit pathogens. We analyzed PR gene expression to determine if riboflavin is capable of triggering a resistance signaling process. We determined resistance and PR gene expression in several plant genetic backgrounds and in the presence of a general inhibitor of protein kinases to reveal the nature of the riboflavin-triggered signal transduction process. Microscopic observation of leaf tissues stained with trypan blue was used to visualize the growth of *Peronospora parasitica* in plant tissues. This method also allowed visualization of microscopic cell death, either as a consequence of riboflavin signaling responses, which could occur within 24 h as usual, or due to possible phytotoxicity of riboflavin, which might occur within several days (23,24,73). Both molecular and phenotypic data showed that riboflavin substantially induced systemic disease resistance and did so by activating a distinct signal transduction pathway.

Practical value of riboflavin as an elicitor of disease resistance. Our studies demonstrate that riboflavin elicits systemic resistance. This conclusion is supported by data showing development of resistance in *Arabidopsis* and tobacco against four pathogens, three of which represent the established spectrum of targets of induced resistance (35,44,66,71). In addition, no direct affect of the vitamin was detected on the growth of the culturable pathogens, suggesting that riboflavin is not likely to directly inhibit pathogens. Riboflavin-induced resistance was effective against *A. alternata*, which has not yet been considered as a target of induced resistance (35,44,66).

As previous studies (25,61,62,74,81) indicate, riboflavin has potential for practical use. Resistance-inducing chemicals have been applied for disease control, but success has been limited thus far (39,44,66). For example, SA and INA are not feasible for dis-

Fig. 7. Effect of K252a on the ability of riboflavin to induce resistance in tobacco and *Arabidopsis*. Plants were treated with water, K252a at 25 mM, riboflavin at 0.5 mM separately, or pretreated with riboflavin at 0.5 mM for 30 min followed by K252a (R+K). **A**, Infection by *Tobacco mosaic virus* (TMV) or *Alternaria alternata* in tobacco; and **B**, infection by *Peronospora parasitica* in *Arabidopsis* was evaluated based on lesion diameters and percentage of infected plants, respectively. Error bars indicate variations from means of three replicates.

Fig. 8. Effect of riboflavin treatment on infection by *Peronospora parasitica* of *Arabidopsis* transgenic NahG and *nim1* mutant plants. Plants were sprayed with water or riboflavin (0.5 mM) and inoculated 5 days after treatment. Degree of infection was evaluated 7 days after inoculation. Bars represent the means of three replicates, each containing 30 to 50 seedlings.

ease control, because the concentration needed to induce resistance is nearly phytotoxic (66). In contrast, our studies suggest that riboflavin is not phytotoxic at concentrations much higher than that needed for resistance induction. The level of riboflavin-induced resistance, though not total, would seem to be useful. In our experiments, treatment of tobacco plants with riboflavin reduced the diameters of brown spot lesions from ≈ 10 to ≈ 3 mm in most cases. This lesion reduction caused an obvious increase in the quality and commercial value of flue-cured tobacco leaves (25). During flue-curing performed after harvest, the necrotic areas of lesions on leaves greatly enlarge. Also, it may alter chemical components, including the levels of sugars, proteins, nicotine, potassium, and chlorides, which are negatively affected by diseases. Even smaller reductions in lesion size on fresh leaves could result in greater commercial value of cured leaves. Riboflavin enhances resistance in *Arabidopsis* and tobacco by 40 to 70%, a level that should provide worthwhile protection to crops against pathogens. Further, riboflavin-induced resistance is persistent, as it continues to be effective against *A. alternata* in tobacco for 40 days. Finally, riboflavin-induced resistance is nonspecific and can provide a basis for protection to a broad range of plants against an array of pathogens. Therefore, riboflavin seems to possess many important characteristics of a safe disease control agent.

Riboflavin activation of a distinct signal transduction pathway. Our studies also indicate that riboflavin triggers resistance signal transduction in a distinct manner. First, expression of PR genes is considered a downstream event of resistance signal transduction that leads to phenotypic resistance (2,6,67,86,88). The genes activated by riboflavin are involved in activation of resistance induced by other factors (66). The kinetics of riboflavin-induced resistance reflect the requirement for accumulation of molecular defense components (32,35,44), thus, PR gene expression took 4 to 5 days to reach its highest level and preceded the greatest phenotypic resistance. This is consistent with previous studies that showed that external application of riboflavin promotes certain physiological processes in plants (30,57). For example, addition of riboflavin to suspension-cultured cells of strawberry enhances synthesis of anthocyanin, a universal plant pigment that has pharmacological effects (57). Second, because K252a, a protein kinase inhibitor, inhibited the ability of riboflavin to induce resistance and defense gene expression, riboflavin appears to act through a signaling process that involves protein kinases (7,27). Finally, because resistance and PR gene expression were induced in NahG transgenic but not in *nim1* mutant plants, responsiveness to riboflavin requires regulation by the *NIM1/NPR1* gene but is independent of SA. Based on these data, we conclude that riboflavin induces nonspecific disease resistance by triggering a novel signal transduction pathway that appears distinct from the typical SAR and ISR pathways.

The riboflavin-initiated signal transduction pathway appears to cross-talk with the ISR pathway at *NIM1/NPR1* and with the SAR pathway from *NIM1/NPR1* to PR gene activation. However, it is distinct from the traditional SAR and ISR pathways in several respects. The SAR signal transduction pathway is characterized by coordinate activation of SAR genes, including PR genes, with the development of resistance (5,53,66,82), and SAR is dependent on SA accumulation (29). ISR is distinct from SAR, although both require regulation by the *NIM1/NPR1* gene and lead to nonspecific resistance (36,51,54,60,75,76). ISR is mediated by different signals and is not associated with expression of the battery of SAR-related genes (63-65,80,85). Although mitogen-activated protein kinases are induced by SA, the contribution of the kinases in SAR and ISR pathways is not clear (87). Unlike many SAR elicitors, such as harpin (23,24), which induces hypersensitive cell death as a necessary step in the development of SAR (18,66), riboflavin does not cause macroscopic and microscopic cell death in plants at concentrations that effectively induce resis-

tance. Thus, riboflavin activates resistance mechanisms in a HR-independent manner.

Possible riboflavin-mediated resistance signal transduction links. The identified signal molecules that mediate resistance development in plants show three characteristics (22,41,42,79,80,84,85): (i) they induce disease resistance when applied exogenously; (ii) they activate a signal transduction process when applied exogenously; and (iii) they are synthesized and mediate resistance signal transduction when plants are infected by pathogens or treated with resistance elicitors. We have shown that external application of riboflavin elicits resistance and initiates a signal transduction process. How could the signal transduction events occur in plants in response to riboflavin, for which physiological roles have been well demonstrated? Further, whether riboflavin functions as a mediator of resistance signal transduction remains an interesting question.

Recent studies of riboflavin (1,3,34,46,49,62,70,74,90) indicate the function of the compound in mediating resistance signal transduction. First, riboflavin is a cofactor of enzyme flavoproteins, some of which catalyze lipid peroxidation (90), a main process in producing ROIs that serve as a signaling network in plant immune responses (3,46). The role of riboflavin in peroxidation is antagonistic to its role in antioxidation (62,74,90). Balance between both reactions should be a part of the signaling mediation and may affect whether programmed cell death occurs. Second, receptors or binding proteins of riboflavin are thought to serve as a storage mechanism for the vitamin in animals and animal products (1,49). Exploration of the existence of similar receptors and their roles as signal transducers in plants responding to riboflavin and other elicitors of resistance would be very worthwhile. Third, glycosylated forms of riboflavin, which are considered unimportant in plant and animals (34,70), may serve as a signal-storage compound. This function may be similar to that of calmodulins (14,45) and glycosylated SA (41), which function in the Ca^{+2} and SA signal storage, respectively. Finally, the universal existence of flavin kinases, required to activate flavoproteins (90), may be linked with protein kinase cascades, which are a typical mode of signal transduction (4,58,89). Therefore, there is a reasonable basis for riboflavin to mediate a distinct signal transduction pathway. We hope that this report will stimulate additional studies of the novel roles of riboflavin and mechanisms of resistance signal transduction in plants.

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