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Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropanoid pathway

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ABSTRACT

Vitamins are plant growth regulators and activators of defense responses against pathogens. The cytomolecular mechanisms involved in the induction of resistance by chemicals especially vitamins on monocotyledonous plants are largely unknown. Here, we show that riboflavin, which acts as a defense activator in rice against economically important sheath blight caused by *Rhizoctonia solani*, primed the expression of lipoxygenase (*LOX*) as a key gene in octadecanoid pathway, and enhanced lignification. Exogenous jasmonic acid (JA) application on rice induces resistance against *R. solani* in a manner similar to riboflavin. Application of jasmonate-deficient rice mutant *hebiba* and using a LOX inhibitor revealed the main role of octadecanoid pathway in riboflavin-induced resistance (IR). In riboflavin-treated inoculated plants, upregulation of phenylalanine ammonia-lyase (*PAL*) expression, as a major marker of phenylpropanoid pathway, was detected downstream of *LOX* upregulation. Co-application of riboflavin and 5, 8, 11, 14-eicosatetraynoic acid (ETYA) on rice leaves revealed no upregulation of *PAL* and no priming in lignification. Furthermore, lower levels of *PAL* transcripts and lignin were detected in *hebiba* compared with control. These findings indicate the role of octadecanoid pathway in the induction of phenylpropanoid metabolism leading to lignification as a novel mechanism of riboflavin-IR in *Oryza sativa–R. solani* pathosystem.

Introduction

Sheath blight disease, caused by anastomosis group 1-IA of the soil-borne necrotrophic fungus *Rhizoctonia solani* (teleomorph *Thanatephorus cucumeris*), is one of the most destructive and widespread rice diseases with yield losses up to 50% (Sridevi et al., 2008). Sheath blight damage has increased in rice-growing regions since the introduction of high-yielding compact semi-dwarf cultivars and the application of high levels of nitrogen fertilizers. The disease symptoms include greenish gray and oval-shaped lesions with yellow to brown margins mostly formed on leaf sheaths; also leaf blades can be infected. Control of rice sheath blight is difficult due to the low inherent level of resistance of rice cultivars against this disease, wide host range of the pathogen, its ability to survive in soil for a long time, and its high genetic variability (Taheri et al., 2007). Although partial genetic resistance to sheath blight in rice has been reported, no major gene responsible for resistance has been found (Kumar et al., 2003), and rice sheath blight is not efficiently controlled by resistance breeding. Most of the traditional cultivars, planted on over 90% of the rice-growing areas, are susceptible to disease. Therefore, an intensive use of other crop protection methods such as application of chemicals seems to be necessary to limit the damage in the fields. The growing concern about the negative environmental effects of fungicides and appearance of fungicide-resistant pathogen strains is motivating research for alternative protection strategies. Among such new strategies, induced resistance (IR) has emerged as a potential supplement in crop protection measures.

IR is a phenomenon by which plants exhibit increased levels of resistance against pathogen attack by activating an efficient signal transduction network that can be triggered by various biotic or abiotic stimuli (Koornneef and Pieterse, 2008). The components of this signaling network including phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and indole acetic acid (IAA) are each involved in the regulation of defense against various pathogens (Domingo et al., 2009; Manavella et al., 2008; Umemura et al., 2009; Wawrzynska et al., 2009). Increasing evidence supports the concept that these signal transduction pathways interact with each other, either

Abbreviations: ABA, abscisic acid; BABA, β-aminobutyric acid; dpi, days post inoculation; dpt, days post treatment; ET, ethylene; ETYA, 5, 8, 11, 14-eicosatetraynoic acid; IAA, indole acetic acid; IR, induced resistance; JA, jasmonic acid; JA-Ile, jasmonoyl-isoleucine; LOX, lipoxygenase; LTGA, lignin thioglycolic acid; PAL, phenylalanine ammonia-lyase; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ROS, reactive oxygen species; SA, salicylic acid; TGA, thioglycolic acid

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synergistically or antagonistically (Pauwels et al., 2008). The interaction between signaling pathways leads to a powerful regulatory potential in plants to defend themselves against biotic or abiotic stresses. Over the recent years, several investigations demonstrated that plant defense responses not only can be activated directly but also can be associated with a primed state of the plant. In the primed conditions, plants are capable of recalling previous infection, root colonization by specific useful microbes, or chemical treatment (Goellner and Conrath, 2008). Consequently, primed plants respond faster and/or stronger to various stresses caused by biotic or abiotic agents. This phenomenon that is known as priming is a part of plant defense reactions. Priming is operative through a complex network of signaling pathways. Investigations on the costs and benefits of priming demonstrated that the fitness cost of priming is lower than that of direct elicitation of defense reactions. Interestingly, the benefits of priming outweigh its costs when disease occurs. Therefore, priming is a fine economic solution to the trade-off dilemma between plant disease protection and costs involved in enhancing defense responses (Conrath et al., 2006).

Various organic and inorganic chemicals are known to be capable of priming plant defense responses especially in dicotyledoneous (dicot) plants (Ahn et al., 2007; Hamiduzzaman et al., 2005; Trouvelot et al., 2008). However, little is known about different chemicals, which are able to induce resistance responses in monocotyledoneous (monocot) crops. The cellular and molecular events associated with the induction of resistance in monocots are less well understood than in dicot plants, and information about signals involved in induction of resistance in rice, as the most important monocot, is still scarce.

Riboflavin, also known as vitamin B_2, is a micronutrient with a key role in maintaining human, animals, and plants health. It is a well-known oral nutritional supplement that is widely used to enhance basal immunity and resistance to infections in human and animals (Sundravel et al., 2003). Riboflavin is reported to be involved in the induction of defense responses in human, animals, plants, and microorganisms, by interfering in antioxidation (Perumal et al., 2005), peroxidation (Nazarul et al., 2006), or activation of several defense mechanisms (Dong and Beer, 2000; Verdrengh and Tarkowski, 2005; Zhang et al., 2009). This vitamin has been shown to be an effective plant defense activator against different fungal, bacterial, and viral pathogens when applied exogenously on dicot plants such as *Arabidopsis* and tobacco. Treatment with riboflavin develops systemic resistance in *Arabidopsis* plants against *Peronospora parasitica* and *Pseudomonas syringae* pv. *tomato*, and in tobacco against *Alternaria alternata* and tobacco mosaic virus (TMV), without inhibiting growth of the culturable pathogens (Dong and Beer, 2000; Zhang et al., 2009). In addition, riboflavin and its dimethylated amino-derivative roseoflavin were effective in inducing systemic resistance against rice blast disease caused by *Pyricularia oryzae* (Aver'yanov et al., 2000). However, the signaling pathways and molecular defense mechanisms involved in riboflavin-IR in monocots are largely unknown. It is preliminarily reported that riboflavin is able to induce defense responses in rice against sheath blight (Taheri and Höfte, 2007). The aim of this study was to determine the molecular mechanisms and signaling pathways involved in riboflavin-IR in monocot model plant rice against infection by *R. solani*. We investigated the effect of riboflavin treatment on generation of H_2O_2 as one of the most important reactive oxygen species (ROS), and its correlation to formation of lignin as a structural barrier. Resistance was evaluated on rice plants pre-treated with riboflavin and compared with plants pre-treated with JA, as a phytohormone capable of activating defense responses. The role of octadecanoid pathway in riboflavin-IR in this pathosystem was investigated not only using 5, 8, 11, 14-eicosatetraynoic acid (ETYA) as a lipoxygenase (LOX) inhibitor but also with application of a jasmonate-deficient rice mutant *hebiba*. The involvement of octadecanoid pathway in riboflavin-IR was further investigated by comparing transcript levels of *LOX*, as a key marker of this pathway, in mock-treated and riboflavin-treated plants. In addition, the role of phenylpropanoid pathway and its correlation with octadecanoid signaling in riboflavin-IR was investigated by determining the *PAL* transcript accumulation.

Materials and methods

Plant material

The susceptible rice (*Oryza sativa*) cultivar induced resistance (IR)-64, which is an elite *indica* cultivar, was routinely used in the experiments. IR-64 seeds were obtained from CIRAD (International Cooperation Center of Agricultural Research for Development, Paris, France). Seeds of jasmonate-deficient *hebiba* mutant (Riemann et al., 2003) and the corresponding wild-type, *japonica* cultivar Nihonmasari, were kindly provided by Dr. Peter Nick (Karlsruhe University, Germany). The seeds were germinated in humid plates ($\geqslant$95% R.H.), at 28 °C for 4 d. Germinated seeds were sown in trays in potting compost (Klassmann-Deilman, Geeste, Germany) and grown in greenhouse conditions as previously described (Taheri et al., 2007). Four-week-old plants were used for inoculations.

Treatment with riboflavin, jasmonic acid (JA), and 5, 8, 11, 14-eicosatetraynoic acid (ETYA)

Riboflavin, JA, and the lipoxygenase (LOX) inhibitor ETYA were purchased from Sigma. Riboflavin was dissolved in water and used at 1 μM concentration, which is known to have the best effect in induction of resistance in rice against *Rhizoctonia solani* (Taheri and Höfte, 2007). JA and ETYA were dissolved in methanol prior to dissolving in water. Equivalent volume of methanol was added to control treatment to ensure that methanol did not interfere with the experiments. Riboflavin and JA were sprayed on the plants until runoff in intact leaf sheath assays at 5 d before inoculation. ETYA was used for treating the leaf segments in detached leaf assays 24 h before inoculation with *R. solani*. To identify the role of the octadecanoid signaling pathway in riboflavin-IR, ETYA was used for pre-treatment of leaf segments (for 2 h) prior to riboflavin treatment (24 h), then the leaf segments were used for inoculation in a detached leaf assay.

Pathogen maintenance and intact leaf sheath inoculation

The virulent *R. solani* AG1-IA isolate NL-84 obtained from symptomatic rice plants (Taheri et al., 2007) were grown on potato dextrose agar (PDA) plates at 28 °C and maintained on PDA slants at 4 °C. Inoculum consisted of toothpicks, 2 cm in length that had been sterilized and inoculated with the above-mentioned isolate as previously *described* (Taheri et al., 2007). Briefly, 15 sterilized toothpicks were placed radially around a mycelial plug of *R. solani*, 5 mm in diameter, which was located at the center of a PDA plate. The mycelial plugs used in all PDA plates were taken from the margine of an actively growing 7-day-old *R. solani* colony on PDA. All of the toothpicks were placed at the same distance from the central mycelial plug and the plates were allowed to incubate at 28 °C. After an incubation period of 5 d, one colonized toothpick was placed into the lowest inner sheath of the main tiller, 5 cm above the soil surface. For each treatment, at least 12 replicate plants were inoculated in a completely randomized