Riboflavin-Induced Disease Resistance Requires the Mitogen-Activated Protein Kinases 3 and 6 in Arabidopsis thaliana

Shengjun Nie, Huilian Xu*

International Nature Farming Research Center, Hata 5632, Matsumoto-city, Nagano 390–1401, Japan

* yf120nsj@163.com

Abstract

As a resistance elicitor, riboflavin (vitamin B2) protects plants against a wide range of pathogens. At molecular biological levels, it is important to elucidate the signaling pathways underlying the disease resistance induced by riboflavin. Here, riboflavin was tested to induce resistance against virulent Pseudomonas syringae pv. Tomato DC3000 (Pst DC3000) in Arabidopsis. Results showed that riboflavin induced disease resistance based on MAPK-dependent priming for the expression of PR1 gene. Riboflavin induced transient expression of PR1 gene. However, following Pst DC3000 inoculation, riboflavin potentiated stronger PR1 gene transcription. Further was suggested that the transcript levels of mitogen-activated protein kinases, MPK3 and MPK6, were primed under riboflavin. Upon infection by Pst DC3000, these two enzymes were more strongly activated. The elevated activation of both MPK3 and MPK6 was responsible for enhanced defense gene expression and resistance after riboflavin treatment. Moreover, riboflavin significantly reduced the transcript levels of MPK3 and MPK6 by application of AsA and BAPTA, an H2O2 scavenger and a calcium (Ca2+) scavenger, respectively. In conclusion, MPK3 and MPK6 were responsible for riboflavin-induced resistance, and played an important role in H2O2- and Ca2+-related signaling pathways, and this study could provide a new insight into the mechanistic study of riboflavin-induced defense responses.

Introduction

To survive in unfavorable environments, plants have evolved sophisticated defense strategies to induce their immune system against pathogens and protect themselves from being infected. Upon appropriate biotic or abiotic stimulation, plants can develop enhanced capacity to express pathogen-induced defense response [1, 2]. The phenomenon has been known as priming, and this priming can result in a faster and/or stronger induction of defense mechanisms when subsequently challenged by pathogens [3, 4]. For example, inoculation with Pseudomonas syringae pv tomato (Pst) strain DC3000 expressing avrRpt2 gene was shown to enhance defense response that were manifested following Pst DC3000 infection [5]. Following infection...
by the pathogen, plants can develop enhanced resistance when subsequently challenged by other pathogens attack. The type of induced resistance is regarded as systemic acquired resistance (SAR) that is mediated by endogenous accumulation of the plant hormone salicylic acid (SA) [6, 7]. In addition, the induced resistance is associated with the accumulation of pathogenesis-related (PR) protein, and in fact, the so-called priming of defense in cells has been known as a component of induced resistance response in plants [5, 8].

In addition to pathogens attack, exogenous application of specific compounds and synthetic chemicals, can also induce this form of resistance in plants [3]. For example, the non-protein amino acid β-amino-butyric acid (BABA) induced resistance against the bacterial pathogen \textit{Pst} DC3000 and the fungal pathogen \textit{B. cinerea} [9, 10], and BABA-induced resistance against \textit{B. cinerea} is based on ABA-dependent priming for callose accumulation [11]. Thiamine is a B-complex that is produced in plants and microbes. Thiamine functions as a plant defense activator, and confers SAR through priming which requires hydrogen peroxide and intact signaling protein NON EXPRESSOR OF PR1 (NPR1) [12, 13]. Thus, plants primed by treatments that induce resistance show a faster and stronger activation of defense response after pathogens attack.

Riboflavin (vitamin B$_2$), as a coenzyme in many physiological processes, is produced by plants and microbes [14–16]. Riboflavin is an essential nutrient for humans. Riboflavin deficiency causes skin and mucosal disorders, including cheilitis and anemia [17]. Riboflavin exists in three forms: free riboflavin and two cofactor forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These forms act as coenzymes in physiological processes, including light sensing, bioluminescence and DNA repair [18]. In addition to riboflavin’s well-known nutritional value and as an enzyme cofactor, recent studies have demonstrated a novel function of riboflavin in disease resistance [19, 20]. Treatment with riboflavin protects tobacco and \textit{Arabidopsis} from fungal and bacterial infections without inhibiting pathogens growth. Riboflavin activates PR genes in \textit{Arabidopsis} which is dependent on the \textit{NPR1} gene and induces SAR to pathogens, suggesting that riboflavin initiates resistance signal transduction. Although priming induced by riboflavin has been known as a component of resistance responses in plants [19, 20], very little is known about the molecular mechanisms of priming defense by riboflavin.

Recently, it has been suggested that priming is associated with enhanced accumulation of cellular signaling proteins when subsequently challenged by stress [3, 9]. Alteration to the phosphorylation state of these signaling proteins plays an important role in signal transduction. Mitogen-activated protein kinase (MAPK) cascades are an important part of the signaling machinery that transduces extracellular stimuli into intracellular responses in all eukaryotic cells [21–26]. This cascade generally consists of three functional kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and a MAPK. In \textit{Arabidopsis}, 20 MAPKs, 10 MAPKKs and 60 MAPKKKs have been identified [21, 27, 28]. Furthermore, MAPKs have been implicated in regulating innate immunity and adverse stress responses in plants and animals [29–31]. Because MAPK cascades play essential roles in cellular signal amplification, MAPKs, MAPKKs and MAPKKKs are excellent candidates for signaling enzymes mediating priming. Recent investigations have verified a MAPK cascade, extending from MEKK1 through MEK4/5 to MPK3/6 in response to the microbe-associated molecular pattern flagellin, or its conserved N-terminal 22-amino-acid epitope flg22 [21]. MPK3 and MPK6 can be activated by various environmental stresses and participate in \textit{Arabidopsis} defense responses [32–37].

Reactive oxygen species (ROS) and calcium (Ca$^{2+}$) are regarded as the key signaling molecules in plant cells [38, 39]. In \textit{Arabidopsis}, application of hydrogen peroxide (H$_2$O$_2$) stimulated Ca$^{2+}$ influx through Ca$^{2+}$-permeable channel [38, 40, 41]. Furthermore, ROS and Ca$^{2+}$ have been observed in numerous plant-pathogen interactions, such as hypersensitive response (HR), and these signaling messengers are up-regulated prior to the expansion of the
local lesions [42–46]. Recent studies have shown that both thiamine- and riboflavin-induced resistance are dependent on H₂O₂ and a functional NPR1 gene in Arabidopsis [12, 13, 19, 20], and thiamine induces priming for pathogen defense through the Ca²⁺-related signaling pathway [12].

Although the involvement of MPK3 and MPK6 in the activation of Arabidopsis defense responses is evident, a clear picture of their contribution to riboflavin-induced resistance to pathogen is still missing. In this work, the roles played by MPK3 and MPK6 in riboflavin-induced resistance to Pst DC3000 were investigated to see whether riboflavin increases the levels of MPK3 and MPK6 transcript and transient expression of PR1 gene and whether or not the activities of MPK3 and MPK6 were strongly enhanced upon infection by Pst DC3000, with effects of riboflavin exerted through H₂O₂- and Ca²⁺-related signaling pathways. Examinations were made to confirm whether MPK3 and MPK6, as important signaling components, could be responsible for riboflavin-induced disease defense in Arabidopsis.

**Materials and Methods**

**Plant materials and chemical treatments**

Plants of Arabidopsis ecotype Col-0 and mutants lacking MAPK genes (mpk3-1, SALK_151594; mpk6-2, SALK_073907; mpk6-3, and SALK_127507; purchased from NASC/ABRC) were used as materials. In the mpk3 and mpk6 mutants (mpk3, mpk6-2 and mpk6-3), they lack detectable transcripts of MPK3 and MPK6, respectively (S1 Fig). All the plants were grown in a growth chamber with photoperiod of 16 h with a over-canopy lighting of photosynthetic photon flux of 120 μmol m⁻² s⁻¹ and 80% relative humidity at 22°C for 2- to 3 weeks. The plants were sprayed with water or 0.6 mM riboflavin in the presence of Silwet L-77 (0.015%) 4 h before pathogen Pst DC3000 was inoculated, unless stated otherwise. Here, the DC3000 strain of *Pseudomonas syringae pv tomato* without any other avirulent genes was lyophilized and stored at -80°C, and cultured on King’s B medium supplemented with appropriate antibiotics before inoculation. AsA, BAPTA-AM and PD98059 were purchased from Sigma-Aldrich China (Shanghai, China).

**Pathogen maintenance and pathogen challenge**

Pst DC3000 was cultivated on the King’s B liquid medium supplemented with 75 μg mL⁻¹ rifampicin at 28°C overnight and shaken until midlog growth phase (OD₆₀₀ = 0.15) was obtained. To inoculate Arabidopsis with Pst DC3000, bacterial cells were retrieved from medium by centrifugation at 3,000 g for 10 min and resuspended in 10 mM MgCl₂, and the concentration was adjusted to 0.01 (OD₆₀₀) in 10 mM MgCl₂. At least 25 plants of Arabidopsis ecotype Col-0 or mutants were inoculated per treatment. Arabidopsis leaves were inoculated with the bacterial suspension of OD₆₀₀ = 0.01 with 1-mL syringes. The inoculated plants were kept in a dew chamber for 16 h at 25°C and 100% relative humidity and then transferred to a growth chamber with a 16-h light/8-h dark regime at 25°C and 80% relative humidity. The bacterial growth was assessed by determining the CFU of 1 g FW (fresh weight) of leaves from five plants through plating appropriate dilutions on King’s B medium containing 75μg mL⁻¹ rifampicin [47].

**Treatment with MAPK cascade inhibitor**

The Arabidopsis leaves were pre-inoculated in a solution containing a MAPK cascade inhibitor PD98059 dissolved in DMSO for 50 min before riboflavin treatment. PD98059 was used to inhibit the activation of MAPK cascade at a final concentration of 150 μM.
Measurements of endogenous riboflavin, FMN and FAD

The leaves were harvested at fresh weight of 100 mg and were frozen with liquid nitrogen to temporarily stop enzyme activity, and then the endogenous riboflavin, FMN and FAD were extracted at the indicated time points as described by Vorwieger et al. [48] and Asai et al. [49].

Total RNA Extraction and Real-time Quantitative RT-PCR

Total RNA was isolated from seedlings frozen in liquid nitrogen with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Concentration of RNA was determined by measuring OD at 260 nm. First-strand cDNA was synthesized with the SuperScript II First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time quantitative PCR (qRT-PCR) analysis was performed using the LightCycler Quick System 350S (Roche Diagnostics K.K.) with SYBR Premix Ex Taq (Takara Bio, Inc.). Each PCR reaction contained 1 × SYBR Premix Ex Taq, 0.2 μM of each primer, and 2 μL of a 1:10 dilution of the cDNA in a final volume of 20 μL. The PCR programme included: the initial denaturation, at 95°C, for 30 s; PCR, of 40 cycles at 95°C, for 5 s, and then at 60°C, for 20 s with a temperature transition rate of 20°C s⁻¹. In melting curve analysis, PCR reactions were denatured at 95°C, annealed at 65°C, then a monitored release of intercalator from PCR products or primer dimmers by an increase to 95°C with a temperature transition rate of 0.1°C s⁻¹. Standard curves were created using PCR products by 10-fold serial dilutions. The MPK3 and MPK6 genes expression profiles were normalized using actin mRNA as an internal control. The primers for Real-time PCR are listed in S1 Table.

GUS Staining

The transgenic PR1:GUS seedlings were grown on MS medium for about 10 days, and then transferred to liquid MS medium with or without 0.6 mM riboflavin, and 4 hours later were inoculated with Pst DC3000 for the indicated times. Histochemical detection of the GUS enzyme activity was analyzed as described by Gust et al. [50]. After staining, the seedlings were boiled in 95% ethanol for about 10 min to remove chlorophyll.

Western blot and MAPK activity assay

Proteins were extracted from frozen leaf samples at the indicated time points after washed with sterile water as described by Liu and Zhang [34]. Protein extracts were separated on a 10% (w/v) SDS-PAGE minigel and then western blot was performed. Plant MAPKs have high homology to mammalian ERK1/2 MAPKs, and ERK1/2 antisera that recognize the dually phosphorylated (pTEpY) forms of activated MAPKs can be used to monitor plant MAPK activity [51]. Hence, endogenous kinase activity of MPK3 and MPK6 after Pst DC3000 inoculation was determined using phospho-P44/42 MAPK antibody (Cell Signaling Technology). Subsequently, blots were washed and incubated with an anti-rabbit horse-radish peroxidase secondary antibody. Antibodies against MPK3 and MPK6 were purchased from Sigma.

Results

Riboflavin induces resistance against Pst DC3000

To evaluate whether riboflavin-induced plant defense is effective against virulent Pseudomonas syringae pv tomato strain DC3000 (Pst DC3000), 2-week-old Arabidopsis ecotype Col-0 plants were treated with either water or riboflavin, and subsequently challenged with Pst DC3000. Most leaves without riboflavin treatment (Control) exhibited light yellow 2 days after challenge inoculation, and finally wilted and died 5 days after inoculation. In contrast, pre-treated with
0.6 mM riboflavin, plants showed no visible symptoms 2 days after inoculation. Following 3 to 5 days, minute spots were observed on the same leaves (Fig 1a and 1b), but no further symptoms of disease were seen thereafter. In addition, compared with water-treated control plants, treatment with 0.6 mM riboflavin induced a statistically significant reduction in bacterial growth from 3 days after challenge inoculation (Fig 1d).

To determine the effects of riboflavin on the growth of *Pst* DC3000, Arabidopsis plants were treated with riboflavin to concentrations ranging from 0 to 1 mM. Riboflavin did not result in any remarkable alterations in the plants. The bacterial number was reduced significantly with 0.4 mM and further by 0.6 mM, and the effect of further higher concentrations (0.8–1 mM) of riboflavin on the bacterial growth was similar to that of 0.6 mM (Fig 1c), indicating that 0.6 mM is sufficient for subsequent experiments in riboflavin-induced resistance against *Pst* DC3000.

### Levels of endogenous riboflavin, and its derivates, FMN and FAD in response to *Pst* DC3000

To investigate whether the endogenous riboflavin biosynthesis was influenced by *Pst* DC3000 infection, we analyzed the expression of genes responsible for the biosynthesis of the lumizine synthase (LS) and riboflavin synthase (RS), which catalyze the last two step responses in the biosynthesis of riboflavin in organisms [48, 52]. A scheme showing the different steps in riboflavin and its derivatives, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) biosynthesis is given in S2 Fig. Results showed that the expression of genes LS and RS were up-regulated in different degrees after challenged with *Pst* DC3000 (Fig 1e). Correlated with the up-regulation of the genes, the changes in riboflavin and its derivatives, FMN and FAD, were also elevated (Fig 1f), suggesting that plants increased riboflavin biosynthesis upon *Pst* DC3000 infection.

### Riboflavin-induced resistance against *Pst* DC3000 is based on MAPK-dependent priming for the expression of *PR1*

The transcriptional expression of PR gene has been regarded as a molecular indicator for the activation of plant defense pathways [53, 54]. In order to investigate the kinetics of riboflavin action, the expression pattern of *PR1* was analyzed. Inoculation of the plants with *Pst* DC3000 induced the expression of *PR1* at 24 h after inoculation. However, riboflavin treatment induced *PR1* expression from 6 h to 48 h after challenge inoculation and the transcript level of *PR1* was peaked at 24 h, which was more rapid and higher than that observed after pathogen inoculation alone (Fig 2a). Alternatively, the transgenic *PR1*:GUS reporter was analyzed for the *PR1* expression. Similar results were detected following the pathogen inoculation (Fig 2b).

To investigate whether MAPK cascades affect the riboflavin-induced priming of defense response in Arabidopsis, PD98059 which is the common inhibitor of MAPK cascade was used. PD98059-pretreatment effectively inhibited the expression of *PR1* at 24 h after challenge inoculation following riboflavin treatment (Fig 2c). In addition, compared to riboflavin-treated plants, pre-treatment with PD98059 caused an increase in bacterial growth 3 d and 5 d after challenge inoculation (Fig 2d). These data indicated that MAPK cascades might be involved in riboflavin-induced resistance and defense gene expression.

### Contribution of MPK3 and MPK6 to riboflavin-induced defense response

As described above, pretreatment with riboflavin protects Arabidopsis plants against *Pst* DC3000 (Fig 1), and this effect appeared to be associated with MAPK cascades. Then the next investigation was focus on the expression and activation of MPK3 and MPK6. Treatment with
Fig 1. Effect of riboflavin on disease development in Arabidopsis infected with *Pst* DC3000. (a) 2-week-old Arabidopsis ecotype Col-0 plants were treated with either water (Control) or riboflavin (0.6 mM) and 4 hours later were inoculated with *Pst* DC3000. Infection was observed 5 days after inoculation. (b) Representative Arabidopsis leaves at 1, 2, 3, 4, and 5 days after infected by *Pst* DC3000. The necrotic lesions on Arabidopsis leaves infected by *Pst* DC3000 are suppressed in riboflavin-pretreated plants. (c)
Numbers of *Pst DC3000* in leaves of Arabidopsis treated with increasing concentrations of riboflavin (0–1 mM) prior to *Pst DC3000* inoculation. Samples were collected 5 days after inoculation. Asterisks indicate significant differences to riboflavin-untreated samples (Student’s t-test, *P* < 0.05).

(d) Inhibitory effect of riboflavin on *Pst DC3000* growth in Arabidopsis. The Arabidopsis leaves were inoculated with *Pst DC3000* 4 hours after riboflavin treatment. Samples were collected during 5 days after inoculation. Asterisks indicate significant differences to control (Student’s t-test, *P* < 0.05).

(e) Changes in biosynthesis genes of riboflavin, including lumazine synthase (LS) and riboflavin synthase (RS) genes, in response to *Pst DC3000* in Arabidopsis.

(f) Quantification of endogenous riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in Arabidopsis inoculation with *Pst DC3000*. Data are means ± SD of three replicates.

---

**Fig 2. Effect of riboflavin on defense priming in Arabidopsis.** (a) Real-time quantitative RT-PCR analyses showing induction of *PR1* gene expression in Arabidopsis plants upon infection with *Pst DC3000* at 0, 6, 12, 24, and 48 hpi. The Arabidopsis ecotype Col-0 plants were sprayed with either water (control) or riboflavin (0.6 mM) in the presence of Silwet L-77 (0.015%) and 4 hours later were challenged with *Pst DC3000* for the different times. Statistical analysis was performed with Student’s t-Test: *, *P* < 0.05 vs control; #, *P* < 0.05 vs *Pst DC3000*. (b) The transgenic *PR1:GUS* seedlings grown on liquid challenged with *Pst DC3000* for different times, and collected the plants for histochemical GUS staining. (c) Effect of MAPK cascade inhibitor (PD98059) on riboflavin-induced *PR1* gene expression in Arabidopsis. Detached leaves were pretreated with or without PD98059 (150 μM), and then treated with 0.6 mM riboflavin for 4 hours and challenged with *Pst DC3000* for 24 hours. Asterisks indicate significant differences between Riboflavin and Riboflavin + PD98059 (Student’s t-test, *P* < 0.05). (d) *Pst DC3000* growth analysis in PD98059-pretreated detached leaves of wild-type plants after riboflavin. Detached leaves were pretreated with or without PD98059, and then treated with 0.6 mM riboflavin for 4 hours and challenged with *Pst DC3000* for 3 days or 5 days. Asterisks indicate significant differences between Riboflavin and Riboflavin + PD98059 (Student’s t-test, *P* < 0.05). Below are representatives of leaves of the indicated genotypes. Data are means ± SD of three replicates.

---

doi:10.1371/journal.pone.0153175.g001
doi:10.1371/journal.pone.0153175.g002
riboflavin induced biphasic accumulation of MPK3 and MPK6 transcripts (Fig 3a and 3b). The expression of both genes was transiently up-regulated during the first 6 h, and peaked 36 h after treatment, and riboflavin-induced transcript level of MPK6 was 1.5 times higher than that of MPK3.

To further investigate the role of MPK3 and MPK6 in riboflavin-induced resistance to Pst DC3000, riboflavin-pretreated plants with induced accumulation of transcripts for MPK3 and MPK6 were challenged with Pst DC3000. Using immunoblot analysis with an anti-phosphoERK 1/2 (anti-pTEpY) antibody, the activation of MPK3 and MPK6 was determined. As shown in Fig 4a and S3 Fig, inoculation with Pst DC3000 led to an activation of MPK3 and MPK6 within 120 min in riboflavin-treated plants (+Riboflavin +Pst DC3000), and it was more intense than that of in riboflavin-untreated plants (-Riboflavin +Pst DC3000). However, the activation of MPK3 and MPK6 were not evident in plants treated with only riboflavin (+Riboflavin +MgCl2) and in riboflavin-untreated plants without Pst DC3000 inoculation (-Riboflavin +MgCl2). No significant increase in kinase activity of MPK3 and MPK6 was observed in Pst DC3000-inoculated leaves of the respective mutants, and immunoblot analysis using anti-MPK3 and anti-MPK6 antibodies confirmed the absence of protein in the respective mutants (Fig 4b; S4 Fig). These results suggested that MPK3 and MPK6 are possible priming components for the riboflavin-induced resistance in Arabidopsis.

Riboflavin-induced resistance against Pst DC3000 is compromised in mpk3 and mpk6 mutants

To confirm whether both MPK3 and MPK6 play a crucial role in priming and resistance induced by riboflavin, the response to Pst DC3000 upon pretreatment with riboflavin was tested in wild-type, mpk3 and mpk6 (mpk6-2 and mpk6-3) plants. Leaves from wild-type or mutant plants were pretreated with water or riboflavin and subsequently inoculated with Pst DC3000. As shown in Fig 5a, treatment with riboflavin induced expression of PRI at 24 h post-inoculation in wild-type. In comparison, the expression level of PRI was reduced in the mpk3 and mpk6 (mpk6-2 and mpk6-3) mutants. As expected, riboflavin-treated wild-type leaves developed significant reduction of bacterial growth with respect to leaves treated with water, whereas the mpk3 and mpk6 (mpk6-2 and mpk6-3) mutants were significantly more susceptible to Pst DC3000 and also failed to develop resistance by riboflavin (Fig 5b and 5c). In sum, these results suggested that MPK3 and MPK6 are required for riboflavin-induced resistance for defense response in Arabidopsis infected with Pst DC3000.

Riboflavin-induced resistance mediating MPK3 and MPK6 through the ROS- and Ca2+-dependent signaling pathways

Previous studies suggested that ROS and Ca2+, as important signal messengers in plants cells, might function in the upstream activation of MAPK cascade under stimuli [12, 39, 51]. Furthermore, among the earliest cellular events in plant-pathogens interactions, H2O2 and ion fluxes across the membrane, such as Ca2+, play important roles [39, 55]. To determine whether ROS and Ca2+ signaling pathways are involved in riboflavin-induced resistance, we tested the effect of riboflavin on the transcript levels of MPK3 and MPK6. As shown in Fig 6a and 6b, upon infection by Pst DC3000, the transcript levels of MPK3 and MPK6 were both up-regulated. However, pretreatment with ROS scavenger AsA and the intracellular Ca2+ scavenger BAPTA-AM effectively arrested riboflavin-induced MPK3 and MPK6 expression, respectively. Furthermore, both MPK3 and MPK6 genes were induced in a time dependent manner in response to 1.5 mM H2O2, and H2O2-induced expression of these two genes was inhibited by AsA. Most importantly, treatment with BAPTA-AM before challenge with H2O2 significantly
reduced MPK3 and MPK6 genes transcripts (Fig 6c). In addition, resistance by riboflavin inhibited disease progression, including the necrotic lesion and bacterial growth, in response to bacterial pathogen Pst DC3000 in Arabidopsis. However, pretreatment with AsA or BAPTA-AM interdicted these effects (Fig 7a and 7b).
In this study, the effectiveness and potential molecular mechanisms of riboflavin-induced resistance against pathogen *Pst* DC3000 have been investigated. The results shown in this work might provide evidence for the cellular signaling cascade which MPK3 and MPK6 were responsible for riboflavin-induced defense response.

Riboflavin endows Arabidopsis with resistance to pathogen *Pst* DC3000 and induces priming for pathogen defense without a direct effect on the causal pathogen [19, 20]. Priming, as one of the various forms of induced resistance in plants and animals, enables cells to show a faster and stronger activation of defense upon a stress stimulus [3, 56–58]. The results presented here showed that riboflavin induced resistance in Arabidopsis to infection by *Pst* DC3000. In addition, riboflavin did not result in any remarkable alterations and phytotoxicity to *Pst* DC3000 at any of the tested concentrations in the Arabidopsis plants (Fig 1). As reported by others, riboflavin affects defense-related gene expression (PR) in tobacco and Arabidopsis [19, 20]. Some chemicals, such as non-protein amino acid BABA [11] and a B-complex vitamin thiamine [13], which both activate resistance, also induce the transient expression of defense-related genes, although their acting sites are different. In our work, it was found that inoculation of *Pst}*
Fig 5. Attenuation of riboflavin-induced resistance in response to *Pst* DC3000 in the *mpk3* and *mpk6* mutant. (a) Expression of *PR1* gene is compromised in the *mpk3* and *mpk6* mutant seedlings. 2-week-old WT and single mutant (*mpk3*, *mpk6-2* and *mpk6-3*) seedlings were treated with water or riboflavin, and then inoculated with *Pst* DC3000 for 24 hours. The total RNAs were extracted at indicated times and analyzed for the expression of *PR1* gene. *Actin2* was used as an internal control. Control represents WT and single mutant seedlings were not treated with riboflavin and *Pst* DC3000. Different letters indicate statistically
DC3000 without riboflavin pretreatment induced transient PR1 gene expression within 24 to 48 hours. However, following infection by the pathogen Pst DC3000, PR1 gene was rapidly and strongly expressed in riboflavin-pretreated plants (Fig 2). Just as a phenomenon in mammalian monocytes [59], riboflavin triggers the priming defense in Arabidopsis plants and alters the plant into a highly competent state in the absence of detectable variations [3]. Thus, this priming of riboflavin-induced defense-related gene may allow the plant to react more effectively to a subsequent invader, such as Pst DC3000.

As priming for enhanced PR1 expression in riboflavin-induced defense, riboflavin is capable of triggering a resistance signaling process [19, 20]. However, the upstream signaling cascades of riboflavin-induced defense-related gene expression remain unclear. MAPKs cascade can be activated by extra- and intracellular stimuli and play essential roles in the process whereby extracellular stimuli are transmited into intracellular responses while at the same time amplified the signal [21, 23–25, 28, 60]. What is more, MAPKs cascade also function as negative regulators in stress responses. For example, a mutation in MKK9 exhibits enhanced stress tolerance [61, 62]. Among these MAPK proteins, MPK3 and MPK6 are activated by various stimuli, including pathogen, UV-B stress, and plant hormones or their functional analogs [32, 63, 64]. In this work, we demonstrated that MAPK cascades participated in the riboflavin-induced resistance against Pst DC3000 challenge (Fig 2c and 2d). In riboflavin-pretreated Arabidopsis, the activations of MPK3 and MPK6 were proved to be responsible for the up-regulation of the defense-related gene (PR1) and the subsequent enhanced resistance (Figs 4 and 5). In the mpk3 and mpk6 mutants (mpk3, mpk6-2 and mpk6-3), which lack detectable transcripts of MPK3 and MPK6, respectively (S1 Fig.), riboflavin-induced priming defense was markedly reduced at the indicated time (Fig 5a and 5b). However, it is not clear whether these activated MPKs have redundant or separate functions in response to riboflavin. Unfortunately, simultaneous knockout of MPK3 and MPK6 is embryolethal [65], such mpk3mpk6 double mutation is not available to more strictly address the roles of MPK3 and MPK6 in stress-induced priming. In addition, although MPK3 and MPK6 seem to have redundant functions, distinct roles are suggested in recent evidences [33, 37].

ROS and Ca²⁺, as the key signaling molecules in plant cells, function upstream of activation of MAPKs [39, 66]. Zhang et al. [19] reported that potentiated ROS production is required for resistance by riboflavin in response to Pst DC3000 challenge. In contrast, riboflavin alone or Pst DC3000 inoculation does not induce ROS production at the same time, which is similar to the phenomena in thiamine–induced priming defense [13]. What is more, among the earliest cellular events in plant–pathogens interactions, H₂O₂ and ion fluxes across the membrane, such as Ca²⁺, also play important roles [39, 55]. Accordingly, ROS accumulation and Ca²⁺ should be one of the defense mechanisms of priming. Our work presented a cellular signal cascade, composed of endogenous ROS production, [Ca²⁺]cyt rise, and MPK3 and MPK6 induction in response to pathogen (S6, S7 and S8 Figs; Fig 6). After challenge inoculation of the riboflavin-pretreated plants with Pst DC3000, the riboflavin-induced accumulation of MPK3 and MPK6 transcripts and proteins was prevented by AsA and BAPTA, an H₂O₂ scavenger and [Ca²⁺]cyt scavenger, respectively (Fig 6; S5 Fig), this was accompanied by abolition of disease progression, including the necrotic lesion and bacterial growth (Fig 7). Furthermore, exogenous H₂O₂-induced expression of MPK3 and MPK6 transcripts was markedly reduced in the present of BAPTA (Fig 6).
Fig 6. Roles of H$_2$O$_2$ and Ca$^{2+}$ in riboflavin-induced expression of MPK3 and MPK6 genes in Arabidopsis seedlings. (a) and (b) Real-time quantitative RT-PCR analyses showing the expression of MPK3 and MPK6 genes measured in WT plants with AsA (1.5 mM) or BAPTA (1 mM) pretreatment in response to Pst DC3000 challenge in 0.6 mM riboflavin-treated Arabidopsis seedlings or not. Actin2 was used as an internal control. Asterisks indicate significant differences to control (student's t-test: *p < 0.05,
Hence, results here suggest that ROS and Ca2+, which functioned in the upstream activation of MPK3 and MPK6, are required for resistance by riboflavin.

Recently, NPR1, as a regulator protein, is required in development of induced resistance induced by pathogen infection [67]. Dong and Beer [20] and Zhang et al. [19] reported that riboflavin functions as a plant defense activator, and induces disease resistance which requires a functional NPR1 in response to virulent 
Pst DC3000, which is just like the effect of thiamine [12,13]. In this work, the npr1 mutant showed more developed chlorotic lesions compared with WT, and riboflavin-pretreated npr1 mutant exhibited no significant improvement on disease progression.

**p < 0.01), and #, P < 0.05 vs Riboflavin. (c) Time course analysis of MPK3 and MPK6 genes expression in response to exogenous H2O2 treatment. Seedlings were pretreated with AsA (1.5 mM) or BAPTA (1 mM), and then incubated with H2O2. Samples were collected at the indicated time points. Asterisks indicate significant differences to control (student’s t-test: *p < 0.05, **p < 0.01), and #, P < 0.05 vs H2O2-treated samples at 6 h.

doi:10.1371/journal.pone.0153175.g006

Fig 7. Effects of AsA and BAPTA on disease development in Arabidopsis treated with riboflavin and challenged with Pst DC3000. (a) The necrotic lesions on representative Arabidopsis leaves at 5 days after infected by Pst DC3000 in AsA- or BAPTA-pretreated plants. Detached leaves were pretreated with AsA (1.5 mM) or BAPTA (1 mM), and then treated with 0.6 mM riboflavin for 4 hours and challenged with Pst DC3000 for 5 days. (b) Pst DC3000 growth analysis in AsA- or BAPTA-pretreated detached leaves of wild-type after riboflavin. FW, fresh weight. Letters indicate statistically significant differences among the samples (P <0.05; Duncan’s multiple range tests). Data are means ± SD of three replicates.

doi:10.1371/journal.pone.0153175.g007
including bacterial growth (S9 Fig). NPR1 moves into the nucleus, where it activates the expression of PRI gene [68] via TGA transcription factors. Riboflavin treatment induced PRI expression after challenge inoculation in WT plants. However, riboflavin pre-treatment did not promote increased PRI transcript in npr1 mutant (S10 Fig). Further, the link of MAPKs and NPR1 after Pst DC3000 inoculation was investigated. Inoculation with Pst DC3000 promoted expression of NPR1 protein in WT plants, whereas the expression of NPR1 was nearly arrested in the mpk3 and mpk6 mutants (S11 Fig). These data indicate that riboflavin induces defense priming through an NPR1-dependent signaling pathway in response to Pst DC3000 and riboflavin-induced this MAPKs signal module may operate upstream of the NPR1 regulator. However, the concrete interaction between MAPKs and NPR1 is still needed to be study in future work.

Taken together, our results demonstrated the contribution of MPK3 and MPK6 to riboflavin-induced resistance to pathogen Pst DC3000 and riboflavin exerted its effect via ROS- and Ca^{2+}-dependent signaling pathways. The results further demonstrated that priming defense and its associated molecular defense mechanisms were induced by riboflavin. According to our experimental results, a potential cascade of cellular events occurred during riboflavin-induced priming defense (Fig 8). Riboflavin increased levels of MPK3 and MPK6 transcript and transient expression of PRI gene. Upon infection by Pst DC3000, the molecular defense-related

![Fig 8. Proposed model for the contribution of MPK3 and MPK6 to riboflavin-induced disease resistance to bacterial pathogen Pst DC3000.](https://doi.org/10.1371/journal.pone.0153175.g008)
responses, including the expression of \textit{PRI} transcript and the activities of MPK3 and MPK6, were strongly enhanced in riboflavin-applied Arabidopsis. These findings add to our understanding of the signaling pathways in disease resistance mediated by riboflavin. In conclusion, defense responses induced by riboflavin could be one of the most economical and effective resistances, just like the resistance induced by thiamine and BABA [9, 12], providing a novel disease control strategy and satisfied environmental regulations.

\textbf{Supporting Information}

S1 Fig. Characterization of \textit{mpk3} and \textit{mpk6} mutants using semi-quantitative RT-PCR. (DOCX)

S2 Fig. Biosynthesis pathway of riboflavin, FMN, and FAD in plants. (DOCX)

S3 Fig. Quantitative analysis of activation (p-MPK6 and p-MPK3) and protein (MPK6) of MPK3/6 proportion shown in Fig 4a. (DOCX)

S4 Fig. Quantitative analysis of activation (p-MPK6 and p-MPK3) and protein (MPK6 and MPK3) of MPK3/6 proportion shown in Fig 4b. (DOCX)

S5 Fig. Roles of ROS/Ca$^{2+}$ in riboflavin-induced activation of MAPKs. (DOCX)

S6 Fig. Effect of priming by riboflavin on O2- and H$_2$O$_2$ generation in Arabidopsis upon \textit{Pst DC3000} inoculation. (DOCX)

S7 Fig. Effect of AsA on H$_2$O$_2$ level in riboflavin-pretreated Arabidopsis upon \textit{Pst DC3000} inoculation. (DOCX)

S8 Fig. Effect of defence priming by riboflavin on Ca$^{2+}$ level in Arabidopsis upon \textit{Pst DC3000} inoculation. (DOCX)

S9 Fig. Effect of riboflavin on disease progression in \textit{npr1} mutant. (DOCX)

S10 Fig. Effect of riboflavin on \textit{PRI} gene expression in \textit{npr1} mutant. (DOCX)

S11 Fig. Effect of riboflavin on the expression of NPR1 protein in WT, \textit{mpk3}, and \textit{mpk6} mutant in response to \textit{Pst DC3000}. (DOCX)

S1 Table. Primers for several genes. (DOCX)

\textbf{Acknowledgments}

We greatly appreciate the criticism and suggestions provided by the colleagues and students in the Xu laboratory during the development of this paper.
Author Contributions

Conceived and designed the experiments: SN HX. Performed the experiments: SN. Analyzed the data: SN HX. Contributed reagents/materials/analysis tools: SN HX. Wrote the paper: SN HX.

References

1. Conrath U, Thulke O, Katz V, Schwindling S, Kohler A. Priming as a mechanism in induced systemic resistance of plants. Eur J Plant Pathol. 2001; 107: 113–119.
2. Conrath U, Pieterse CM, Mauch-Mani B. Priming in plant-pathogen interactions. Trends Plant Sci. 2002; 7: 210–216. PMID: 1192826
3. Conrath U, Beekers GJ, Flors V, Garcia AP, Jakab G, Mauch F, et al. Priming: getting ready for battle. Mol Plant Microbe Interact. 2006; 19: 1062–1071. PMID: 11891259
4. Conrath U. Molecular aspects of defense priming. Trends Plant Sci. 2011; 16: 524–531. doi:10.1016/j.tplants.2011.06.004 PMID: 21782492
5. Kohler A, Schwindling S, Conrath U. Benzothiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in Arabidopsis. Plant Physiol. 2002; 128: 1046–1056. PMID: 11891259
6. Dong X. SA, JA, ethylene, and disease resistance in plants. Curr Opin Plant Biol. 1998; 1: 316–323. PMID: 10066607
7. Durrant WE, Dong X. Systemic acquired resistance. Annu Rev Phytopathol. 2004; 42: 185–209. PMID: 15283665
8. Jung HW, Tschapinski TJ, Wang L, Glazebrook J, Greenberg JT. Priming in systemic plant immunity. Science 2009; 324: 89–91. doi: 10.1126/science.1170025 PMID: 19342588
9. Zimmerli L, Jakab G, Metraux JP, Mauch-Mani B. Potentiation of pathogen-specific defense mechanisms in Arabidopsis by β-aminobutyric acid. Proc Natl Acad Sci, USA. 2000; 97: 12920–12925. PMID: 11058166
10. Zimmerli L, Metraux JP, Mauch-Mani B. β-aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus Botrytis cinerea. Plant Physiol. 2001; 126: 517–523. PMID: 11402183
11. Tonn J, Mani BM. β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. 2004; 38: 119–130. PMID: 15053765
12. Ahn IP, Kim S, Lee YH. Vitamin B1 functions as an activator of plant disease resistance. Plant Physiol. 2005; 138: 1505–1515. PMID: 15980201
13. Ahn IP, Kim S, Lee YH, Suh SC. Vitamin B1-induced priming is dependent on hydrogen peroxide and the NPR1 gene in Arabidopsis. Plant Physiol. 2007; 143: 838–848. PMID: 17195883
14. Gastaldi G, Laforenza U, Gasirola D, Ferrari G, Tosco M, Rindi G. Energy depletion differently affects membrane transport and intracellular metabolism of riboflavin taken up by isolated rat enterocytes. J Nutr. 1999; 129: 406–409. PMID: 10024619
15. Gregory JF 3rd. Nutritional properties and significance of vitamin glycosides. Annu Rev Nutr. 1998; 18: 277–296. PMID: 9786648
16. Zubay G. Biochemistry. Brown Publishers, Dubuque, IA. 1998.
17. Combs GF. The vitamins: fundamental aspects in nutrition and health. 3rd Ed. BURLINGTON, MA: Elsevier Academic Press. 2008.
18. Fischer M, Bacher A. Biosynthesis of flavocoenzymes. Nat Prod Rep. 2005; 22: 324–350. PMID: 16010344
19. Zhang SJ, Yang X, Sun MW, Sun F, Deng S, Dong HS. Riboflavin-induced priming for pathogen defense in Arabidopsis thaliana. J Integr Plant Biol. 2009; 51(2): 167–174. doi: 10.1111/j.1744-7909.2008.00763.x PMID: 19200155
20. Dong H, Beer SV. Riboflavin induces disease resistance in plants by activating a novel signal transduction pathway. Phytopathol. 2000; 90: 801–811.
21. Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, et al. MAP kinase signaling cascade in Arabidopsis innate immunity. Nature 2002; 415: 977–983. PMID: 11875555
22. Chang LF, Karin M. Mammalian MAP kinase signalling cascades. Nature 2001; 410: 37–40. PMID: 11242034
23. Davis RJ. Signal transduction by the JNK group of MAP kinases. Cell 2000; 103: 239–252. PMID: 11057897
24. Nakagami H, Pitzschke A, Hirt H. Emerging MAP kinase pathways in plant stress signaling. Trends Plant Sci. 2005; 10: 339–346. PMID: 15953753
25. Tena G, Asai T, Chiu WL, Sheen J. Plant mitogen-activated protein kinase signaling cascades. Curr Opin Plant Biol. 2001; 4: 392–400. PMID: 11597496
26. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999; 79: 143–180. PMID: 9922370
27. Colcombet J, Hirt H. Arabidopsis MAPKs: A complex signaling network involved in multiple biological processes. Biochem J. 2008; 413: 217–226. doi: 10.1042/BJ20080625 PMID: 18570633
28. Ichimura K, Shinozaki K, Tena G, Sheen J, Henry Y, Champiom A, et al. Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci. 2002; 7: 301–308. PMID: 12119167
29. Hirt H. Connecting oxidative stress, auxin, and cell cycle regulation through a plant mitogen-activated protein kinase pathway. Proc Natl Acad Sci, USA. 2000; 97: 2405–2407. PMID: 10716978
30. Sessa G, Martin GB. Signal recognition and transduction mediated by the tomato Pto kinase: A paradigm of innate immunity in plants. Microbes Infect. 2000; 2: 1591–1597. PMID: 11113378
31. Xing Y, Jia WS, Zhang JH. AtMKK1 mediates ABA-induced CAT1 expression and H2O2 production via AtMPK6-coupled signaling in Arabidopsis. Plant J. 2008; 54:440–451. doi: 10.1111/j.1365-313X.2008.03433.x PMID: 18249592
32. Galletti R, Ferrari S, De Lorenzo G. Arabidopsis MPK3 and MPK6 play different roles in basal and oligo-galacturonide- or flagellin-induced resistance against Botrytis cinerea. Plant Physiol. 2011; 157: 804–814. doi: 10.1104/pp.111.174003 PMID: 21803860
33. Han L, Li GJ, Yang KY, Mao G, Wang R, Liu Y, et al. Mitogen-activated protein kinase 3 and 6 regulate Botrytis cinerea-induced ethylene production in Arabidopsis. Plant J. 2010; 64: 114–127. doi: 10.1111/j.1365-313X.2010.04318.x PMID: 20659280
34. Liu Y, Zhang S. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell 2004; 16: 3386–3399. PMID: 15539472
35. Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S. Phosphorylation of a WRKY transcription factor by AtMPK6 compromises disease resistance in Arabidopsis. Plant Cell 2004; 16: 897–907. PMID: 15020743
36. Ren D, Liu Y, Yang KY, Han L, Mao G, Glazebrook J, et al. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. Proc Natl Acad Sci, USA. 2008; 105: 5638–5643. doi: 10.1073/pnas.0713011105 PMID: 18378893
37. Rentel MC, Knight MR. Oxidative stress-induced calcium signaling in Arabidopsis. Plant Physiol. 2004; 135: 1471–1479. PMID: 15247375
38. Romeis T, Ludwig AA, Martin R, Jones JD. Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. 2001; 20: 5556–5567. PMID: 11979999
39. Levine A, Pennell R, Palmer R, Lamb CJ. Calcium-mediated apoptosis in a plant hypersensitive response. Curr Biol. 1996; 6: 427–437. PMID: 8723347
40. Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, et al. Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. Nature 2000; 406:731–734. PMID: 10963598
41. Bowler C, Fluur R. The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends Plant Sci. 2000; 5: 241–246. PMID: 10838614
42. Chung E, Park JM, Oh SK, Joung YH, Lee S, Choi D. Molecular and biochemical characterization of the Capsicum annuum calciumdependant protein kinase 3 (CaCDPK3) gene induced by abiotic and biotic stresses. Planta 2004; 220: 286–295. PMID: 15449060
43. De Jong CF, Laxalt AM, Bargmann BO, de Wit PJ, Joosten MH, Munnik T. Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction. Plant J. 2004; 39: 1–12. PMID: 15200638
44. Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J. The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. 2000; 23: 441–450. PMID: 10972870
45. Kachroo A, He Z, Patkar R, Zhu Q, Zhong J, Li D, et al. Induction of H2O2 in transgenic rice leads to cell death and enhanced resistance to both bacterial and fungal pathogens. Transgenic Res. 2003; 12: 577–586. PMID: 14601656
47. Mishina TE, Zeier J. Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. Plant J. 2007; 50: 500–513. PMID:17419843

48. Vorwieger A, Gryczka C, Czihal A, Douchkov D, Tiedemann J, Mock HP, et al. Iron assimilation and transcription factor controlled synthesis of riboflavin in plants. Planta. 2007; 226: 147–158. PMID:17260143

49. Asai S, Mase K, Yoshioka H. A key enzyme for flavin synthesis is required for nitric oxide and reactive oxygen species production in disease resistance. Plant J. 2010; 62: 911–924. doi: 10.1111/j.0960-7412.2010.04206.x PMID: 20230506

50. Gust AA, Biswas R, Lenz HD, Rauhut T, Ranf S, Kemmerling B, et al. Bacteria-derived peptidoglycans constitute pathogen-associated molecular patterns triggering innate immunity in Arabidopsis. J Biol Chem. 2007; 282: 32338–32348. PMID: 17761682

51. Wang PC, Du YY, Li Y, Ren DT, Song CP. Hydrogen peroxide-mediated activation of MAP Kinase 6 modulates nitric oxide biosynthesis and signal transduction in Arabidopsis. Plant Cell 2010; 22: 2981–2998. doi: 10.1105/tpc.109.072959 PMID: 20870959

52. Roje S. Vitamin B biosynthesis in plants. Phytochem. 2007; 68, 1904–1921.

53. Kim S, Ahn IP, Park C, Park SG, Park SY, Jwa NS, et al. Molecular characterization of the cDNA encoding an acidic isoform of PR-1 protein in rice. Mol Cells. 2001; 11: 115–121. PMID: 11266113

54. Van Loon LC. Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol. 1997; 103: 753–765.

55. Blume B, Numberter R, Nass N, Scheel D. Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 2000; 12: 1425–1440. PMID: 10948260

56. Hayes MP, Enterline JC, Gerrard TL, Zoon KC. Regulation of interferon production by human monocytes: requirements for priming for lipopolysaccharide-induced production. J Leukocyte Biol. 1991; 50:176–181. PMID: 1649241

57. Jakab G, Cottier V, Toquín V, Rigoli G, Zimmerli L, Metraux JP, et al. b-Aminobutyric acid-induced resistance in plants. Eur J Plant Pathol. 2001; 107; 293–37.

58. Pham LN, Dionne MS, Shirasu-Hiza M, Schneider DS. A specific primed immune response in Drosophila is dependent on phagocytes. PLoS Pathog. 2007; 3:e26. PMID: 17352533

59. Hayes MP, Zoon KC. Priming of human monocytes for enhanced lipopolysaccharide response: expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. Infect Immun. 1993; 61: 3222–3227. PMID: 8335353

60. Herskowitz I. MAP kinase pathways in yeast: for mating and more. Cell 1995; 80: 187–197. PMID: 7834739

61. Alzwiya IA, Moris PC. A mutant in the Arabidopsis MAP kinase kinase 9 gene results in enhanced seedling stress tolerance. Plant Sci. 2007; 173:302–308.

62. Zhou CJ, Cai ZH, Guo YF, Gan SS. An Arabidopsis mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. Plant Physiol. 2009; 150: 167–177. doi: 10.1104/pp.108.133439 PMID: 19251906

63. Anderson JC, Bartels S, Gonzalez Bestriro MA, Shahollari B, Ulm R, Peck SC. Arabidopsis MAP Kinase phosphatase 1 (AtMKP1) negatively regulates MPK6-mediated PAMP responses and resistance against bacteria. Plant J. 2011; 67: 259–268. doi: 10.1111/j.1365-313X.2011.04588.x PMID: 21447069

64. González Bestriro MA, Bartels S, Albert A, Ulm R. Arabidopsis MAP Kinase phosphatase 1 and its target MAP Kinases 3 and 6 antagonistically determine UV-B stress tolerance, independent of the UVBR photoreceptor pathway. Plant J. 2011; 68:727–737. doi: 10.1111/j.1365-313X.2011.04725.x PMID: 21790814

65. Wang HC, Ngwenyama N, Liu YD, Walker JC, Zhang S. Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell 2007; 19: 63–73. PMID: 17252529

66. Kouvun Y, Chiu WL, Tena G, Sheen J. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci, USA. 2000; 97: 2940–2945. PMID: 10717008

67. Kohler A, Schwindling S, Conrath U. Benzothiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in Arabidopsis. Plant Physiol. 2002; 128: 1046–1056. PMID: 11891259

68. Mou Z, Fan W, Dong X. Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell. 2003; 113: 935–44. PMID: 12837250