RESEARCH ARTICLE

Rutin-Mediated Priming of Plant Resistance to Three Bacterial Pathogens Initiating the Early SA Signal Pathway

Wei Yang1☯, Xiaonan Xu1,3☯, Yang Li1, Yingzi Wang2, Ming Li1, Yong Wang1, Xinhua Ding1*, Zhaohui Chu1*

1 State Key Laboratory of Crop Biology, Shandong Provincial Key Laboratory of Agricultural Microbiology, Shandong Agricultural University, Taian 271018, China, 2 Institute of Plant Protection, Yantai Academy of Agricultural Science, Yantai 265500, Shandong, China, 3 Tianjin Entry-Exit Inspection and Quarantine Bureau, Tianjin 300300, China

☯ These authors contributed equally to this work.
* zchu@sdau.edu.cn (ZC); xhding@sdau.edu.cn (XD)

Abstract

Flavonoids are ubiquitous in the plant kingdom and have many diverse functions, including UV protection, auxin transport inhibition, allelopathy, flower coloring and insect resistance. Here we show that rutin, a proud member of the flavonoid family, could be functional as an activator to improve plant disease resistances. Three plant species pretreated with 2 mM rutin were found to enhance resistance to *Xanthomonas oryzae* pv. *oryzae*, *Ralstonia solanacearum*, and *Pseudomonas syringae* pv. *tomato* strain DC3000 in rice, tobacco and *Arabidopsis thaliana* respectively. While they were normally propagated on the cultural medium supplemented with 2 mM rutin for those pathogenic bacteria. The enhanced resistance was associated with primed expression of several pathogenesis-related genes. We also demonstrated that the rutin-mediated priming resistance was attenuated in *npr1*, *eds1*, *eds5*, *pad4-1*, *ndr1* mutants, and *NahG* transgenic Arabidopsis plant, while not in either *snc1-11*, *ein2-5* or *jar1* mutants. We concluded that the rutin-priming defense signal was modulated by the salicylic acid (SA)-dependent pathway from an early stage upstream of NDR1 and EDS1.

Introduction

Flavonoids belong to an important class of secondary metabolites in plants, which can be divided into several subgroups by the diversity of chemical radical groups [1]. They exhibit broad biological functions including defense (antibacterial activity), UV protection, auxin transport inhibition, allelopathy, energy transfer, control of respiration and photosynthesis and flower coloring in plant [2]. Rutin is one of the huge families of flavonoids which was broadly distributed in fruits, vegetables and other plant food sources [3, 4]. Even in tobacco leaves, the content of rutin is approximately reached to 80 μg g⁻¹ fresh weight [5]. Rutin has anti-inflammatory and strong antioxidant properties too. It was reported to attach to metal ions and
prevent reactions that form free radicals [6], to maintain the level of collagen in the skin which prevents signs of aging [7]. In addition, rutin have good anti-diabetic activity on type II diabetic rats [8], and it is potentially an excellent source of functional antihypertensive product which has an inhibitory effect on angiotensin-converting enzyme activity [9]. However, it was not clear whether it could improve itself immunity system in plant.

Plants can acquire enhanced resistance to pathogens after treatment with quiet a few so-called activators such as salicylic acid (SA) and its synthetic analogs, 2,6-dichloroisonicotinic acid (INA), benzo (1,2,3) thiadiazole-7-carbothioic acid (BTH), chitin, ß-aminobutyric acid (BABA), lipopolysaccharide (LPS), azelaic acid, hexanoic acid, Vitamin B1 (thiamine), Vitamin B2 (riboflavin) and many other natural or synthetic compounds [10–15]. They will result as activating or priming activation of various cellular defense responses in plants, included early reactive oxygen species (ROS) burst, rapidly induced ion transport changes at the plasma -membrane, callose deposition, the synthesis and secretion of phytoalexins, and the accumulation of transcripts for various pathogenesis-related (PR) genes [10, 16]. Most of those activation defense responses were identified to generally dependent on some known resistance signaling pathways, such as systemic acquired resistance (SAR) and induced systemic resistance (ISR) pathways, which use endogenous hormone salicylic acid (SA) and jasmonic acid (JA) as the signal transduction molecules, respectively. However, the activated plant defense by ß-aminobutyric acid (BABA) was reported to mediate another signaling mechanism that differs from SAR and ISR [17].

Recently studies were shown that flavonoids are also involved in plant protection. In vivo a multitude of studies have revealed a structure-related activity with respect to the anti-fungal effect of flavonoids [1]. For instance, Pyricularia oryzae, a fungal pathogen causing blast in rice, has demonstrated a differential sensitivity of growth inhibition against naringenin, kaempferol, quercetin and dihydroquercetin [18]. Including rutin, flavonoids were also found to moderately effective in inhibiting aerobic bacteria causing plant disease [19, 20]. Otherwise, flavonoids were reported to involve in plant immunity as well. A nonsense mutation in the structural gene encoding dihydroflavonol reductase was reported to accumulate the small amounts of dihydroquercetin as well as repress the hyphae penetration for Fusarium on testa layers of barley [21]. Spraying quercetin was also identified to prime defense against virulent strain Pseudomonas syringae pv. tomato DC3000 (Pst) in Arabidopsis thaliana. It was mediated by H2O2 burst and the SA signal pathway [22].

Previously study demonstrated that the overexpression of a member of MYB transcriptional factor, the AtMYB12 gene, resulted in accumulation of flavonoids, especially for the component of rutin in tobacco [5, 23]. The transgenic tobacco was also identified to enhance the resistance to Ralstonia solanacearum SD as well as strongly activated many defense-related genes post pathogen inoculation. These results has been borne in mind that rutin might activated the plant immunity directly. In this study, we demonstrated that rutin was acted as a general elicitor which could enhance resistance against bacteria in different hosts, such as rice against Xanthomonas oryzae pv. oryzae causing rice bacterial blight, Nicotiana benthamiana against Ralstonia solanacearum SD causing tobacco bacterial wilt and Arabidopsis thaliana against Pst DC3000. The expressions of PR genes were investigated. We also manifested that the rutin-priming defense was modulated by the SA-dependent signal pathway, and it was initiated upstream of the NDR1 and EDS

Materials and Methods
Pathogen culture

Bacterial strains Xanthomonas oryzae pv. oryzae PXO99, Xanthomonas oryzae pv.oryzicola RH3 were first grown in potato dextrose agar (PSA) liquid medium at the early logarithmic
phase. Cells were collected by centrifugation and resuspended in distilled water to form a gradient concentration of 10^6, 10^7 and 10^8 CFU ml⁻¹ (equal to approximate 0.2 OD). The bacterial suspensions were grown on PSA solid medium containing 0 to 4 mM rutin which purchased from Sangon Biotech (Shanghai, CN), at 28°C for 24 h.

Pathogenic bacteria *Ralstonia solanacearum* SD (Isolated from Shandong Province, China at the year of 2011) and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) were cultured as described above and the medium were replaced with Nutrient Agar (NA) medium and King’s B (KB) medium, respectively. All the pathogens were only studied in the lab and greenhouse. There has no specific permissions were required for these locations/activities.

**Plant material and pathogen inoculation**

Rice Mudanjiang 8 (*Oryzae sativa* cv. japonica) plants were grown in the greenhouse at 28°C, 70% relative humidity and with a 12 h photoperiod. Each of five plants at booting stage were sprayed with a solution containing different concentrations of rutin diluted in distilled water, which was supplemented with 0.02% Tween 20. The control plants were sprayed with 0.02% Tween 20 only. The plants were inoculated with PXO99 (Philippine race 6) by the leaf clipping method after three days of pre-spraying with rutin, as described previously [24]. The disease was scored by measuring the lesion length at 7 and 14 days after inoculation. The results show average values of triple experiments.

*N. benthamiana* were grown in the greenhouse under a 16 h light/8 h dark cycle at 25°C, with 70% relative humidity. Eight-week-old plants were sprayed with different concentrations of rutin diluted in distilled water containing 0.02% Tween 20. The control plants were sprayed with 0.02% Tween 20. The plants were inoculated with 10^8 CFU ml⁻¹ of *R. solanacearum* SD through hypodermic injection with a syringe after three days of treatment [25]. Different growth stages of *R. solanacearum* SD were detected after inoculation to draw the growth curve. Bacteria in leaves was counted by determining the CFU of 1 g leaves (fresh weight) either pretreated or untreated with rutin on NA medium [20]. At least three plants for each time point were inoculated through leaf injection with the bacterial suspension. The same experiment was repeated in triplicate at the greenhouse.

Seeds of the Columbia (Col-0) ecotype, *npr1-1* (CS3726), *jar1-1* (CS8072), and *pad4-1* (CS3806) were obtained from the Arabidopsis Information Resource (http://www.arabidopsis.org). *NahG*, *snc1-11*, *eds5*, *ein2-5*, *eds1*, *ndr1-1* mutants were kindly provided by other contributors. Seeds were chilled at 4°C for 3 days and sown in 50 cm³ pots containing a mixture of vermiculite and potting soil. Arabidopsis was incubated in a growth chamber with 16 h (200 μmol/m²/s) of illumination daily at 20°C and 70–90% relative humidity for 4 weeks before treatments and/or *Pst* DC3000 inoculation. *Pst* DC3000 was stored at -80°C, and cultured on KB medium incubated with 50 μg/ml rifampicin (Rif) at 28°C for 36 to 48 h, adjusting the concentration to 10^8 CFU ml⁻¹ for spray inoculation. The method of rutin treatment is the same as above. The pathogen inoculation is as described previously [26].

**RNA extraction and qRT-PCR**

Total RNA was isolated from 100 mg plant tissue with TRI reagent according to the manufacturer’s instructions (T9424, Sigma-Aldrich, USA). 0.5 μg RNA was used for first-strand cDNA synthesis using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, CN). Quantitative PCR was performed with SYBR® Premix **Ex Taq**™ (Tli RNaseH Plus) (Takara, Dalian, CN) on the IQ5 Real-Time PCR System (Bio-Rad, USA). The following PCR program from the reference was used [24]: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 30 s. A heat dissociation curve (55–95°C) following the final cycle of the
PCR was checked to test the specificity of the PCR amplification. OsActin of rice, NbEF1α of tobacco and AtActin2 of Arabidopsis were used as internal control to standardize the results. We used NCBI database to get the gene sequences and Primer Premier 5 to design the primers. For each gene, qRT-PCR assays were repeated at least twice with triplicates runs. Relative expression levels were measured using the 2^{-\Delta\Delta Ct} analysis method. The sequence of each primer for all detected genes is listed in Table 1.

### Data treatment
All experiments were performed in three replicates with similar results. Each replicate contained at least three plants. The quantitative data were performed by Student’s t test (two-tail t
test with equal variances; Microsoft Excel) to evaluate the significance in differences between CK and other individuals or treatments. (P < 0.05) are indicated by an asterisk or (P < 0.01) are indicated by two asterisks.

**Results**

Rutin has limited antibacterial action to all tested bacterial pathogens at 2 mM or lower concentration

To evaluate the effects of rutin against bacterial pathogens, four strains represented as three bacteria species were growth on cultural medium plus with different concentration of rutin. Among four strains, PXO99 and RH3 were belong to typical strain of *Xanthomonas oryzae* pv. *oryzae* (Xoo) and *Xanthomonas oryzae* pv. *oryzicola* (Xoc), they were shown no clear growth inhibition on PSA contains either 0.5 mM, 1.0 mM or 2mM rutin as well as *R. Solanacearum* SD and *Pst* DC3000 (Fig 1). The growth inhibition of all four pathogens was only observed with 4 mM rutin supplemented in PSA when their inoculum titrations were lower as 10⁶ CFU ml⁻¹. Compared with rutin, the quercetin demonstrated a better growth inhibition capacity to all four tested pathogens (S1 Fig). These results were indicative of limited antibacterial ability of rutin for tested bacteria.

Rutin promoted resistance against *Ralstonia solanacearum* in *Nicotiana benthamiana*

Previous studies described that AtMYB12-overexpressing tobacco was resistant against *R. solanacearum* SD as well as enriched rutin. To test whether rutin could directly activated the plant resistance, in this study, we investigated the effect of rutin on the defense response against *R. solanacearum* SD in *N. benthamiana*. Most leaves in the control group showed water-soaked symptoms, wilted post three days inoculation, as shown in Fig 2a. However, the wilted symptoms were attenuated in *N. benthamiana* leaves when it was pretreated with rutin from 1 mM to 4 mM. The stronger attenuation disease symptoms were observed to associate with rutin concentration on inoculation plants (Fig 2a). In addition, rutin hardly inhibit bacteria growth at a concentration of 2 mM in the cultural medium (Fig 1). Therefore, this concentration was chosen for subsequent experiments. The bacterial growth curve indicated that pre-sprayed rutin could remarkably protect *N. benthamiana* from *R. solanacearum* SD infection at a concentration of 2 mM (Fig 2b). Compared to pretreatment with 2 mM rutin, more than 4.82 folds of bacteria had been evaluated on control plant at 48 hpi (Fig 2b). Additionally, in spite of less antibacterial ability than quercetin, pretreated with 2 mM rutin presented better resistance to *R. solanacearum* SD (S2a Fig).

**Fig 1.** The influence of different consistence of rutin on bacterial pathogens. *Ralstonia solanacearum* SD was cultured on NA medium; *Xanthomonas oryzae* pv. *oryzae* PXO99 and *Xanthomonas campestris* pv. *oryzicola* RH3 were cultured on PSA medium and *Pst* DC3000 was cultured on KB medium with 50 mg/L rifampicin (Rif). The photographs were taken after 24 h incubation at 28°C.

doi:10.1371/journal.pone.0146910.g001
We also analyzed the transcription level of PR genes: NbPR1a, NbNOA1 (nitric oxide-associated 1) which is related to NO production and to defense responses [27], and NbrbohB (respiratory burst oxidase homologs B) which is related to active oxygen species generation [28]. Without inoculation of R. Solanacearum SD, the transcription level of NbPR1a was slightly up-regulated one day after spraying with rutin and turn to down-regulation at 3 dpi compared with spraying with water in N. benthamiana (Fig 2c). So we selected three days as the interval time between spraying rutin and R. solanacearum SD inoculation to balance the weak activation defense caused by spraying rutin. We observed there was a more rapid and strong increased expression levels of PR genes, including NbPR1a, NbNOA1 and NbrbohB in rutin-pretreated plants than in control plants when R. solanacearum SD was inoculated (Fig 2d). The transcript levels reached their maximum value at 6 hpi for NbNOA1 (7.26-fold higher than the control) and NbrbohB (3.33-fold higher than the control), and 24 hpi for NbPR1a (2.44-fold higher than the control) in rutin-pretreated leaves, respectively. These results suggested that rutin primed the expressing activation of several PR genes in challenged N. benthamiana.

Suppressed the proliferation of Xanthomonas oryzae pv. oryzae by pre-spraying rutin on rice

To test whether rutin could enhance resistance against bacterial pathogen in other host, we have evaluated the efficacy of rutin against PXO99 which caused bacterial blight disease in rice. The plants were inoculated with PXO99 three days later after sprayed with different concentrations of rutin as 1 mM, 2mM and 4 mM respectively. The lesion length of rice leaves was
measured post 14 days inoculation. It was averaged to 14.32 ± 3.75 cm for control plant which pre-spraying with 0.02% Tween 20 only. And the average lesion length was reduced to 9.76 ± 2.65 cm, 7.79 ± 2.19 cm and 6.94±0.57 cm for pretreatment with 1 mM, 2mM and 4 mM of rutin respectively (Fig 3a). Statistical data also suggested that the lesions caused by PXO99 were suppressed in rutin-pretreated Mudanjiang 8 (Fig 3a). As 2 mM rutin inhibited little or nothing to PXO99 in vitro, and it has dramatically reduced the lesion length in rice after pre-spraying 2 mM rutin (Fig 1), therefore, we chose 2 mM rutin for subsequent experiments. Capture our attentions, compared with the control, the lesion length was dramatically reduced for rutin pre-spraying rice leaves since 7 d-post-inoculation (Fig 3b). Interestingly, the reductive lesion length was almost similar with each other between rutin- and quercetin- pretreated leaves at 14 dpi (S2b Fig). To investigate whether the pre-spraying 2 mM rutin affected the proliferation of PXO99 in rice leaves or not, we conducted a growth curve experiments in rice. Compared to spray 0.02% Tween 20 only, the number of colonies from spraying rutin leaves has no clear difference post 2 days inoculation, while it was regarding to 5.01 times and 26.92 times reduction at 4 dpi and 6 dpi, respectively (Fig 3c). These results were suggested that pretreated with rutin could enhance rice to against PXO99.

Because enhanced plant disease resistance is usually related to the expression of PR genes, to elucidate the rutin-mediated resistance, the expression pattern of several PR genes was investigated in rice. The results demonstrated that all six PR genes included PR-1a, PR-1b, PR-10, CAT, POX, PAL, LOX genes were up-regulated expression after inoculation with PXO99 both for pre-spraying with 0.02% Tween 20 and 2 mM rutin. But the expression of chloramphenicol acetyl transferase (CAT) was not significant changed post rutin treatment compared to the control. (Fig 3d). The expression levels of PR-
1a, PR-1b PR-10 and POX genes were all reached their maximum levels at the 12 h post inoculation, and were approximately 4.98-, 5.05-, 3.39- fold and 4.23-fold higher than the control, respectively. The maximum transcription level of the PAL gene was obtained at 24 h post inoculation. The transcription of LOX was also induced more highly in treated plants compared to the control plants after inoculation. It was reached high values at the 12 h and 48 h time points after the initiation of inoculation which was approximately 5.71- and 8.23-folds higher than the control plants, respectively.

Enhanced the resistance against to Pst DC3000 in Arabidopsis thaliana

In addition to the above mentioned rice-Xoo and N. benthamiana-R. solanacearum investigation, we also tested the function of rutin in Arabidopsis thaliana. The results demonstrated that rutin also protected susceptible Arabidopsis ecotype Columbia-0 (Col-0) against the virulent Pseudomonas syringae pv. tomato strain DC3000 (Pst DC3000). After inoculation with Pst DC3000, typical wilting and chlorotic symptoms was observed on the leaves without pre-spraying with rutin at 3 dpi. However, attenuated disease symptoms were observed on Arabidopsis leaves pre-sprayed with 1, 2 and 4 mM rutin (Fig 4a). The proliferation of Pst DC3000 was indicated that the growth had been inhibited in leaves which were pretreated with 2 mM rutin (Fig 4b). More than 111.78 folds of Pst DC3000 had been identified in control leaves.

To understand the mechanisms involved in rutin-mediated resistance in Arabidopsis, we analyzed the expression patterns of four PR genes including AtPR1, AtPR2, AtPR5, and AtPAL, which are involved in defense responses to pathogen attack (Fig 4c). Similar with our observation in N. benthamiana-R. solanacearum and rice-Xoo interactions, four PR genes was shown more rapidly and stronger expressing activation in rutin-pretreated plants than in control plants after inoculation with Pst DC3000 (Fig 4c). These results suggested that rutin had the function of primed resistance in a broadly range of host, including Arabidopsis.

Rutin-mediated priming is dependent on the SA signal pathway in Arabidopsis

Plant hormones were known as the signals of plant defense. To explore the resistance signal transduction pathway mediated by rutin, a set of Arabidopsis mutants were used for investigation which was involved in SA, JA and ethylene (ET) dependent pathway. The NahG transgenic plant abolishes the accumulation of SA, and the Arabidopsis mutant npr1 was a typical mutant of SA-dependent pathway. jar1-1 and ein2-5 were typical mutants of JA- and ET-dependent pathway. If the rutin-mediated priming defense is dependent on one of them, the inhibition growth of Pst DC3000 by pretreatment with 2 mM rutin will be attenuated. The results demonstrated that it was still able to inhibit the growth of Pst DC3000 in jar1-1 and in ein2-5, while not in npr1-1 and NahG plants (Fig 5). It was indicated that the rutin-mediated plant resistance is dependent on the SA signal pathway in Arabidopsis and independent on the JA and ET pathways.

Rutin-mediated signaling initiated from upstream of NDR1, PAD4 and EDS1

To obtain more details about the signals of rutin-mediated resistance, we had investigated the growth of Pst DC3000 in several other mutants involved in SA signaling pathway, including with snc1-11, pad4-1, ndr1, eds5 and eds1 [29]. SNC1 is encoded an interleukin-1 receptor-like nucleotide-binding site leucine-rich repeat type of resistance (R)-like gene residing in the RPP5 gene cluster which possibly mediates race-specific disease resistance [30–32]. EDS1 and PAD4
belong to two lipase-like proteins [33], NDR1 is a putative membrane-binding protein [34] and EDS5 is an MATE-like SA transporter which pumped the SA from the chloroplast to the cytoplasm [35]. These proteins belong to three upstream components responsible for the transduction of SA signals and for downstream pathways triggered by the R protein. Except in snc1-11, the inhibition growth of *Pst* DC3000 by pretreatment with 2 mM rutin had been attenuated in *pad4-1*, *ndr1*, *eds 5* and *eds1* (Fig 6). These results were further suggested that the rutin-mediated resistance was dependent on the SA signal pathway, which was initiated upstream of NDR1, PAD4 and EDS1.

![Diagram](image)

**Fig 4. Primed the resistance against *Pst* DC3000 in *Arabidopsis thaliana* (Col-0).**

- **a** Typical necrotic lesions normally caused on *Arabidopsis* ecotype Col-0 by the *Arabidopsis* pathogen *Pst* DC3000 were suppressed in rutin-treated plants. The photographs were taken 3 days post inoculation.
- **b** The growth of *Pst* DC3000 in control and in rutin-pretreated *Arabidopsis thaliana* (Col-0) plants. Samples were collected from 10 plants at 0, 1, 2, 3, 4 and 5 days post inoculation. The values are means ±SE.
- **c** Relative expression levels of resistance-related genes (*PR1*, *PR2*, *PR5* and *PAL*) in control and in rutin-pretreated *Arabidopsis thaliana* (Col-0) plants. Samples were collected from 10 plants at 0, 3, 6, 12 and 24 hours post inoculation. The values are means ±SE.

doi:10.1371/journal.pone.0146910.g004
Rutin, classified as a polyphenolic substance, had also shown to exhibit bactericidal and fungicidal activity in vitro assay. The antibacterial activity of rutin was reported to specific bacteria species, such as Xanthomonas campestris, Agrobacterium tumefaciens, Xylella fastidiosa etc [19, 20]. The possible mechanism of action is presumably as follows: first, such polyphenolic substances most likely disrupt the cell wall and the cell membrane integrity of microbial cells, which leads to the release of intracellular components and causes the electron transfer at the membrane, the repression of nucleotide synthesis and ATP activity, thereby inhibiting the growth of microorganisms [36]; Second, rutin excessively scavenges the reactive oxygen species of microbes, leading to a reduction in the normal physiological function of reactive oxygen [37]. But rutin were effective in inhabiting bacteria causing the plant disease at relative high minimum inhibitory activity (MIC) which means to weaker bactericidal activity than other phenolic compounds [19, 20]. In this study, we have measured the inhibition efficiency of rutin against four plant bacterial pathogens, including R. solanacearum SD, Xanthomonas oryzae pv. oryzae (PXO99), Xanthomonas oryzae pv. oryzicola (RH3) and Pst DC3000. The results demonstrated that rutin was only functional in very high concentration over 4 mM (Fig 1). Our other study demonstrated that the AtMYB12-overexpressing tobacco had approximately enriched the averaged concentration of rutin as 1.43 mM in fresh weight. It was also enhanced resistance against R. solanacearum (Li et al., unpublished data). Together, the conclusions of this work were consistent with previous studies that rutin has demonstrated weak antibacterial activity to against three additional species of plant gram-negative bacterial pathogens.

In vitro assays, 2 mM rutin hardly inhibit the growth of R. solanacearum SD, PXO99 and Pst DC3000 in medium. Causing we hardly quantify the concentration of rutin for intercellular space, we couldn’t completely eliminate the direct inhibition caused by the antibacterial agent. However, spraying 2 mM of rutin dramatically reduced the growth of those bacteria in each
host plant, which implied that other resistance mechanisms had been triggered (Figs 2 and 3). Notably, the foliar application of 2 mM rutin almost rarely affected the expression of the SA-responsive PR1a gene on N. benthamiana (Fig 2c). This was indicated that rutin couldn’t directly activates the basal plant defense. Interestingly, when challenged with a pathogen, the plants pre-spraying rutin show a faster and stronger expression of PR1a than control as well as other PR genes (Figs 2d, 3d and 4c). The delay of occurrence resistance was indicated that rutin promotes disease resistance by a priming mechanism. In addition, exogenous application of rutin simultaneously enhanced the expression of genes which involved into SA, reactive oxygen species and nitric oxide signal pathway (Figs 2 and 3), indicating the ownstream signaling activated by rutin was complex.

Many chemicals or plant metabolic components have also been reported to induce or prime plant defense responses that are dependent on the SA signal transduction pathway. However,
most of these studies primarily focused on the characterization of the effects of these components using NahG and npr1 mutants [12,13,15], except for azelaic acid which has been analyzed to induce plant defense responses dependent on NDR1 and PAD4, which are two importance components involved in the upstream signals of SA [14]. Rutin-stimulated plant resistance was compromised in many defective SA pathway mutants (NahG, npr1, eds5), Arrows point to event fluids.

Fig 7. Working model for rutin-primed signal transduction pathway. The components that are relevant to this study are shown. The resistance signal activated by rutin is located upstream of NDR1, PAD4 and EDS1, and probably specifically affect R proteins or some downstream components of the SNC1 and other R protein. Rutin-primed plant resistance was compromised in the defective SA pathway mutants (NahG, npr1, eds5), Arrows point to event fluids.

doi:10.1371/journal.pone.0146910.g007
including the CC-NBS-LRR type and the TIR-NBS-LRR type, and they represent an important node acting upstream of SA in PTI [38, 39]. Interestingly, we have determined that snc1-11 mutant did not affect the rutin-primed resistance, given the possibility that rutin may specifically affect the other R proteins or downstream components of the SNC1 and other R protein. Based on these results, the possible work model for rutin-primed defense is described (Fig 7), which temporally suggests that the resistance signal is initiated upstream from NDR1, PAD4 and EDS1, followed by activating SA signal transduction. Even though we did not decipher the beginning signals or the targeted receptor of rutin in plants, nevertheless, this study it still offers a new research insight into this newly characterized plant activator.

Flavonoids play a critical role in preventing human diseases and have been evolved as a protective mechanism for different plants. In this study, we also found that rutin as a component of flavonoids which could involve into plant immunity with a broad range of host. Together with the quercetin [22], it is feasible suggestion of a conserve mechanism for priming the plant immunity with other components of flavonoids. As rutin was functional at a relative high concentration and economical cost, it is formidable to use directly as a purify bactericide. However, there has increasingly growing of reports that the high content of rutin could be regulated synthesis and accumulated in plant by several transcriptional factors, including with AtMYB11, AtMYB12 and AtMYB111 [23, 40–42]. It was provided opportunity to promote the use of rutin by reducing the economic cost in future. Additionally, AtMYB12-expression tobacco was also reported to be resistant to insects, such as aphid, whitely. Spodoptera litura and Helicoverpa armigera, by the high-level accumulation of rutin [23, 40]. And our previous study showed that the flavonol enriched AtMYB12-expression tobacco enhanced the resistance against pathogens, such as R. solanacearum, Colletotrichum nicotianae Avena and Alternaria alternata. The priming resistance identified with rutin would be helpful to understand the resistance generated by AtMYB12-expression tobacco. It was opens the opportunity to make the daily nutrient and biosafety bactericide with overcapacity simultaneously by transgenic method.

Supporting Information

S1 Fig. The influence of different concentration of quercetin on bacterial pathogens. The growth condition of various bacterial on the plate with different concentration of quercetin. The photographs were taken after 24 h incubation at 28°C. (TIF)

S2 Fig. The comparison of anti-bacterial activity of rutin and quercetin. a The growth curve of R. solanacearum SD in N. benthamiana after 2mM rutin and 1mM quercetin pretreatment. The data represent the mean ±SE of 5 plants. b The lesion length causing by Xanthomonas oryzae strain PXO99 after 2mM rutin and 1mM quercetin pretreatment. The data were collected at 7 and 14 days post inoculation. The data represent the mean ±SE of 5 plants. (TIF)

Acknowledgments

We thank Dr Yujun Wang for kindly providing R. solanacearum SD strains as well as Dr. Yongqing Li, Dr. Jun Fan, Dr. Brad Day and Dr. Shengyang He for providing several Arabidopsis mutants.

Author Contributions

Conceived and designed the experiments: ZC XD. Performed the experiments: WY XX YL Yingzi Wang ML. Analyzed the data: WY Yong Wang XD. Wrote the paper: WY ZC.
References

1. Treutter D. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant Biol. 2005; 7: 581–591 PMID: 16388461

2. Cushnie TP, Lamb AJ. Antimicrobial activity of flavonoids. Int J Antimicrob Agents. 2005; 26: 343–356 PMID: 16323269

3. Middleton E, Kandaswami C, Theoharis TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev. 2000; 52:673–751 PMID: 11121513

4. Tapas AR, Sakarkar DM, Kakde RB. Flavonoids as nutraceuticals: a review. Trop J Pharm Res. 2008; 7:1089–1099

5. Misra P, Pandey A, Tiwari M, Chandrashekar K, Sridhru OP, Asif MH, et al. Modulation of transcriptome and metabolism of tobacco by Arabidopsis transcription factor, AtMYB12, leads to insect resistance. Plant Physiol. 2010; 152:2258–2268 doi: 10.1104/pp.110.150979 PMID: 20190095

6. Kuntic V, Filipovic I, Vujicic Z. Effects of rutin and hesperidin and their Al(III) and Cu(II) complexes on in vitro plasma coagulation assays. Molecules. 2011; 16:1378–1388 doi: 10.3390/molecules16021378 PMID: 21301410

7. Erel SB, Karaalp C, Bedir E, Kaehlig H, Gisl S, Khan S, et al. Secondary metabolites of Centaurea calolepis and evaluation of cnicin for anti-inflammatory, antioxidant, and cytotoxic activities. Pharm Biol. 2011; 49:840–849 doi: 10.3109/13880209.2010.551538 PMID: 21612369

8. Hunyadi A, Martins A, Hsieh T-J, Seres A, Zupko I. Chlorogenic acid and rutin play a major role in the in vivo anti-diabetic activity of Morus alba leaf extract on type II diabetic rats. PLoS One. 2012; 7(11): e50619. doi: 10.1371/journal.pone.0050619 PMID: 23185641

9. Guerrero L, Castillo J, Quinones M, Garcia-Valverde S, Arola L, Pujadas G, et al. Inhibition of angiotensin-converting enzyme activity by flavonoids: structure-activity relationship studies. PLoS One. 2012; 7(11): e49493. doi: 10.1371/journal.pone.0049493 PMID: 23185345

10. Conrath U, Pieterse CMJ, Mauch-Mani B. Priming in plant-pathogen interactions. Trends Plant Sci. 2002; 7:210–216 PMID: 11992626

11. Conrath U, Beekers GJM, Flors V. Priming: getting ready for battle. Mol Plant Microbe Interact. 2006; 19: 1062–1071 PMID: 17022170

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. Zhang S, Yang X, Sun M, Sun F, Deng S, Dong H. Riboflavin-induced priming for pathogen defense in Arabidopsis thaliana. J Integr Plant Biol. 2008; 51:167–174

14. Jung HW, Tschapilinski TJ, Wang L, Glazebrook J, Greenberg JT. Priming in systemic plant immunity. Science. 2009; 324: 89–91 doi: 10.1126/science.1170025 PMID: 19342588

15. Scalschi L, Vicedo B, Camañes G, Fernandez-Crespo E, Lapeña L, González-Bosch C, et al. Hexanoic acid is a resistance inducer that protects tomato plants against Pseudomonas syringae by priming the jasmonic acid and salicylic acid pathways. Mol Plant Pathol. 2013; 14:342–355 doi: 10.1111/mpp.12010 PMID: 23279078

16. Goellner Katharina, Conrath Uwe. Priming: it’s all the world to induced disease resistance. Eur J Plant Pathol. 2008; 121:233–242

17. Ton J, Mauch-Mani B. β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. Plant J. 2004; 38:119–12010 PMID: 15053765

18. Padmavati M, Sakhthivel N, Thara KV, Reddy AR. Differential sensitivity of rice pathogens to growth inhibition by flavonoids. Phytochemistry. 1997; 46:499–502

19. Taguri T, Tanaka T, Kouno I. Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biol Pharm Bull. 2006; 29:2226–2235 PMID: 17077519

20. Maddox CE, Laur LM, Tian L. Antibacterial activity of phenolic compounds against the phytopathogen Xylella fastidiosa. Curr Microbiol. 2010; 60: 53–58 doi: 10.1007/s00284-009-9501-0 PMID: 19813054

21. Skadhauge B, Thomsen K, von Wettstein D. The role of barley testa layer and its flavonoid content in resistance to Fusarium infections. Hereditas. 1997; 126:147–160

22. Jia ZH, Zou B, Wang XM, Qiu J, Ma H, Gou Z, et al. Quercetin-induced H2O2 mediates the pathogen resistance against Pseudomonas syringae pv. tomato DC3000 in Arabidopsis thaliana. Biochem Biophys Res Commun. 2010; 396:522–527 doi: 10.1016/j.bbrc.2010.04.131 PMID: 20434432

23. Luo J, Butelli E, Hill L, Parr A, Niggegeweg R, Bailey P, et al. AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol. Plant J. 2008; 56, 316–326 doi: 10.1111/j.1365-313X.2008.03597.x PMID: 18643978
24. Li N, Kong L, Zhou W, Zhang X, Wei S, Ding X, et al. Overexpression of Os2H16 enhances resistance to phytopathogens and tolerance to drought stress in rice. Plant Cell Tiss Org Cult. 2013; 115: 429–441
25. Kay E, Vogel TM, Bertolla F, Nalin R, Simonet P. In situ transfer of antibiotic resistance genes from transgenic (transplastomic) tobacco plants to bacteria. Appl Environ Microb. 2002; 68:3345–3351
26. Katagiri F, Thilmory R, He SY. The Arabidopsis thaliana-Pseudomonas syringae interaction. The Arabidopsis Book. 2002; 1:e0039 doi: 10.1199/tab.0039 PMID: 22303207
27. Kato H, Asai S, Yamamoto-Katou A, Yoshioka H, Doke N, Kawakita K. Involvement of NbNOA1 in NO production and defense responses in INF1-treated Nicotiana benthamiana. J Gen Plant Pathol. 2008; 74:15–23
28. Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, et al. Nicotiana benthamiana gp91phox homologs NbrbohA and NbrbohB participate in H2O2 accumulation and resistance to Phytophthora infestans. Plant Cell. 2003; 15:706–718 PMID: 12615943
29. Li YQ, Yang SH, Yang HJ, Hua J. The TIR-NB-LRR gene SNC1 is regulated at the transcript level by multiple factors. Mol Plant Microbe Interact. 2007; 20:1449–1456 PMID: 17977156
30. Noël L, Moores TL, van der Biezen EA, Parniske M, Daniels MJ, Parker JE, et al. Pronounced intraspecific haplotype divergence at the RPP5 complex disease resistance locus of Arabidopsis. Plant Cell. 1999; 11:2099–2111 PMID: 10559437
31. Li Y, Chen M, Wang S, Ning J, Ding X, Chu Z. AtMYB11 regulates caffeoylquinic acid and flavonol synthesis in tomato and tobacco. Plant Cell Tiss Organ Cult. 2015; 122:309–319