Silencing COI1 in Rice Increases Susceptibility to Chewing Insects and Impairs Inducible Defense

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Abstract

The jasmonic acid (JA) pathway plays a key role in plant defense responses against herbivorous insects. CORONATINE INSENSITIVE1 (COI1) is an F-box protein essential for all jasmonate responses. However, the precise defense function of COI1 in monocotyledonous plants, especially in rice (Oryza sativa L.) is largely unknown. We silenced OsCOI1 in rice plants via RNA interference (RNAi) to determine the role of OsCOI1 in rice defense against rice leaf folder (LF) Cnaphalocrocis medinalis, a chewing insect, and brown planthopper (BPH) Nilapavata lugens, a phloem-feeding insect. In wild-type rice plants (WT), the transcripts of OsCOI1 were strongly and continuously up-regulated by LF infestation and methyl jasmonate (MeJA) treatment, but not by BPH infestation. The abundance of trypsin protease inhibitor (TrypPI), and the enzymatic activities of polyphenol oxidase (PPO) and peroxidase (POD) were enhanced in response to both LF and BPH infestation, but the activity of lipooxygenase (LOX) was only induced by LF. The RNAi lines with repressed expression of OsCOI1 showed reduced resistance against LF, but no change against BPH. Silencing OsCOI1 did not alter LF-induced LOX activity and JA content, but it led to a reduction in the TrypPI content, POD and PPO activity by 62.3%, 48.5% and 27.2%, respectively. In addition, MeJA-induced TrypPI and POD activity were reduced by 57.2% and 48.2% in OsCOI1 RNAi plants. These results suggest that OsCOI1 is an indispensable signaling component, controlling JA-regulated defense against chewing insect (LF) in rice plants, and COI1 is also required for induction of TrypPI, POD and PPO in rice defense response to LF infestation.

Introduction

Plants are frequently exposed to herbivorous insect attack and microbial pathogen infection in the natural environment. Different defense mechanisms are activated in response to potential enemies via several interacting signaling pathways, including the jasmonate (JA), salicylate (SA) and ethylene (ET) pathways. Jasmonates (JAs) are derived from linolenic acid and characterized by a pentacyclic ring structure [1,2]. The jasmonate pathway plays a key role in plant defense responses against herbivorous insects. In many plant species, insect feeding activates a wide variety of genes that are responsive to JA and related octadecanoids, including methyl jasmonate (MeJA) and 12-oxo-phytodienoic acid (OPDA) [3]. It has been well studied that feeding damage by herbivorous insect elicits a rapid burst of octadecanoid signals in dicotyledonous plants, such as Arabidopsis thaliana, tobacco (Nicotiana attenuata), and tomato (Lycopersicon esculentum) [4-6], to trigger production of defense compounds and anti-nutritive substances that deter further insect damage [7-11]. The jasmonate pathway also regulates production of volatiles in tomato plants, which can attract natural enemies of herbivorous insects [12].

In dicotyledonous plants, mutants impaired in JA biosynthesis and perception have been examined for effects on plant-herbivore interactions. Coronatine, a phytotoxin produced by the plant pathogen Pseudomonas syringae, acts as a molecular mimic of jasmonoyl-isoleucine (JA-Ile) and activates JA signaling [13-16]. CORONATINE INSENSITIVE1 (COI1) is an F-box protein and has been implicated in jasmonate-regulated defense responses [17]. COI1 interacts with multiple proteins to form the SCFCOI1 E3 ubiquitin ligase complex and recruits JASMONATE ZIM-DOMAIN (JAZ) proteins for degradation by the 26S proteasome. The physical interaction of COI1 with the JAZ protein is promoted by an Ile-conjugated form of jasmonic acid (JA-Ile) to serve as a receptor for jasmonate and activate the JA signaling pathway [18-21]. COI1 is required for expression of approximately 84% of 212 JA-induced genes in Arabidopsis [22].

Our current understanding of JA function in dicotyledonous plants mainly derives from analyses of mutants with alteration in either JA biosynthesis or signal transduction. Recently, mutants defective in the perception of JA including coi1, jar1, jin1, and jin4 have been widely used in study of JA signaling [17,23,24]. Of these characterized JA-insensitive mutants, coi1 is the least responsive to...
JA and has been extensively used to study the effects of JA signaling in various plant processes. The coi1 mutant is male-infertile, and insensitive to JA-mediated root growth inhibition [25–27]. Likewise, coi1 mutants are more sensitive to insects in Arabidopsis, tobacco and tomato plants [11,28,29,30]. For example, COI1-silenced tobacco plants do not activate nicotine biosynthesis genes after jasmonate treatment or wounding on leaves, which lead to reduced resistance against larvae of Manduca sexta [31]. The two-spotted spider mite (Tetranychus urticae) preferred the tomato coi1 mutant over WT plants in choice assays, and laid more eggs on the mutant plants [15]. Recent studies have found that COI1 involves inositol polyphosphates [32] and ethylene-induced root growth inhibition in the light in Arabidopsis thaliana [33]. In Solanum nigrum, COI1 controls jasmonate metabolism and the production of a systemic signal against insect attack [34].

Interestingly, plants activate different signaling pathways in response to different insect feeding styles, leading to the production of different defensive compounds [35–37]. In general, chewing herbivorous insects induce JA-regulated defense [38,39], whereas piercing-sucking insects tend to trigger expression of genes and the synthesis of defense compounds similar to those activated by fungal or bacterial pathogens [40–45]. Aphid feeding, for instance, induces the transcription of genes regulated by SA signaling pathways [36–48].

Relative to dicots, COI1-mediated resistance to herbivorous insects in monocots is largely unknown. Hu et al. [49] firstly isolated a putative OsCOI1 gene (accession: AY168645) from rice with 74% sequence identity to COI1 gene in Arabidopsis, and its expression has been confirmed to be regulated by JA. Later, Mukesh et al. [50] identified 687 potential F-box proteins from rice and classified them into 10 subfamilies based on their domain composition. Two F-box proteins (Os05g37690, Os01g63420) represent the closely related orthologs of Arabidopsis COI1 and thus may perform similar functions in rice. These two genes show 65% and 100% sequence identity to the gene isolated by Hu et al. [49]. Mei et al. [51] have successfully silenced OsCOI1 gene in rice plants by using RNA interference technology. However, the function of COI1 in rice plants remains unknown.

In the present study, to elucidate the role of OsCOI1 in insect-induced defense responses in rice plants, we silenced the gene OsCOI1 (accession: AY168645) isolated by Hu et al. [49] via RNA interference technology. The relative expression levels of defense related genes, activities of defense-related enzymes (PPO, POD, LOX), production of TrypPI, JA and SA levels were compared between OsCOI1 RNAi lines and wild-type plants (WT) in response to brown planthopper (BPH) Nilaparvata lugens, a phloem-feeding insect, and rice leaf folder (LF) Cnaphalocrosis medinalis, a chewing insect. We also examined the differential performance of the two insects on WT and OsCOI1 RNAi plants.

Results

OsCOI1 transcripts induced by insect infestation and MeJA treatment in WT plants

To determine transcript response of OsCOI1 to insect infestation and exogenous MeJA application in WT rice plants, we performed a time-course real-time PCR analysis. Leaf tissue (or leaf sheath tissue) was harvested from individual plants at different time points after infestation by LF (or BPH) or application of 1 mM MeJA. OsCOI1-specific qRT-PCR revealed that OsCOI1 transcripts were up-regulated by MeJA and LF infestations. OsCOI1 transcripts accumulated to 1.88-, 2.41- and 1.98-fold higher levels in response to LF infestation at 6, 12 and 24 h, respectively (F1, 29 = 17.8, P < 0.01) (Fig. 1A). OsCOI1 transcripts were induced approximately 1.99-, 2.04- and 1.68-fold by MeJA treatment at 6, 12 h and 24 h, respectively (F1, 29 = 0.951, P = 0.338) (Fig. 1B). These results suggest that OsCOI1 may only be involved in JA-related rice defense to chewing insects.

Differential induction of TrypPI level and enzymatic activities by LF and BPH

Enzymatic activity analyses revealed that activity of lipoxygenase (LOX), which catalyzes the initial reaction in JA biosynthesis pathway [52], increased by 48.7% in LF-infested WT plants compared with non-infested WT plants, while BPH feeding did not change LOX activity (Fig. 2A). Activities of polyphenol oxidase (PPO), which oxidizes phenolics to highly toxic quinones [53], and peroxidase (POD), which catalyzes the formation of lignin and other oxidative phenols to prevent insect consumption [54], were enhanced by 21.3% and 72.3%, respectively in response to LF

![Figure 1. Transcript level of OsCOI1 in wild-type (WT) rice plants. (A) WT plants treated with methyl jasmonate (MeJA) and rice leaf folder (LF). (B) WT plants treated with brown planthopper (BPH). qRT-PCR was used to detect the transcript levels. Values are mean ± standard error of three biological replicates. For each time point, asterisks indicate significant difference in treated plants compared to untreated control plants respectively (*P < 0.05, **P < 0.01 according to Student’s t-test). doi:10.1371/journal.pone.0036214.g001](Image)
feeding in WT plants. Likewise, BPH infestation increased activities of PPO and POD by 30.6% and 119.1%, respectively (Fig. 2B, C). These results showed that PPO and POD are induced by both LF and BPH infestation, while LOX is only induced by LF.

Protease inhibitors (PIs) have been implicated in plant defense against lepidopteran herbivorous insects via interfering with their digestive process [55,56]. In our study, we found that trypsin protease inhibitor (TrypPI) levels in WT plants were induced by 107% and 130% by BPH and LF infestation (respectively), compared with un-infested control plants (Fig. 2D).

Silencing OsCOI1 reduces rice resistance to LF but not to BPH

Southern blot analysis showed that a single copy of the OsCOI1 RNAi construct was inserted into the genome of *Oryza sativa* L. in RNAi lines (Fig. S4A). In addition, RT-PCR analysis showed that OsCOI1 expression was significantly down-regulated in RNAi lines, and could not be recovered by JA treatment (Fig. S4B). The OsCOI1 RNAi lines showed earlier and less tillering compared with WT, and most RNAi lines yielded empty grain (Fig. S5), suggesting a role of COI1 in rice fertility. There were few seeds in each line. Therefore T2 seeds were pooled for functional analysis for most experiments.

qRT-PCR analysis revealed different expression levels of OsCOI1 in 30 RNAi plants. According to the expression level of OsCOI1, these 30 plants were divided into five groups, which transcripts level were only 7.4% to 39.5% of that in WT plants (F5, 34 = 14.24, P<0.01) (Fig. 3A). In each group, there were six plants with approximately equal expression level of OsCOI1. Additionally, 15 WT plants served as control. Two second instar LF larvae were placed individually on the node 3 and 4 leaves of WT and RNAi plants. By day 3, the mean weight gain percentage (%) of LF larvae feeding on the RNAi group 3, 4 and 5 were 1.47-, 1.79- and 1.78-times of those feeding on WT plants (F5, 89= 40.62, P<0.01) (Fig. 3B). Additionally, larvae on MeJA-treated WT plants got 51.7% less weight gain compared with those feeding on untreated control plants (Fig. 3C). In contrast, BPH showed no significant difference between WT and RNAi lines. The amounts of honeydew secreted per day by a BPH female adult, an indicator of the amount of food intake, did not show a significant difference between those feeding on WT and RNAi lines (Fig. 4A). Also, the survival rate of BPH nymphs feeding on WT plants had no significant difference from those feeding on RNAi lines (Fig. 4B). The obvious differences in LF weight gain between those feeding
on WT and RNAi lines demonstrate the important role of OsCOI1 in rice resistance against LF. The result that BPH showed no difference between WT and RNAi lines suggests that the reduced expression of OsCOI1 does not negatively affect rice resistance against BPH, in agreement with the weak induction of OsCOI1 transcript by BPH (Fig. 1B).

**JA-mediated insect-induced responses are OsCOI1-dependent**

To determine the exact role of OsCOI1 in response to LF, we evaluated JA and SA concentration, as well as the TrypPI content and enzymatic activities of LOX, POD, and PPO in WT plants and RNAi plants with or without LF infestation.

Results showed that JA levels were significantly higher 3 and 8 h after LF infestation in both WT plants (F1, 47 = 4.318, P = 0.044) and RNAi plants (F1, 47 = 5.582, P = 0.022) as compared to the non-infested control. JA levels in RNAi plants were not significantly lower at 3 and 8 h compared to those in WT plants (F1, 47 = 0.085, P = 0.967) (Fig. 5A), suggesting that LF-induced JA level was not affected by OsCOI1 silencing. BPH infestation did not increase JA level in both WT and RNAi plants (F1, 47 = 1.56, P = 0.21) (Fig. 5B).

SA levels in BPH-infested WT plants were significantly higher 1.5, 3 and 9 h after insect infestation than those in the non-infested WT plants (F1, 47 = 45.81, P < 0.01). LF-infested RNAi plants showed the same trend (F1, 47 = 16.94, P < 0.01) (Fig. 5D). There was no significant difference in SA levels between WT and OsCOI1 RNAi plants 1.5, 3 and 8 h after BPH infestation (F1, 47 = 1.67, P = 0.23), suggesting that OsCOI1 silencing does not change BPH-induced SA levels. LF infestation also increased SA levels in WT plants 8 h after treatment (F1, 47 = 11.29, P < 0.01). Silencing OsCOI1 in rice did not reduce LF-induced SA levels (F1, 47 = 24.59, P < 0.01) (Fig. 5C).

LF infestation strongly induced the transcripts of OsCOI1 (F3, 41 = 19.44, P < 0.01) and enhanced TrypPI level in WT plants (Fig. 6A). However, OsCOI1 silencing impaired the inducibility of OsCOI1 transcripts and TrypPI by LF, and there was no significant change in OsCOI1 transcripts and TrypPI level in RNAi lines after LF feeding (Fig. 6A). The TrypPI level in RNAi lines was only 38.7% of that in infested WT plants (F3, 23 = 30.77, P < 0.01) (Fig. 6B).

There was no significant difference in LOX activity between WT plants and RNAi plants with LF infestation (Fig. 6C), indicating that silencing OsCOI1 did not change LOX activity. However, the suppressed expression of OsCOI1 resulted in 50.9% reduction in POD activity compared to that in non-infested WT plants. LF-induced POD activity was significantly reduced (by 48.5%) in OsCOI1 RNAi plants compared to that in LF-infested WT plants (F3, 23 = 20.65, P < 0.01) (Fig. 6D). PPO activity in RNAi plants did not differ significantly from that in WT plants without LF infestation, but PPO activity in LF-infested RNAi plants was significantly reduced by 27.2% compared to that in LF-infested WT plants (F3, 23 = 10.26, P < 0.01) (Fig. 6E). These results suggest that OsCOI1 is required for the induction of POD, PPO and TrypPI activities in the rice defense response to LF.

Additionally, transcripts of OsCOI1 in WT plants were induced by 89.2% with exogenous MeJA application (Fig. 7A). TrypPI, LOX, POD and PPO activities were enhanced by MeJA by 322.2%, 54.6%, 42.9% and 71.6% respectively (Fig. 7B–E). However, MeJA-induced TrypPI and POD activities were reduced by 57.2% and 48.2% in RNAi plants compared to those of MeJA-treated WT plants (Fig. 7B, D). These results demonstrate that OsCOI1 is required for MeJA-induced rice defense, including POD and TrypPI activities.

**Discussion**

In this study, a comparison of resistance against chewing and phloem-feeding insects between wild-type (WT) and OsCOI1 silenced RNAi plants provides new insight into the role of COI1 in rice defense against herbivorous insects. We found that LF infestation and MeJA application strongly and constantly enhanced the transcript levels of OsCOI1 in WT plants (Fig. 1A), but BPH only slightly induced OsCOI1 transcripts (Fig. 1B). Lipoxygenase enzyme (LOX), a key component in JA biosynthesis [52], was significantly induced by LF but not by BPH (Fig. 2A). In addition, LF induced higher levels of JA production in WT plants (Fig. 5A), while BPH infestation significantly increased SA in WT plants. Therefore, OsCOI1 is required for the induction of JA and SA levels in response to LF and MeJA. Furthermore, OsCOI1 silencing impaired the inducibility of OsCOI1 transcripts and TrypPI by LF, indicating that OsCOI1 expression is strongly induced by LF and MeJA.
plants (Fig. 5D). These results suggested that COI1 and the JA signaling pathway are involved in rice resistance against LF but not BPH.

Insect infestation elicits a burst of JA signaling in plants [10,36], resulting in JA accumulation [18,22,48]. Our study showed that silencing OsCOI1 did not reduce LF-induced LOX activity (Fig. 6C) and JA levels (Fig. 5A), nor did it reduce MeJA-induced

Figure 4. Honey dew and survival rate of BPH feeding on wild-type (WT) and OsCOI1 RNAi rice lines. (A) Amount of honeydew per day secreted by three female BPH adults and (B) Survival rate of BPH nymphs after 5 days feeding on each line. Values are mean ± standard error of ten replicates, asterisks indicate significant differences in RNAi lines compared to WT (*P<0.05 according to Student’s t-test).

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Figure 5. JA and SA levels in wild-type (WT) and OsCOI1 RNAi rice plants infested with LF (A, C) and BPH (B, D). Values are mean ± standard error of six biological replicates. For each time point, letters indicate significant differences among treatments (P<0.05 according to Tukey’s multiple range test).

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LOX activity (Fig. 7C), suggesting that OsCOI1 is not involved in JA biosynthesis in rice plants. It is likely that COI1 acts as a receptor in the JA signal pathway in monocots, as its counterparts in dicots [18,33,57].

Increases in activities of PPO [53], POD [12,54], and TrypPI [55,56] are the most prominent systemic responses against insect feeding in plants. All of these proteins have been demonstrated to reduce the nutritive value of plant foliage to herbivorous insects. Our study confirmed that POD, PPO activities and TrypPI production in rice were all increased in response to both LF and BPH infestation (Fig. 2B–D), implicating their roles in rice resistance to LF and BPH.

Silencing OsCOI1 in rice led to improved performance of the chewing insect LF (Fig. 3A and B). Meanwhile, it decreased LF-induced TrypPI levels (Fig. 6B), POD and PPO enzymatic activities (Fig. 6D, E), demonstrating that OsCOI1 plays a crucial role in rice defense against LF, and that OsCOI1 is required for induction of POD, PPO and TrypPI in rice responses to LF. Indeed, POD, PPO and TrypPI have been demonstrated to be involved in rice defense responses to LF [58,59]. Hence the reduction of herbivore resistance in OsCOI1 RNAi plants may be partially caused by decreased induction of POD, PPO and TrypPI. Likewise, the enhanced rice resistance to LF (Fig. 3C) by MeJA application can partially be explained by an increase in MeJA-induced TrypPI, POD, and PPO activities (Fig. 7B, D, E).

The weakened induction of POD and TrypPI activities in OsCOI1 RNAi plants by MeJA treatment (Fig. 7B, D and E) indicates that COI1 is a key regulator of MeJA-induced defense [22,31].

Plants have evolved complex strategies to protect themselves against pests. Phloem-feeding insects tend to induce SA-mediated resistance as pathogens do [46,47]. BPH infestation induces PAL and NPR1 genes, which are the key regulators of SA-dependent systemic acquired resistance. Likewise, some PR genes regulated by the SA pathway are induced by BPH [60,61]. Our results reveal that BPH infestation induced higher levels of SA (Fig. 5D) but not JA (Fig. 5B). Silencing OsCOI1 did not alter the amount of honeydew (Fig. 4A) or survival rate (Fig. 4B) of BPH, implying that rice resistance to BPH, a homopteran phloem feeder of rice, is OsCOI1-independent.

It is generally assumed that JA and SA signaling pathways are mutually antagonistic in plant defense [62,63]. Impaired JA signaling by suppressed expression of OsHI-LOX leads to increased SA-dependent resistance to BPH [59]. However, in this study silencing OsCOI1 did not increase BPH-induced SA levels (Fig. 5D), nor it increased rice resistance to BPH (Fig. 4A, B). No antagonistic interaction between the two signaling pathways was found. The possible reason could be that silencing OsCOI1 did not affect JA accumulation, and thereby the antagonism did not occur.

In addition to the role in herbivore resistance, COI1 plays a central role in fertility. In tomato plants, silencing COI1 results in

Figure 6. Levels of OsCOI1 transcripts (A), TrypPI (B) and three defence-related enzymes in wild-type (WT) and OsCOI1 RNAi rice plants infested with LF. Three defence-related enzymes included: (C) lipoxygenase (LOX), (D) peroxidase (POD), (E) polyphenol oxidase (PPO). qRT-PCR was used to detect the transcript levels. Values are mean ± standard error of six biological replicates. Letters above bars indicate significant differences among four treatments (P<0.05 according to Tukey’s multiple range test).

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defective maternal control of seed maturation, as well as altered the trichome shape and number [15]. In *N. attenuata* and *Arabidopsis*, the sterility is mainly caused by defective dehiscence [17] and shorter stamens in flowers [30]. It appears that the suppressed expression of COI1 leads to different flower phenotypes in dicotyledonous plants. In rice, silencing *OsCOI1* resulted in earlier and less tillering compared to WT plants. Most *OsCOI1*-deficient plants yielded empty grain (Fig. S5), suggesting that COI1 is essential for development of fertile flowers and viable seeds in rice, although its mechanism remains to be examined.

Based on the results that *OsCOI1* is responsive to LF infestation and MeJA, and that COI1 silencing in rice increases susceptibility to chewing insects and impairs the inducibility of TrypPI, PPO and POD, we conclude that the JA signal transduction pathway plays a key role in rice defense against chewing insects, and COI1 is specifically required for the regulation of JA-mediated insect defense in response to the chewing insect LF, but not for SA-mediated defense in response to BPH. Moreover, TrypPI, POD, PPO and LOX are JA-induced defense responses to the chewing insect LF. TrypPI, POD and PPO are all *OsCOI1*-mediated (Fig. 8). We therefore propose that rice plants can recognize different signals induced by chewing insects and phloem-feeding insects. In response to chewing insects, rice plants activate the JA signaling pathway leading to increases in LOX activity, increased JA level, and up regulation of *OsCOI1*. *OsCOI1* serves as a receptor of the JA signal and activates the JA signal transduction pathway, thereby increasing enzymatic activities of PPO and POD as well as TrypPI production, which lead to increased rice resistance against chewing insects (Fig. 8).

![Figure 7. Levels of *OsCOI1* transcripts (A), TrypPI (B) and three defence-related enzymes in wild-type (WT) and *OsCOI1* RNAi rice plants treated with MeJA. Three defence-related enzymes included: (C) lipoxygenase (LOX), (D) peroxidase (POD), (E) polyphenol oxidase (PPO). qRT-PCR was used to detect the transcript levels. Values are mean ± standard error of six biological replicates. Letters above bars indicate significant differences among four treatments (*P*<0.05 according to Tukey’s multiple range test). doi:10.1371/journal.pone.0036214.g007](image1)

![Figure 8. Schematic summary of the crucial role of *OsCOI1* in JA-regulated defence responses to chewing insect (LF, rice leaf folder). doi:10.1371/journal.pone.0036214.g008](image2)
Materials and Methods

Generation of transgenic plants

In the present study, we silenced OsCOI1 (accession: AY168645) in rice isolated by Hu et al. [49], encoding a putative protein containing an F-box motif and 16 leucine-rich repeats (LRRs). To construct the OsCOI1 RNAi vector, the 617 bp cDNA fragment of OsCOI1 was amplified by RT-PCR with primers 5'-gcagctgacctg-gcatggctgacagga-3' and 5'-gatcaagacgctcaagctgt-3' derived from conserved LRR cDNA regions of OsCOI1 (Fig. S1). Restriction sites BamH I and HindIII were incorporated into the primers, respectively. PCR conditions were 1 min initial denaturation at 94°C, followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 52°C and extension for 8 min at 72°C. Vector pRNAi.5 (Fig. S2, kindly provided by Professor Yao-Guang Liu, College of Life Sciences, South China Agricultural University) was digested by BamH I and HindIII enzymes, and the OsCOI1 fragment was then inserted into BamH I and HindIII restriction sites. Both PCR with the specific primers and restriction enzyme digestion verified that the fragment had been correctly inserted into the vector. This first round-ligated vector was then used as the template to amplify a second sequence with two unique restriction sites in both ends (RNAi-Mid: 5'-cactgctgacctgggtgt-tatcttgccgg-3'; RNAi-Pst: 5'-actgaagacgctgacagctgac-3'). The second sequence was subsequently cloned between Pst I and Mid I, resulting in an opposite orientation in contrast to the first sequence. Restriction digestion showed that the second target fragment had been correctly inserted into the vector. Finally, the DNA sequencing further confirmed the correct orientations sequences 100% identical to that reported in GenBank (accession: AY168645) (Fig. S3 A and B).

Rice (Oryza sativa L. cv. Shishoubaimao) was used for transformation. The construct containing the invert OsCOI1 sequence repeats driven by the 35S promoter was transferred into rice callus according to an Agrobacterium (strain EHA105)-mediated transformation procedure [64]. Calluses were cocultured for 2 d, and were then screened twice for hygromycin resistance at 40 mg L⁻¹, each for 20 d. The selected resistant calluses were put on pre-redifferentiated medium for 15 d and then transferred to redifferentiation medium until the callus produced shoots, and the shoots rooted by transfer to rooting medium. The plantlets were transplanted to soil. Twenty days later, leaves of different T₀ lines were harvested for analyses of the copy number of OsCOI1 RNAi construct by Southern hybridization, two homozygous T₀ lines (L1 and L2) were identified, each harboring a single insertion (Fig. S4A). OsCOI1 transcripts in these T₀ lines were not induced by JA (Fig. S4B). The seeds harvested from L1 and L2 were germinated and grown in complete Kimura B nutrient solution, then transferred to normal soil conditions to grow until the seeds were harvested. The relative expression of OsCOI1 in T₁ lines was analyzed by qRT-PCR (Fig. S4C). The well silenced individuals were used for seed production. T₂ seeds were used for the functional analyses in this study.

Southern blot analysis

Genomic DNA was extracted from the leaves using a cetyl trimethyl ammonium bromide procedure [65]. DNA was digested using HindIII restriction enzymes, separated on a 0.8% w/v agarose gel, and transferred to a nylon membrane (Hybond -N°, Amersham, United Kingdom). To determine the copy number of OsCOI1 RNAi construct in transgenic plants, a PCR fragment of the hygromycin phosphotransferase gene amplified by gene-specific primers Hpt-F (5'-tcggctgacctgcaagcagta-3') and Hpt-R (5'-ctgaacctgcgcaagctgtg-3') was used as a probe for detection in Southern hybridization. α-32P dCTP was used to label the probe using the manufacturer’s protocol for the TakaRa random primer labeling kit (TAKARA, http://www.takara-bio.co.jp). Hybridization conditions were as follows: pre-hybridization at 65°C with hybridization buffer (0.25 M NaHPO₄, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA) for 60 min, hybridization at 65°C for 1 h, and washing with 2× SSC and 0.1% SDS twice (30 min for each), and then washing with new 2× SSC and 0.1% SDS for 15 min. After autoradiography on a storage phosphor screen, images were scanned using a FX scanner (BIO-RAD).

Plant growth

Rice seeds of WT and OsCOI1 RNAi lines were surface-sterilized with 10% H₂O₂ and rinsed three times with sterile distilled water. The seeds were presoaked in sterile distilled water for 1 d, pre-germinated for 3 d, and grown in plastic buckets in a greenhouse for 20 d. Seedlings were then transplanted to small plastic pots (diameter 10 cm, height 12 cm), and each pot contained one plant. The soil for plant growth was obtained from the rice fields on the campus of South China Agricultural University in Guangzhou, China. Plants were watered daily, and each pot was supplied with 20 ml of nutrient solution (urea, 1 g L⁻¹) every week. All plants were grown in a greenhouse at 28±2°C with a 12 h light phase and 80% relative humidity. Plants were used for experiments 25–30 days after transplanting.

Insects

BPH and LF larvae were originally obtained from rice fields of Dafeng Base of Guangdong Academy of Agricultural Sciences, Guangzhou, China, and maintained on WT plants in a climate-controlled room (26±2°C, 80% relative humidity, and 12 h light phase). The BPH nympha of the third generation and third instars of LF were used for bioassays and feeding treatments.

Plant treatments

Two third instar LF larvae that had been starved for 2 h were placed on leaves at node 3 and 4 of each individual plant (the youngest fully expanded leaf was defined as leaf node 1). Non-infested control plants were not manipulated. For BPH treatment, each plant was individually infested by 15–20 gravid BPH contained in two paraffin bags (6×5 cm), each bag was then fixed to upper and lower positions on the stems. Two empty bags were fixed to control (non-infested) plants.

Plants (one plant per pot) were individually sprayed with 1 ml of MeJA (1 mM) with 0.01% Tween 20 for 48 h. Control plants were sprayed with 1 ml of the buffer with 0.01% Tween 20.

For LF and MeJA treatments, node 4 of leaves was harvested for analyses of gene expression, TrypPI content, enzyme activities and JA level analysis. In the BPH treatment, leaf sheathes were harvested for analysis. There were six biological replicates for each treatment.

Quantitative real-time PCR analysis

Differential expression of selected genes was verified by quantitative real-time PCR (qRT-PCR) using the RNA samples isolated from rice tissues obtained from different treatments. The actin gene was used as a reference gene. Total RNA from rice leaves was extracted according to the method as described by Kiefer [66] including a DNase (Promega, Madison, USA) treatment. First strand cDNA was synthesized from 1 μg of total RNA using ImProm-II™ Reverse transcription system (Promega, Madison, USA) according to the manufacturer’s instructions. The primers for target gene OsCOI1 were designed by Primer 3.0.
LOX activity was measured as conjugated diene formation [69]. Leaf samples (0.1 g) were ground in liquid nitrogen and extracted with 1 ml of ice-cold 0.5 M Tris-HCl buffer (pH 7.6) and centrifuged at 12000 g for 15 min at 4°C. The supernatant was kept at 4°C until used. The substrate contained 1.6 mM linoleic acid and 0.5% (v/v) Tween 20 in 0.1 M phosphate buffer (pH 7.6). The reaction was initiated by the addition of 0.2 ml of crude extract in 4.8 ml of the substrate. Diene formation was followed as increase of absorbance at 234 nm.

TrypPI analyses

TrypPI activity was measured using a colorimetric assay which uses the protein chromophore azocasein as a substrate [70]. In our modified assay, leaf tissue (0.1 g) was ground in 0.2 M Tris-HCl buffer (pH 8.0) with 0.1% Tween 20, samples were centrifuged at 12000 g for 20 min at 4°C and the supernatant was collected. Each reaction contained 200 μl plant extract. 500 μl of 0.1 μg/μl trypsin was added to each reaction, mixed and left at room temperature for 10 min. 100 μl of 25 mg/ml azocasein was added, the reaction mixed and incubated at 37°C for 40 min. Samples were centrifuged at 12000 g for 10 min, then 200 μl of supernatant was mixed with 200 μl of 0.5 M NaOH and the absorbance measured at 450 nm. The amount of protease inhibitor as nmol in each sample was calculated based on a standard curve, and results were expressed as nm protease inhibitor per mg protein, with protein determined by the Bradford assay [71] using BSA as standard.

JA and SA analyses

Plants (one per pot) were randomly assigned to LF and non-infested treatments. The leaves were harvested at 0, 1.5, 3 and 8 h after treatment. Leaf samples were immediately frozen in liquid nitrogen and stored at −80°C. For each time point and treatment, six plants were sampled. JA and SA content was measured by GC analyses using external JA and SA standards (Sigma-Aldrich, St. Louis, MO, USA) as described by Song et al. [72]. Samples were extracted by mixture of acetone and citric acid (50 mmol L⁻¹) (v/v = 7/3), and ethyl acetate. Then the supernatant was dried by N₂ and subsequently methylated with trimethylsilyldiazomethane. The volatilized compounds were collected by using headspace-solid phase microextraction (HS-SPME) on Tenax adsorbents' and subsequently methylated with trimethylsilyldiazomethane. The final chromatographic peaks of JA and SA in the samples were identical to the authentic compounds (Fig. S6). 25 μl 80 μg/ml JA and 125 μl 160 μg/ml SA were mixed, and after the step of extraction and methylation with trimethylsilyldiazomethane as samples, 100 μl n-hexane was used to elute the MeJA and MeSA collected in Tenax by HS-SPME, the mixed MeJA (20 μg/ml) and MeSA (200 μg/ml) were diluted into several concentration to be used as stands to quantify JA and SA levels of samples. In addition, mixed standard MeJA (18 μg/ml) and MeSA (40 μg/ml) (Sigma-Aldrich, St. Louis, MO, USA) were used to confirm the recovery rate of JA and SA. The method resulted in a high level of recovery, reproducibility, and linearity in the quantification of JA and SA [Fig. S7; Table S1].

Statistical analysis

SPSS 14.0 (SPSS, Chicago, IL, USA) package for Windows was used for statistical analysis. Differential gene expression, enzymatic activities, and TrypPI level of LF- or BPH-infested and their respective non-infested WT control plants were determined using Student’s t-test. Differential OsCOI expression caused by LF,
MeJA or BPH treatment at each time point as compared to control plants respectively was determined using Student’s t-test. For LF performance on exogenous MeJA-treated and untreated control WT plants and BPH performance on WT and RNAi lines, Student’s t-tests were used. Differences in LF performance on WT and RNAi lines, LF-induced gene expression, enzymatic activities, and TrypPI level, JA and SA levels at each time point on WT and RNAi lines were evaluated by Tukey post-hoc test one-way ANOVA at \( p = 0.05 \).

Supporting Information

Figure S1 Nucleotide sequence and amino acid sequence of targeted OsCOI1 gene (accession: AY168645) and RNAi target region of the hairpin-forming RNAi transgene cassette used in the present study. F-box motif is indicated by double underline. Leucine rice repeats (LRRs) were marked by single underline. RNAi target region is shown in red, the primers (OsCOI1 5’ and OsCOI1 3’) derived from conserved domains in LRRs region are indicated below the nucleotide sequence.

Figure S2 Rice transformation vector pRNAi-COI1 with HPT as plant selectable marker gene.

Figure S3 (A) cDNA sequence of amplified OsCOI1 fragment. (B) Identity analyses of amplified OsCOI1 fragment.

Figure S4 (A) DNA gel-blot analysis of two T0 OsCOI1 RNAi lines and one WT line. (B) RT-PCR analysis of transcriptional expression of OsCOI1 from the T0 OsCOI1 RNAi lines and WT plants (C: Control, no manipulation; T: Treated with 100 nmol L\(^{-1}\) JA). (C) Relative expression of OsCOI1 in WT and T1 RNAi lines.

Figure S5 Growth phenotype of OsCOI1 RNAi lines and WT rice plants.

Figure S6 Profiles of GC chromatography of authentic MeJA, MeSA and JA derived MeJA and SA derived MeSA in rice leaves.

Figure S7 Recovery rates of jasmonic and salicylic acids in the GC analysis.

Table S1 Retention time, linear regression equation and limit of detection of JA and SA detected by GC-FID.

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Author Contributions

Conceived and designed the experiments: MY RSZ. Performed the experiments: MY YYS. Analyzed the data: MY SMIL KZ. Contributed reagents/materials/analysis tools: JFX YFL TX YL. Wrote the paper: MY RSZ KZ.

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