Level of Endogenous Jasmonate is Critical for Durable Broad-Spectrum Disease Resistance Against Rice Blast in a Japonica Rice Variety, Ziyu44

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Research Article

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Abstract

Background: The molecular mechanism of durable and broad-spectrum resistance to rice blast disease in *japonica* rice variety is still very little known. Ziyu44, a local *japonica* rice variety in Yunnan Province of China, has shown durable broad-spectrum blast resistance for more than 30 years, and provides an opportunity for us to explore the molecular basis of broad-spectrum resistance to rice blast in *japonica* rice variety.

Methods and Results: We conducted a comparative study of mycelium growth, aposporium formation, the accumulation of salicylate (SA), jasmonate (JA) and \( \text{H}_2 \text{O}_2 \), the expression of SA- and JA-associated genes between Ziyu44 and susceptible variety Jiangnanxiangnuo (JNXN) upon *M. oryzae* infection. We found that appressorium formation and invasive hyphae extention were greatly inhibited in Ziyu 44 leaves compared with that in JNXN leaves. Both Ziyu 44 and JNXN plants maintained high levels of baseline SA and did not show increased accumulation of SA after inoculation with *M. oryzae*, while the levels of baseline JA in Ziyu 44 and JNXN plants were relatively low, and the accumulation of JA exhibited markedly increased in Ziyu 44 plants upon *M. oryzae* infection. The expression levels of key genes involving JA and SA signaling pathway *OsCOI1b*, *OsNPR1*, *OsMPK6* as well as pathogenesis-related (PR) genes *OsPR1a*, *OsPR1b* and *OsPBZ1*, were markedly up-regulated in Ziyu44.

Conclusions: The level of endogenous JA is critical for synchronous activation of SA and JA signaling pathway, up-regulating PR gene expression and enhancing disease resistance against rice blast in Ziyu44.

Introduction

Rice blast, caused by the fungus *Maganaporthe oryzae*, is the most devastating rice disease resulting in the worldwide annual loss of 10–30% of the rice harvest, amount enough to feed approximately 60 million people. Deployment of resistance (*R*) genes in rice is considered as the best practice to manage diseases and curtail the environmental damage by reducing the use of agrochemicals[1]. To date, more than 100 *R* genes have been identified in rice, and 25 of them have been cloned and characterized[2]. Although some *R* genes conferring strain-specific resistance have been applied in plant breeding, their effect can be rapidly overcome by the emergence of compatible blast isolates[1]. In addition, because pyramiding *R* genes to develop resistant cultivars is an extremely time-consuming process, rice production is still facing a huge threat caused by the fast evolution of pathogenic blast fungi[3]. Therefore, characterizing the molecular mechanism of durable and broad-spectrum resistance is important for guiding rice resistance breeding. Gumei 4 (GM4) and Digu, two Chinese *Indica* rice varieties, display high and durable blast resistance[4, 5]. Recent studies have shown that *PigmR* gene encoding nucleotide-binding leucine-rich repeat (NLR) receptor confers broad-spectrum resistance in GM4[4], and a single base change (SNP33-G) in the *bsr-d1* (a natural allele of a C2H2-domain transcription factor gene) promoter enhances binding to MYBS1 and confers broad-spectrum resistance to rice blast in Digu[5].
The cultivated rice (*Oryza sativa* L.) is divided into two main subspecies: *Japonica* and *indica*. Because *japonica* rice has better taste quality than *indica* rice, the planting area in China is expanding year by year. However, compared with *indica* rice, the blast resistance of *japonica* rice is generally very poor[6]. In fact, most of the identified broad-spectrum blast resistance resources are *indica* rice, like Digu and Gumei, and the molecular mechanisms on broad-spectrum blast resistance characterized so far are limited to *indica* rice and these genes cloned from *indica* rice materials. However, the resistance resources of *japonica* rice, especially those with broad-spectrum blast resistance, are relatively scarce, and the broad-spectrum resistance mechanism of *japonica* rice is still very little known, which is very unfavorable to the overall understanding of the molecular mechanism of broad-spectrum resistance to rice blast.

Plants employ a two-tier innate immunity system to protect them from a wide range of pathogens: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), and effector-triggered immunity (ETI)[7, 8]. PTI contributes to host defense against infections by a broad range of pathogens, activation of PTI leads to various immune responses, including calcium influx, the deposition of callose, a rapid burst of reactive oxygen species (ROS), and expression of defense genes[9]. These responses confer effective and broad-spectrum defense against the majority of potential pathogens. Downstream of PTI activation, the activation of complex phytohormones signaling networks is critical for stimulating plant innate immunity[10]. Salicylic acid (SA) and jasmonate (JA) are recognized as the most important hormones in plant immune responses and are believed to represent the hormonal backbone of defense against pathogens[11]. In general, the SA pathway is crucial for immune responses against biotrophic and hemibiotrophic pathogens, whereas the JA pathway is involved in defense against necrotrophic pathogens acquiring nutrients from the decaying host tissue[12, 13]. Moreover, interaction between these two types of defense is mostly antagonistic[12, 14, 15]. This reciprocal antagonistic crosstalk between the SA and JA pathways, initially demonstrated in *Arabidopsis*[16, 17], is present also in other plant species[18]. Nonetheless, evidence deviating of the antagonism between SA and JA also exists, particularly in monocotyledonous plants[19–22].

Ziyu44, a *japonica* rice variety of Yunnan Province, has broad-spectrum resistance to 16 physiological races (ZA1, ZA49, ZA57, ZA61, ZB1, ZB13, ZB17, ZB25, ZC1, ZC3, ZC13, ZC15, ZE1, ZE3, ZF1 and ZG1) from Yunnan province[23]. Over the past 30 years, field-cultivated Ziyu44 has displayed high and durable resistance to rice blast[24]. Our previous studies have identified a number of major and minor resistance genes in Ziyu44[24–26] and suggested that durable broad-spectrum resistance to rice blast in this cultivar may reflect a combined effect of multiple loci. However, the functions of SA and JA in regulating immunity in Ziyu44 are unclear. Specifically, the spatiotemporal dynamics of SA and JA during the interaction between Ziyu44 and *M. oryzae*, and the relative contribution of each hormone to the defense response of Ziyu44 remain unknown. Therefore, the objective of the present study was to compare appressorium formation, hypha growth, endogenous SA and JA content, and expression of SA- and JA-associated genes in Ziyu44 and JNXN rice varieties in response to *M. oryzae* infection. The obtained results revealed that the accumulation of JA and activation of the SA-JA defense signaling at the early stages of *M. oryzae* infection in the durably resistant rice Ziyu44 is essential for the resistance to rice blast.
Materials And Methods

Rice cultivars, fungal isolates

Ziyu44 and Jiangnanxiangnuo (JNXN), two Chinese Japonica (Oryza sativa L. subsp. geng) rice varieties, used in this study. Ziyu44, a landrace of Yunnan Province, exhibits broad-spectrum and durable blast resistance, while the rice cultivar JNXN is susceptible to rice blast. The *M. oryzae* isolates of Zhong-10-8-14 (an *M. oryzae* strain tagged with enhanced green fluorescent protein (eGFP)) and LP33 were kindly provided by Prof. Lihuang Zhu (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) and Prof. Yueqiu He (Yunnan Agricultural University), respectively.

Fungal growth on rice sheath

The durably resistant rice Ziyu44 and the susceptible rice JNXN were grown in a growth chamber at 28°C and 75% humidity in a 12 h light/12 h dark photoperiod. For microscope monitoring of fungal development, *M. oryzae* strains Zhong-10-8-14 and LP33 were grown on Potato sucrose medium for 2 weeks (at 26°C and natural light), then conidial spores were collected via flooding oat-tomato agar medium with sterile water. 5×10⁵ conidia/mL spore suspension were used to inoculate the detached rice sheaths from 4-week-old Ziyu44 and JNXN as described previously[27, 28]. The images of conidial germination, appressorium development, and invasive hyphae growth were recorded using an Olympus fluorescent microscope. The microscopic evaluation of the infected sheath was performed in three independent experiments, more than 30 cells were counted in each replicate.

**H₂O₂ accumulation**

Seedlings of Ziyu44 and JNXN rice with 3–4 leaves were spray-inoculated with *M. oryzae* strain LP33. The spore concentration in the suspension was adjusted to approximately 5 × 10⁵ conidia/mL with 4‰ gelatin. H₂O₂ accumulation was monitored by staining with 3,3′-diaminobenzidine (DAB, Sigma), as previously described[29]. Briefly, leaf sections were placed in 1 mg mL⁻¹ DAB, incubated at 22°C for 10 h under illumination, and the formation of a brown precipitate of oxidized DAB was observed under a microscope.

RNA isolation and qRT-PCR

Total RNA was extracted using the MiniBEST Plant RNA Extraction Kit (TaKaRa, Dalian, China) according to the manufacturer's protocol. cDNA was synthesized using the ReverTra Ace Qpcr RT Master Mix with gDNA Remove kit (TOYOBO, Shanghai, China). The real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was conducted using a Bio-Rad (Hercules, CA, USA) CFX96 Real-Time System coupled to a C1000 Thermal Cycler. The expression of the ubiquitin (Ubq) gene was used as a reference for the normalization of all qRT-PCR data[28]. The relative levels of gene expression were calculated using the 2⁻ΔΔCT method, and each determination was done in triplicate[30]. Sequences of the primers are listed in Table 1.
Table 1
Primers used in the study.

| Primer name | Forward sequence (5' to 3') | Reverse sequence (5' to 3') |
|-------------|-----------------------------|----------------------------|
| OsUbi       | GCCCAAGAAGAAGATCAAGAAC      | AGATAACAACGGAAGCATAAAAAGTC |
| OsNPR1      | TGAGAGTCTACGAGGAAGTTTG     | CGTTGCTTTTCAGGAGGTGGAT     |
| OsPAL1      | AGGAGCTCGGCTGCGTATT        | ATGCCGAGGAACACCTTGTT       |
| OsAOS2      | CCCTAGCGTTGACAACAAGCA      | CGGAGGTGAAGCTTTTGAGTA      |
| OsPR1a      | TCGTATGCTATGCTAGTGGTT     | CACTAAGCAAATACGGCTGACA     |
| OsPR1b      | GGCAACTTCTCGTGAGACAGA      | CCGTGGACCTTGTACATT        |
| OsPBZ1      | CCCTGCCGAATACGCCTAA        | CTCAACGCCAGGAGAATT        |

SA, JA, and jasmonoyl-isoleucine (JA-Ile) assays

SA, JA, and JA-Ile were prepared and quantified as previously described[31]. The levels of SA, JA and JA-Ile were measured by gas chromatography–mass spectrometry (GC-MS) using labeled internal standards. Three biological replicates were performed for the statistical analysis.

Results

Response to M. oryzae infection in Ziyu44 and JNXN leaves

The resistance response of rice plants to the M. oryzae infection is thought to be dependent on the stage of development of the fungus [32]. To characterize the resistance responses of Ziyu44 against blast fungus during early infection stages, we inoculated the leaf sheath of Ziyu44 with M. oryzae strains, Zhong 10-8-14 (tagged by eGFP) and LP33. JNXN, a susceptible variety, was used as a control. At the early invasion stage (3 hours post-infection, hpi), the conidial germination and germ tube extension of Zhong 10-8-14 and LP33 showed no obvious difference on the leaf sheaths of Ziyu44 and JNXN rice (Fig. 1). However, the development of M. oryzae was greatly affected at 10–48 hpi in the resistant Ziyu44 rice compared with the susceptible rice JNXN. Although appressorium formation was observed on both JNXN and Ziyu44 at 10 hpi, its frequency was approximately 10% lower in Ziyu44 than in JNXN rice (Fig. 1). On JNXN, the invasive hyphae formed at 22 hpi and extended to the neighboring cells at 34 hpi. In contrast, on Ziyu44, the invasive hyphae were mostly restricted to the primary infected leaf sheath cells at 34 hpi and disappeared in some cells at 48 hpi (Fig. 1). Additionally, extensive yellow autofluorescence was observed on Ziyu44. Therefore, we conclude that the appressorium formation, invasion hyphae growth and further extension were greatly inhibited in Ziyu44 leaves.

Accumulation of H$_2$O$_2$ in M. oryzae-infected leaf cells
Reactive oxygen species (ROS) play important roles in both the first line of defense termed as pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI) and the second line of defense related to effector-triggered immunity (ETI)[33, 34]. To determine the generation of ROS during the infection, the leaves of the resistant Ziyu44 rice and the susceptible JNXN rice were inoculated with \textit{M. oryzae} strain LP33 and stained with DAB. At 36 and 48 hpi, the infected leaf cells of Ziyu44 produced much darker staining than cells of JNXN, reflecting a higher concentration of \textit{H$_2$O$_2$} (Fig. 2). This result indicates that Ziyu44 may protect itself against the \textit{M. oryzae} infection by enhancing the generation of ROS.

**The response of JA and SA in Ziyu44 and JNXN plants to \textit{M. oryzae} infection**

Plant hormones, such as SA and JA, have conserved and divergent functions in fine-tuning immune responses in rice[21, 35]. Typically, rice plants maintain a high level of free SA that is only weakly responsive to the pathogen attack[36]. Consistently with this report, our results showed that both Ziyu44 and JNXN plants maintained a high baseline levels of endogenous SA and did not show obvious changes of endogenous SA level after inoculation with \textit{M. oryzae} (Fig. 3a). Although both Ziyu44 and JNXN plants maintained low levels of endogenous JA and jasmonoyl-isoleucine (JA-Ile), the levels of endogenous JA and JA-Ile were constitutively markedly increased only in Ziyu44 plants after inoculated with \textit{M. oryzae}, which was ~ 8.2 and ~ 20.2 times higher than those in JNXN at 48 hpi, respectively (Fig. 3b,c). These results indicate that increased accumulation of endogenous JA in Ziyu44 plants is important to mediate resistance to \textit{M. oryzae}.

**Expression analysis of SA- and JA-associated genes**

To understand the roles of SA and JA signaling pathways in resistance of Ziyu44 against \textit{M. oryzae} infection, the transcriptional levels of several SA- and JA-associated genes were examined using qRT-PCR. \textit{OsPAL1} encodes phenylalanine ammonia lyase, a key enzyme in the SA biosynthetic pathway[37], we observed that the expression levels of \textit{OsPAL1} were low in both JNXN and Ziyu44 pants, and even down-regulated in Ziyu44 pants inoculated with \textit{M. oryzae} LP33 at 24 hpi(Fig. 4a). \textit{OsAOS2} encodes allene oxide synthase which is a key enzyme in the JA biosynthetic pathway[38], the transcriptions of \textit{OsAOS2} were significantly up-regulated in Ziyu44 and JNXN after inoculation with \textit{M. oryzae} LP33 (Fig. 4b). The expression patterns of \textit{OsAOS2} and \textit{OsPAL1} were consistent with the accumulation of endogenous SA (Fig. 3a) and JA (Fig. 3b) in Ziyu44 and JNXN plants upon \textit{M. oryzae} infection.

\textit{OsCOI1b} is a JA receptor and play key roles in JA signaling[39], \textit{OsNPR1} is a key regulator of SA signaling pathway[40], and \textit{OsCOI1}-mediated JA pathway is indispensable for the disease resistance conferred by \textit{OsNPR1}[21]. \textit{OsMPK6} is a major MAP kinase of SA pathway[41]. \textit{OsCOI1b}, \textit{OsNPR1} and \textit{OsMPK6}, as well as PATHOGENESIS-RELATED (PR) genes, such as \textit{OsPR1a}[42], \textit{OsPR1b} and \textit{OsPBZ1}[43], play a fundamental role in a plant's response to pathogen challenge. So we measured the expression of \textit{OsNPR1}, \textit{OsMPK6}, \textit{OsCOI1b}, \textit{OsPR1a}, \textit{OsPR10a} and \textit{OsPR1b} six genes in Ziyu44 and JNXN plants challenged with \textit{M. oryzae} LP33. We observed that the expression levels of these six genes are remarkably up-regulated in both varieties plants at 24 hpi, the expression levels of them were significantly
higher in resistant rice Ziyu44 plants than that in susceptible JNXN plants (Fig. 4). Therefore, we conclude that the increased accumulation of endogenous JA and synchronous activation of SA and JA signaling pathways may be critical in mediating rice Ziyu44 defense responses to *M. oryzae* infection.

**Discussion**

Ziyu44, a *japonica* rice variety of Yunnan Province, has exhibited durable broad-spectrum resistance to rice blast for over 30 years. Previous studies have identified multiple major and minor blast resistance genes and suggested that their combination underlies the broad-spectrum and durable resistance of this cultivar[24–26]. According to the resistance manifestations, the host's resistance to pathogens can be divided into resistance to contact, resistance to infection, and resistance to spread. Although 24 hpi is considered the critical point in *M. oryzae* invasion[44], the significant differences of *M. oryzae* development between Ziyu44 and JNXN was observed as early as 10 hpi (Fig. 1b). At this developmental stage, the tip of the germ tube differentiated into a dome-shaped structure called the appressorium (Fig. 1a). Invasive hyphae formed on JNXN at 22 hpi and spread to neighboring cells at 34 hpi. In contrast, on Ziyu44, invasive hyphae were still restricted in the primary infected cells at 34 hpi and disappeared in some cells at 48 hpi (Fig. 1a). We suggest that a series of early defense responses in Ziyu44 inhibit key aspects of *M. oryzae* development, including the formation and maturation of appressorium, primary infection, and the extension of invasive hyphae, which play important roles in conferring durable resistance to *M. oryzae* on Ziyu44.

Production of reactive oxygen species (ROS) during PTI occurs is critical for successful activation of immune responses against pathogen infection [45]. Rice plants modulate their activities of ROS generating and scavenging enzymes, mainly on NADPH oxidase OsRbohB, by different signaling pathways to accumulate ROS against rice blast [34]. Treating rice plants with SA or methyl-jasmonate (MeJA) can induce ROS accumulation and enhance the resistance against rice blast [46, 47]. The current work documented that Ziyu44 and JNXN plants contain low baseline levels of JA, and that the levels of JA and JA-Ile are dramatically increased after inoculation with *M. oryzae* in Ziyu44 compared with JNXN (Fig. 3b and 3c), the production of H$_2$O$_2$ was also higher in the inoculated leaf cells of Ziyu44 than JNXN, resulting in darker DAB staining at 36 and 48 hpi (Fig. 2), indicating that ROS play an important role in inhibiting the extension of invasive hyphae in Ziyu44. The autofluorescence of rice cells is considered to be associated with plant resistance to rice blast [48]. It is also notable that autofluorescence was also visible in leaf cells of Ziyu44 inoculated with *M. oryzae* Zhong-10-8-14 (a GFP-tagged *M. oryzae* strain) at 22–48 hpi (Fig. 1a). This is probably because the infection of *M. oryzae* caused the accumulation of callose in the cell wall of rice Ziyu44. This speculation needs further experimental verification.

Phytohormones are small molecules produced by plants that govern diverse physiological processes, including the defense against pathogens. Among them, SA, JA, and ethylene (ET) are the archetypal defense hormones and their importance in the hard wiring of the plant innate immune system is well
documented in the model plant *Arabidopsis thaliana*, suggesting that plant innate immunity follows a binary model with SA and JA–ET having opposing influences[12, 14, 18]. In contrast to the simple binary SA versus JA–ET defense model in *A. thaliana*, disease resistance in rice appears to be controlled by a more complicated signaling network that does not support a dichotomy between the effectiveness of the SA, JA, and ET pathways and the lifestyle of a given pathogen[19], JA plays a significant role in *PR* gene induction and defence against *M. oryzae* (a hemibiotrophic pathogen). Such as, exogenous application of JA was able to activate defense gene expression and local induced resistance in rice seedlings against the rice blast fungus[20, 38]; OsBAG4 is a BAG (Bcl-2-associated athanogene) family protein of rice, EBR1 (the rice E3 ubiquitin ligase) targets OsBAG4 and negatively regulates rice immunity, the transcript levels of many *PR* genes involved in rice PTI and the SA and JA pathways were decreased in OsBAG4-RNAi protoplasts, while excessive accumulation of OsBAG4 triggers autoimmunity and enhances rice blast disease resistance in the *ebr1* mutant or OsBAG4-OX (plants overexpressing OsBAG4-Flag), SA and JA signals were synchronously magnified in both of these lines[49]; Mitogen-activated protein kinase (MAPK) cascades play central roles in response to biotic and abiotic stresses. The *mpk15* (knock-out mutant of OsMPK15) mutant lines exhibited an increased resistance to *M. oryzae*, phytohormones SA and JA were accumulated, and the expression of SA- and JA-pathway associated genes were significantly upregulated in *mpk15* mutant rice[50]. In the present study, we observed increased accumulation of endogenous JA, *OsCOI1b* (encodes a JA receptor), *OsNPR1* (encodes a key regulator of SA-signaling pathway), *OsMPK6* (encodes a major MAP kinase of SA pathway), as well as a number of PR genes *OsPR1a*, *OsPR1b* and *OsPBZ1* were markedly up-regulated in resistant rice Ziyu44 upon *M. oryzae* infection, implying the endogenous JA level may be critical in mediating rice Ziyu44 defense responses to *M. oryzae* infection. To increase the level of endogenous JA by genetic manipulation may be effective in improving the resistance to rice blast in *japonica* rice varieties.

**Declarations**

**Author contributions** QL was responsible for research design, funding acquisition and project administration. AXY and HZ conducted out the experiment. QL and AXY performed writing the manuscript and editing. JLL participated in PCR data analysis and drawings. RY participated in RNA isolation and qRT-PCR. QCZ had the idea for the article. MW critically revised the work. AXY and QL were responsible for interpretation of results. All authors read and approved the final manuscript, as well as in the final approval of the version to be published. AXY and HZ contributed equally.

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Figures
Figure 1

Microscopic analysis of early infection events in sheaths of Ziyu44 (ZY44) and JNXN inoculated with M. oryzae isolates. (a) Development of M. oryzae on leaf sheaths of resistant rice Ziyu44 and susceptible rice JNXN. Sheaths were inoculated with spore suspensions of M. oryzae Zhong-10-8-14 (top) and LP33 (bottom), respectively. The inoculated leaf sheaths were examined under a fluorescence microscope 3, 10, 22, 34 and 48 hpi, as indicated. Arrows indicate appressorium. (b) Statistics of fungal infection at 3, 10, 22, 34, and 48 hpi. At least 100 single-cell interaction sites were examined per time point. The scale bar is 20 μm

Figure 2

DAB staining at infection sites of Ziyu44 (ZY44) and JNXN leaves. Tawny shading indicates accumulation of H2O2. The scale bar is 20 μm
Figure 3

The levels of endogenous SA, JA and JA-ile in Ziyu44 (ZY44) and JNXN plants upon M. oryzae infection. CK, untreated control, H2O-24, inoculated with sterile water at 24 hpi, M-24, inoculated with M. oryzae LP33 at 24 hpi, H2O-48, inoculated with sterile water at 48 hpi, M-48, inoculated with M. oryzae LP33 at 48 hpi.

Figure 4

Expression of genes involving in SA and JA biosynthesis in Ziyu44 (ZY44) and JNXN inoculated with M. oryzae LP33. Different letters above the bars indicate significant differences as indicated by ANOVA (P < 0.05). Error bars represent mean ± SD of three independent repeats.
Figure 5

Expression of pathogenesis-related genes in Ziyu44 (ZY44) and JNXN inoculated with M. oryzae LP33. Different letters above the bars indicate significant differences as indicated by ANOVA (P < 0.05). Error bars represent mean ± SD of three independent repeats.