Osa-miR162a balances rice yield and resistance to *Magnaporthe oryzae*

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Abstract

MicroRNAs (miRNAs) play essential roles in rice immunity against Magnaporthe oryzae, the causative agent of rice blast disease. *Osa-miR162a* targets *Dicer-like 1* (*DCL1*) genes, which play vital roles in miRNA biogenesis and act as negative regulators in rice immunity. Here we demonstrate that *Osa-miR162a* improves rice immunity against *M. oryzae* and balances the trade-off between rice yield and resistance. Overexpression of *Osa-miR162a* compromises rice susceptibility to *M. oryzae* accompanying enhanced induction of defense-related genes and accumulation of hydrogen peroxide (H_2O_2). In contrast, blocking miR162 by overexpressing a target mimic of miR162 enhances susceptibility to blast fungus associating with compromised induction of defense-related gene expression and H_2O_2 accumulation. Moreover, the transgenic lines overexpressing *Osa-miR162a* display decreased seed setting rate resulting in reduced yield per plant, whereas blocking miR162 leads to an increased number of grains per panicle, resulting in increased yield per plant. Altered accumulation of miR162 had limited impact on the expression of *OsDCL1*. Together, our results indicate that *Osa-miR162a* improves rice blast resistance and plays a role in the balance of trade-off between resistance and yield.

Background

Plants mount a multi-layered immune system in fighting against the invasion of pathogens, and small RNAs are essential regulators in this process (Katiyar-Agarwal and Jin 2010; Weiberg et al. 2014). miRNA is a subset of non-coding small RNAs of 20–24 nucleotides (nt). miRNAs play vital roles in the regulation of gene expression either by chromatin methyl modification, mRNA cleavage, or translational inhibition (Yu et al. 2017). In plants, miRNAs are transcribed from miRNA genes and subsequently cleaved by the endoribonucleases Dicer-like proteins (DCLs) and processed into approximately 20–24 nt miRNAs (Kurihara and Watanabe 2004). Finally, the mature miRNAs are recruited into ARGONAUTE (AGO) proteins to form miRNA-Induced Silence Complex (miRISC), the critical component required for suppression of target genes (Vaucheret et al. 2004). Obviously, DCLs act as core components during miRNA biogenesis. In turn, the expression of *DCL1* is regulated by miR162 (Wu et al. 2009; Zhou et al. 2010a).

miR162 is a conserved miRNA family involved in multiple abiotic stress responses in plants. For example, miR162b was induced by ABA treatment to improve adaptation to drought stress through suppressing *OsTRE1* (*Trehalase precursor 1*; Tian et al. 2015). In maize, miR162 was responsive to salt stress with enhanced accumulation at 0.5 hours post-treatment of salt, whereas decreased at five and 24 hours post-treatment (Ding et al. 2009). In switchgrass (*Panicum virgatum*), the accumulation of miR162 was significantly changed under drought stress (Sun et al. 2012). In cotton (*Gossypium hirsutum L.*), miR162 was responsive to salinity (Salih et al. 2018). Besides, miR162 also participates in biotic stress responses. For example, virus suppressors, such as *Tombusvirus* p19 and *Cucumovirus* 2b, had a binding preference for miR162 to counteract host antiviral RNA silencing, a plant immune response during viral infection initiation (Pertermann et al. 2018).

In Arabidopsis and rice, miR162 was reported to suppress the expression of *DCL1*, which plays essential roles in miRNA biogenesis and metabolism (Tijsterman and Plasterk 2004; Xie et al. 2004; Zhou et al. 2010b). In Arabidopsis, the loss-of-function mutants of *AtDCL1* showed multiple altered phenotypes associating with the reduction of miRNA levels (Park et al. 2002). Besides, *AtDCL1* was required for miR393-mediated anti-bacterial defenses (Navarro et al. 2006). In rice, strong loss of function of *OsDCL1* led to developmental arrest at the seedling stage, whereas weak loss of function of *OsDCL1* resulted in pleiotropic developmental defects, including dark green, dwarfism, and different leaves and root phenotypes in comparison with control (Liu et al. 2005). Besides the roles in rice development, *OsDCL1* also regulates rice resistance against pathogens. For example, the silence of *DCL1* enhanced resistance to the virulent Magnaporthe *oryzae* strains with improved defense responses, including hydrogen peroxide accumulation and cell death at the infected sites (Zhang et al. 2015). In contrast, activation of *OsDCL1a* enhances susceptibility to fungal pathogen *Fusarium fujikuroi* and *M. oryzae* accompanying with compromised pathogen-inducible defense responses and impacts miRNA network (Salvador-Guirao et al. 2019). These results indicate that *OsDCL1* regulates rice growth and immunity against *M. oryzae*. Although miR162 suppresses the expression of Os*DCL1* (Zhou et al. 2010b), the effect of *Osa-miR162a* on rice blast resistance and yield is unclear. It is also unknown whether there are other target genes of miR162 involved in these processes.

In rice, the miR162 family has two isoforms, namely *Osa-miR162a* and *Osa-miR162b*. Previously, we found that *Osa-miR162a* was responsive to *M. oryzae* in both susceptible and resistance accessions (Li et al. 2014). To elucidate the roles of miR162 in rice growth and blast disease resistance, we constructed transgenic lines overexpressing miR162a and a target mimic of miR162, respectively. We examined the blast disease phenotypes and yield traits in these transgenic lines. Our data demonstrate that overexpression of miR162a led to enhanced resistance but penalized yield, whereas blocking miR162 via a target mimic resulted in suppressed resistance but improved yield. Besides, altered accumulation of miR162 showed limited impact on *OsDCL1* expression. Overall, our results indicate that *Osa-miR162a* positively regulates rice resistance agianst *M. oryzae* and the trade-off between resistance and yield via *OsDCL1*.

Results

miR162a is up-regulated in both susceptible and resistant accessions by *M. oryzae*

To explore the involvement of *Osa-miR162a* in rice blast resistance, we examined the amounts of *Osa-miR162a* in a highly susceptible accession Lijiangxin Tuan Heigu (LTH) and a resistant accession IRBLKm-Ts. LTH is sensitive to over 1,300 regional isolates of *M. oryzae* worldwide and often used as a susceptible reference in blast disease assay (Lin et al. 2001). IRBLKm-Ts carries a resistance (*R*) locus *Pi-km* and exhibits resistance to *M. oryzae* isolates carrying *Avr-Pikm* (Tsumematsu et al. 2000). We identified the disease phenotype of LTH and IRBLkm-Ts by spray-inoculation of *M. oryzae* strain Guy11. LTH showed susceptible phenotype, whereas IRBLkm-Ts showed resistance (Fig. 1a). We then examined the expression of miR162a in both accessions. LTH displayed unchanged or decreased miR162a abundance following the inoculation of Guy11 in comparison with mock samples. In contrast, IRBLKm-Ts showed reduced accumulation at 12 and 48 hpi but increased at 24 hpi (Fig. 1b), indicating that miR162a was involved in rice-*M. oryzae* interaction and possibly played positive roles in rice blast disease resistance.

Overexpression of *Osa-miR162a* enhances rice resistance to *M. oryzae*

To explore the roles of miR162a in rice blast resistance, we constructed transgenic lines overexpressing *Osa-miR162a* (OX162). We got over 20 transgenic lines displaying increased miR162 abundance in comparison with wild type (WT) control. We selected two lines showing moderate accumulation of miR162 and producing seeds, namely OX162-11 and OX162-24, for subsequent study (Fig. 2a). We then examined blast disease phenotype of these lines following the punch-inoculation of virulent strains, namely GFP-tagged Zhong 8-10-14 (GZ8) and 97-27-2. OX162 lines displayed compromised susceptibility with smaller disease lesions than control (Fig. 2b). Consistently, the fungal biomass in OX162 was significantly less than that in control (Fig. 2c). Then we examined the disease phenotype of OX162 by spray-inoculation of GZ8 and got the results similar to that by punch-inoculation (Additional file 1: Figure S1). Moreover, we also identified the resistance of OX162 against four *M. oryzae* strains isolated by punch-inoculation. Similarly, OX162 displayed smaller disease lesions than control (Additional file 2: Figure S2). These results indicate that miR162a improves rice blast resistance.

To understand how OX162 lines suppress fungal growth, we observed the infection process of GZ8 in the leaf sheath. At 24 hpi, more than 60% of spores formed invasive hyphae in sheath cells of WT. However, in OX162, we only observed many appressoria (Fig. 2d). At 36 hpi, the hyphae started to infect the neighbor cells near the local infected cell of WT but were limited in the local infected cells of OX162 (Fig. 2d). The quantification assay confirmed that overexpression of miR162 delayed the infection progress (Fig. 2e).

To understand why overexpression of miR162 delayed the infection of *M. oryzae*, we examined immune responses, such as hydrogen peroxide (H_2O_2) accumulation and expression of resistance-related genes in OX162 in comparison with control. OX162 showed greatly more H_2O_2 than WT upon GZ8 inoculation (Fig. 3a). Four defense-related genes, namely *ENT-KA RENE SYNTHASE 4* (*KS4*), *NAC DOMAIN-CONTAINING PROTEIN 4* (*NAC4*), *PATHOGENESIS-RELATE GENG 1A* (*PR1a*), and *PR10b*, were examined by RT-qPCR. *KS4* is an early-induced basal defense-related gene (Park et al. 2012). *NAC4* is involved in plant cell death and highly expressed in a lesion mimic mutant spl11 at the lesion forming period (Yin et al. 2000). *PR1a* and *PR10b* are pathogenesis-related genes (Yamaguchi et al. 2013). The expression of *KS4* and *NAC4* was induced to higher levels in

OX162 than in control at 12 hpi (Fig. 3b and c). Similarly, the expression of *PR1a* and *PR10b* was significantly induced to higher levels at 12 and 48 hpi than that in control (Fig. 3d and e). These data indicate that overexpression of miR162a improves the immune responses triggered by *M. oryzae.*

Blocking miR162a leads to increased blast disease susceptibility

To confirm the roles of miR162a in rice immunity again blast fungus, we constructed the transgenic lines overexpressing a target mimic of miR162a (MIM162). MIM162 contained a sequence reversely complementary to miR162a with three nucleotides insertion between 10 to 11 nucleotide. Therefore, the target mimic acted as a sponge to absorb miR162 and block its binding to target genes (Additional file 3: Figure S3). We then examined miR162 abundance and *OsDCL1a* mRNA amounts in MIM162. MIM162 displayed significantly less accumulation of miR162 than control (Fig. 4a). Next, we conducted a disease assay on MIM162. As expected, MIM162 showed enhanced susceptibility to *M. oryzae* with larger disease lesions and supported more fungal growth than control by punch- and spray-inoculation, respectively (Fig. 4b and c, and Additional file 1: Figure S1). We also examined the disease phenotypes of MIM162 against four *M. oryzae* strains by punch-inoculation. MIM162 displayed larger disease lesions and supported more fungal growth than control (Additional file 2: Figure S2).

Moreover, the infection process of GZ8 in MIM162 was faster than that in control. In WT plants, a few of spores formed invasive hyphae in sheath cells at 24 hpi and started to infect the neighbor cells at 36 hpi. In contrast, in MIM162, many hyphae had invaded into neighbor cells at 24 hpi, and more than 60% hyphae invaded more cells at 36 hpi than that in WT (Fig. 4d and e). These results indicate that blocking Osa-miR162a facilitates the *M. oryzae* invasion.

Blocking miR162 also led to compromised immune responses. WT plant showed visible H_2O_2 accumulation in leaves and cells where *M. oryzae* appressorium located, whereas MIM162 displayed little H_2O_2 amounts at the invaded sites (Fig. 5a). The induction of defense-related genes, *KS4*, *NAC4*, *PR1a*, and *PR10b*, was lower in MIM162 than that in WT. These data indicate that blocking miR162 compromises immune responses against *M. oryzae* (Fig. 5b-d).

miR162a regulates rice yield traits

Besides blast disease resistance, we found that miR162a also regulated agronomic traits. Both OX162 and MIM162 exhibited comparable phenotypes with WT, including gross morphology, panicle number per plant, and panicle length (Fig. 6a and Table 1). However, OX162 showed narrower seeds, resulting in lower seed weight than control, and significantly lower seed setting rate, leading to lower grain number than control (Fig. 6b and Table 1). Substantially, OX162 displayed a lower yield per plant (Table 1). In contrast, MIM162 exhibited similar seed size as control (Fig. 6b), slightly lower seed weight (Table 1), lower seed setting rate, but more grains per plant in comparison with control (Fig. 6c and Table 1). As a result, MIM162 showed higher yield per plant than control (Table 1). These results indicate that miR162a is involved in the regulation of rice yield traits and negatively regulates rice yield, and miR162 acts as a regulator in the trade-off between rice blast disease resistance and yield.

miR162 regulates rice blast resistance by suppressing *OsDCL1*

Rice miR162 was reported to target *LOC_Os03g02970* (Zhou et al. 2010b) and was predicted to target *LOC_Os03g15230* (http://plantgrn.noble.org/psRNATarget/). *LOC_Os03g02970* encodes OsDCL1a, and *LOC_Os03g15230* encodes an expressed protein OsDUF292 (domains of unknown function protein 292; Additional file3：Figure S3b). We examined the expression of miR162 and the two genes in OX162 and MIM162. We tested 11 independent OX162 lines and 11 independent MIM162 lines, respectively. Eight of the 11 tested OX162 lines showed significantly higher miR162 accumulation (Additional file3：Figure S3c). Conversely, the mRNA amounts of *OsDCL1a* decreased in the eight OX162 lines (Fig. 7a). Besides, among the 11 tested MIM162 lines, ten lines displayed lower miR162 abundance (Additional file3：Figure S3d). Conversely, the mRNA amounts of *OsDCL1a* increased in eight of the 11 MIM162 lines, except MIM162-22 and MIM162-23 showing unchanged *OsDCL1a* accumulation (Fig. 7b). These results confirmed the regulation of miR162a on *OsDCL1*. Intriguingly, the mRNA levels of *OsDCL1a* decreased slightly in all the eight lines overexpressing miR162 (Fig. 7a). Even in the four OX162 lines showing over 40-fold amounts of miR162a (Additional file3：Figure S3c), the mRNA levels of *OsDCL1a* only decreased less than 40% of that

of control, indicating that the down-regulation of *OsDCL1a* is limited by increasing the abundance of miR162. However, the mRNA levels of *OsDUF292* were markedly higher in both OX162 and MIM162 (Additional file3：Figure S3e and f), suggesting miR162 may not directly target *OsDUF292*.

In a previous report, the silencing of *OsDCL1* led to enhanced rice resistance to blast fungus and increased H_2O_2 accumulation and cell death at the infection sites, suggesting OsDCL1 negatively regulates rice blast disease resistance (Zhang et al. 2015). In this study, we confirm the role of *OsDCL1* in rice blast resistance by inoculation of GZ8 on an *OsDCL1* RNA inference line (DCL1i, Liu, 2005 #1062). DCL1i showed significantly lower *OsDCL1* mRNA amounts than control (Fig. 7c). Consistent with the previous report, DCL1i exhibited compromised susceptibility to GZ8 with smaller disease lesions and supported less fungal growth (Fig. 7d and e). These data imply that miR162 positively regulates rice blast disease resistance via suppressing the expression of *OsDCL1*. To further explore the roles of *OsDCL1* in rice resistance, we examined the expression of *OsDCL1* in susceptible accession LTH and resistance accessions IRBLKm-Ts and Yahui2115 upon *M. oryzae* infection, respectively. Yahui2115 is an elite hybrid restorer line carrying several blast resistance genes and widely used in breeding programs (Shi et al. 2015). The mRNA amounts of *OsDCL1* increased in LTH at 24 and 48 hpi of GZ8, whereas decreased or unchanged in IRBLKm-Ts and Yahui2115 (Fig. 7f-h), indicating that down-regulation of *OsDCL1* contributed to resistance in the two accessions. These results indicate that miR162a positively regulates rice immunity against blast fungus by suppressing the expression of *OsDCL1*.

Discussion

MiRNAs play important roles in controlling plant developmental progress and responses to biotic or abiotic stresses (de Lima et al. 2012; Seo et al. 2013). In recent years, rice-*M. oryzae* interaction has become a model in the study of plant-fungi interaction (Liu and Wang 2016). More than 70 miRNAs were responsive to *M. oryzae* or its elicitors and eleven of which have been identified as regulators in rice-*M. oryzae* interaction (Li et al. 2019b; Zhou et al. 2019). For example, seven miRNAs were identified as negative regulators in rice immunity against *M. oryzae* by disease assays on overexpressing or silencing transgenic lines, namely miR164a, miR169a, miR167d, miR319, miR396, miR444, and miR1873 (Li et al. 2017; Xiao et al. 2017; Xiao ZY 2017; Chandran et al. 2018; Wang et al. 2018; Zhang et al. 2018a; Zhang et al. 2018b; Zhao et al. 2019; Zhou et al. 2019). In contrast, four miRNAs were characterized as positive regulators, namely miR160a, miR166k-miR166h, miR398b, and miR7695 (Campo et al. 2013; Li et al. 2014; Salvador-Guirao et al. 2018; Li et al. 2019a). Here we characterized *Osa-miR162a* as a positive regulator in rice immunity against *M. oryzae*. First, the expression of *Osa-miR162a* was unchanged or decreased in susceptible accession LTH but up-regulated in resistance accession IRBLKm-Ts upon *M. oryzae* infection (Fig. 1). Second, overexpression of *Osa-miR162a* improved rice resistance against *M. oryzae* with higher induction of defense responses (Fig. 2 and 3). In contrast, blocking *Osa-miR162a* by expressing a target mimic compromised rice resistance associating with the suppression of defense responses (Fig. 4 and 5).

Besides the regulation of blast disease resistance, miR162 also regulates rice yield and yield traits. The trade-off between yield and disease resistance is a common phenomenon in crop production. For example, the mutation in *OsSPL28* (*SQUAMOSA promoter-binding protein-like transcription factors 28*) showed enhanced resistance to *M. oryzae* but led to a reduction in rice yield (Qiao et al. 2010). miRNAs may act as the crucial fine-tuning regulators to balance rice blast resistance and yield by regulating the expression of their target genes. For example, miR396 fine-tunes rice blast disease resistance and yield by controlling the expression of *Growth Regulating Factor* (*GRF*) transcription factor genes. The transgenic lines overexpressing miR396 showed enhanced susceptibility to *M. oryzae*, whereas the transgenic lines blocking miR396 exhibited compromised rice susceptibility but increased yield (Chandran et al. 2019). In this study, we demonstrated that *Osa-miR162a* balanced the trade-off between rice blast resistance and yield. Overexpression of *Osa-miR162a* improved rice blast disease resistance but compromised yield. In contrast, blocking of miR162 facilitated the infection of *M. oryzae* but enhanced yield, indicating that the accurate accumulation of miR162a is required for the development of yield traits and manipulation of immune responses.

miR162a may affect the whole miRNA network by target *DCL1*. OX162 displayed blast resistance similar to *OsDCL1* RNAi lines: higher blast disease resistance (Fig. 7) with increased induction of H_2O_2 and cell death at infected sites (Liu et al. 2005; Zhang et al. 2015). In the weak *OsDCL1*-knocking down mutant, more than ten miRNAs were identified as *OsDCL1*-regulated miRNAs for their differential expression in *OsDCL1* RNAi lines and control (Liu et al. 2005; Zhang et al. 2015). For example, the accumulation of eight miRNAs was abolished or greatly reduced in a weak loss of function of *DCL1*-knocking down transformants, including miR156, miR159, miR166, miR167, miR168, miR168-3p, miR396, and miR528 (Liu et al. 2005). Besides, in another *OsDCL1* RNAi lines, the abundance of 12 miRNAs was decreased significantly, including miR156, miR166, miR167, miR168, and miR396 (Zhang et al. 2015). Intriguingly, among these miRNAs, four miRNAs were characterized as regulators in rice-*M. oryzae* interaction, namely miR166, miR167, miR169, and miR396; moreover, four miRNAs were involved in rice development and growth, namely miR156, miR166, miR167 and miR396 (Tang and Chu 2017), suggesting that miR162a might regulate rice development and rice-blast fungus interaction by controlling these miRNAs via *OsDCL1*.

OsDCL1 is required for rice development. *OsDCL1* RNAi lines with heavy loss of function showed developmental arrest at the seedling stage, including shoot and root abnormalities at an early developing stage, wilting of leaves and early senescence at a later stage, and eventually died without seed production (Liu et al. 2005). The lines with weak loss of function also displayed pleiotropic developmental defects but with seed production (Liu et al. 2005). In this study, we found that the OX162 lines with seed production all showed slightly decreased expression of *OsDCL1*. In the OX162 lines showing over 40-fold amounts of miR162a (Additional file3：Figure S3c), the expression of *OsDCL1a* decreased less than 40% of WT (Fig. 7a), whereas, in the MIM162 lines showing great decreasing of miR162 (Additional file3：Figure S3d), the expression of OsDCL1 was increased less than four-fold of that of WT (Fig. 7b). These results indicate that the regulation of *Osa-miR162a* on *OsDCL1* is limited, and certain amount of OsDCL1 is required for rice development and growth, because too lower expression of OsDCL1a is possibly fatal. We selected two OX162 lines showing an appropriate 10% to 20% decrease of *DCL1* amounts and two MIM162 lines displaying less than a 2-fold increase of *OsDCL1a* mRNA levels in comparison with that of control for this study. The two OX162 also showed slight defects in yield traits but enhanced blast disease resistance, suggesting that we could control the abundance of *Osa-miR162a* to manipulate resistance without significant loss of yield.

Conclusions

Collectively, our results shows that *Osa-miR162a* improves rice resistance to *M. oryzae* and balances the trade-off between rice yield and resistance. Overexpression of *Osa-miR162a* leads to improved rice resistance to *M. oryzae* but compromised yield per plant slightly, whereas blocking miR162 by expressing a target mimic of miR162 results in susceptibility but increased yield per palnt. Further study reveals that miR162 had limited impact on the expression of *OsDCL1*. Silencing OsDCL1 leads to the similar resistance phenotype as that caused by overexpression *Osa-miR162a*. Thus, *Osa-miR162a* improves rice blast resistance via *OsDCL1* and can be used to manipulate resistance without significant loss of yield.

Materials And Methods

Plant materials and growth conditions

Rice plants used in this study included susceptible accession Lijiangxin Tuan Heigu (LTH), resistance accessions IRBLkm-Ts and Yahui2115. *Japonica* accession NPB (Nipponbare) was used to construct transgenic lines overexpressing *Osa-miR162a* (OX162) or overexpressing a target mimic of *Osa-miR162a* (MIM162a), respectively. For transformation, fungal inoculation, and defense responses assay, rice plants were planted at a growth room at 26℃ and 70% relative humidity with 14/10-h of day/night light. For yield straits detection, rice plants were planted in paddy yard at a regular season in Wenjiang district, Sichuan Province, China.

Plasmid construction and genetic transformation

The transgenic plants were generated following previous protocols (Li et al. 2017). The sequence of the *Osa-miR162a* gene containing approximate 400 bp upstream and downstream sequences was amplified from NPB genomic DNA with primers

Osa-miR162a-F and *Osa-miR162a*-R (Additional file 4: Table S1). Then the amplified fragment was cloned into *Kpn*I-*Sal*I sites of binary vector 35S-pCAMBIA1300 and got the overexpression construct p35S:miR169a. To construct the target mimicry of *Osa-miR162a*, target mimic sequences of *Osa-miR162a* were formed by annealing with primers MIM162a-F and MIM162a-R (Additional file 4: Table S1) and the annealing double-strand product was inserted into Arabidopsis *IPS1* gene to substitute miR399 target site as described previously (Franco-Zorrilla et al. 2007; Li et al. 2017). The reconstructed *IPS1* sequence was cloned into *Kpn*I-*Sal*I sites of the binary vector pCAMBIA1300, resulting in overexpressing construct p35S: MIM162. The vectors were transformed into NPB by *Agrobacterium* strain EHA105, and the positive transgenic lines were screened with Hygromycin B.

Pathogen infection and microscopy analysis

M. oryzae strains Guy11, 97-27-2, eGFP-tagged Zhong8-10-14(GZ8) were cultured in oatmeal/tomato media at 28°C with 12-h/12-h light/dark cycles. Two weeks later, the hyphae were scratched, and the plates were continuously cultured at 28°C with 24-h light for sporulation. After four days post-inoculation (dpi), spores were collected, and the inoculum concentration was adjusted to 5×10^5 spore ml^{-1} for punch- or spray-inoculation. Three-leaf-stage seedlings were used for spray inoculation following previous reports (Qu et al. 2006). The disease symptom was observed at five dpi. The relative fungal biomass was measured using the DNA amounts of fungal *Mopot2* against rice ubiquitin DNA amounts by reverse transcription-quantitative PCR (Li et al. 2017). GZ8 spores with concentration 2×10^5 ml^{-1} were inoculated on the leaf sheaths to observe the infection process of *M. oryzae* as described previously (Kankanala et al. 2007). The epidermal layer was excised for observation at 24 and 36 hours post-inoculation (hpi). The fungal growth process was analyzed by Laser Scanning Confocal Microscopy (Nikon A1), and the infestation stage was analyzed as described previously (Li et al. 2014). 3, 3'-Diaminobenzidine (DAB) was used to stain H_2O_2 and fungal structures at 48 hpi (Xiao et al. 2003). Leaves were placed in DAB (1 mg /ml, Sigma, ALORICH, USA) and shook at room temperature for 12 h in a dark place, and then decolorized in 90% ethyl alcohol at 65°C for several times. H_2O_2 accumulation and fungal structures were observed with a microscope (Zeiss imager A2).

RNA Extraction and Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) Analysis

Total RNA was extracted from leaves using Trizol reagent (Invitrogen). The RNA quality and quantity were determined by a spectrophotometer (NanoDrop2000 Uvevis). First-strand cDNA was synthesized using the qPCR RT Master Mix with gDNA Remover (TOYOBO). Stem-loop pulse RT-qPCR was performed to analyze the amounts of miR162 with universal reverse primer and specific forward primer listed in Additional file 4: Table S1 following previous reports (Chen et al. 2005; Varkonyi-Gasic et al. 2007). U6 snRNA was used as an internal reference to normalize miRNA levels (Turner et al. 2013). qPCR was performed with specific primers for the detection of gene expression (Additional file 4: Table S1), and the rice ubiquitin (UBQ) gene was selected as an internal reference gene.

Abbreviations

M. *oryzae*:Magnaporthe *oryzae*; miRNAs: MicroRNAs; *DCL1*: *Dicer-like 1*; H_2O_2: hydrogen peroxide; nt: nucleotides; AGO: ARGONAUTE; miRISC: miRNA-Induced Silence Complex; *OsTRE1*: trehalase precursor 1; LTH: Lijiangxin Tuan Heigu; *R:* resistance; OX162: transgenic lines overexpressing *Osa-miR162a*; GZ8: GFP-tagged Zhong 8-10-14; *KS4*: *ENT-KA RENE SYNTHASE 4*; *NAC4*: *NAC DOMAIN-CONTAINING PROTEIN 4*; *PR1a*: *PATHOGENESIS-RELATE GENG 1A*; MIM162: the transgenic lines overexpressing a target mimic of miR162a; OsDUF292 domains of unknown function protein; DCL1i: *OsDCL1* RNA inference line; *OsSPL28*: SQUAMOSA promoter-binding protein-like transcription factors 28; *GRF*: *Growth Regulating Factor*.

Declarations

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Authors' contributions

YL and WW designed the research; XL, XM, HW, YZ, and XL conducted the experiments; TL, YZ, JZ, JZ, YH, MP, HF and JF analyzed the data; YLwrote the article; WW supervised and complemented the manuscript. All authors read and approved the manuscript.

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The data sets supporting the conclusions of this article are included within the article and its addition files.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Table

Table 1: the agronomic traits of OX162 and MIM162

Strain	Plant height (cm)	Panicle number	Panicle length (cm)	Setting Rate (%)	Grain number	1000-grain weight (g)	Yield per plant (g)
NPB	91.4±2.4[a]	9.2±0.44[a]	19.6±0.74[a]	92.7±0.93[a]	833±45[a]	26.9±0.63[a]	22.3±2.35[ab]
OX-11	90.8±2.16[a]	9.4±1.34[a]	19.5±0.63[a]	87.4±1.52[a]	850±74[a]	23.4±0.56[c]	20.7±3.90[b]
OX-24	89.6±2.88[a]	10.6±1.5[a]	19.6±0.7[a]	60.7±7.06[a]	839±65[a]	22.8±0.38[c]	18.3±2.76[b]
MIM-1	94.8±4.02[a]	9.4±0.89[a]	19.8±0.89[a]	90.5±3.14[a]	987±36[a]	25.9±0.38[ab]	24.1±3.35[a]
MIM-16	93.7±4.4[a]	10.4±0.89[a]	19.2±0.94[a]	85.9±0.77[a]	999±71[a]	25.4±0.3[b]	24.4±3.75[a]

Notes: Data are shown as mean ± SD (n = 5). Means labeled with different letters indicate significant difference at 5% level via Tukey-Kramer test.

Figures

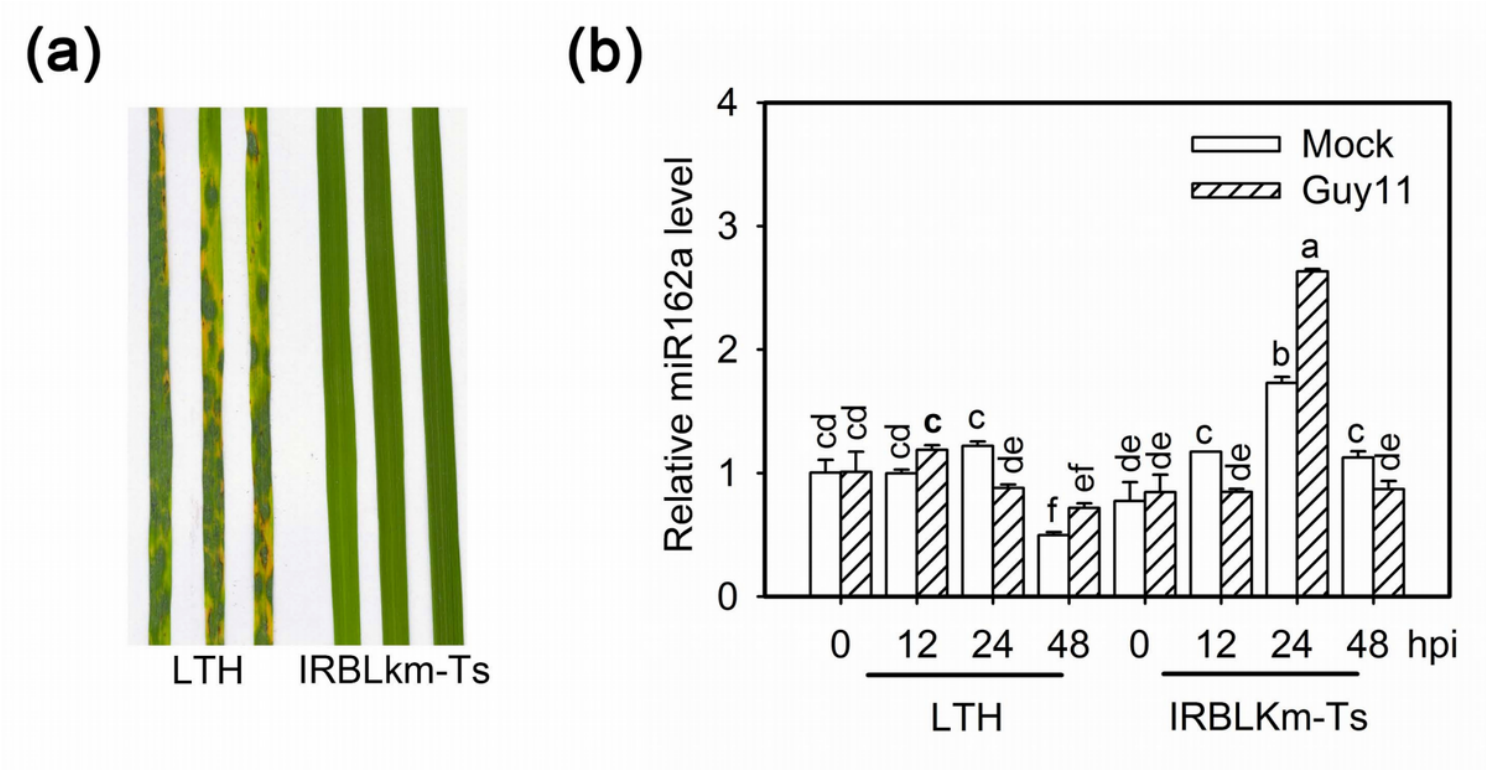

Figure 1

Expression patterns of miR162a is different in the susceptible and resistance accessions. (a) Blast disease phenotype on leaves of susceptible accession LTH and resistant accession IRBLKm-Ts following spray-inoculation of Magnaporthe oryzae Guy11 (1×105spore/ml concentration) at 5 days post-inoculation (dpi), scale bar =1cm. (b) Accumulation of miR162a in indicated accessions with or without Magnaporthe oryzae infection. RNA was extracted at the indicated time points for Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis. SnRNA U6 served as an internal reference. Error bars indicate SD. Different letters above the bars indicate significant difference ($P<0.05$) as determined by a one-way ANOVA analysis. Similar results were obtained in at least two independent experiments.

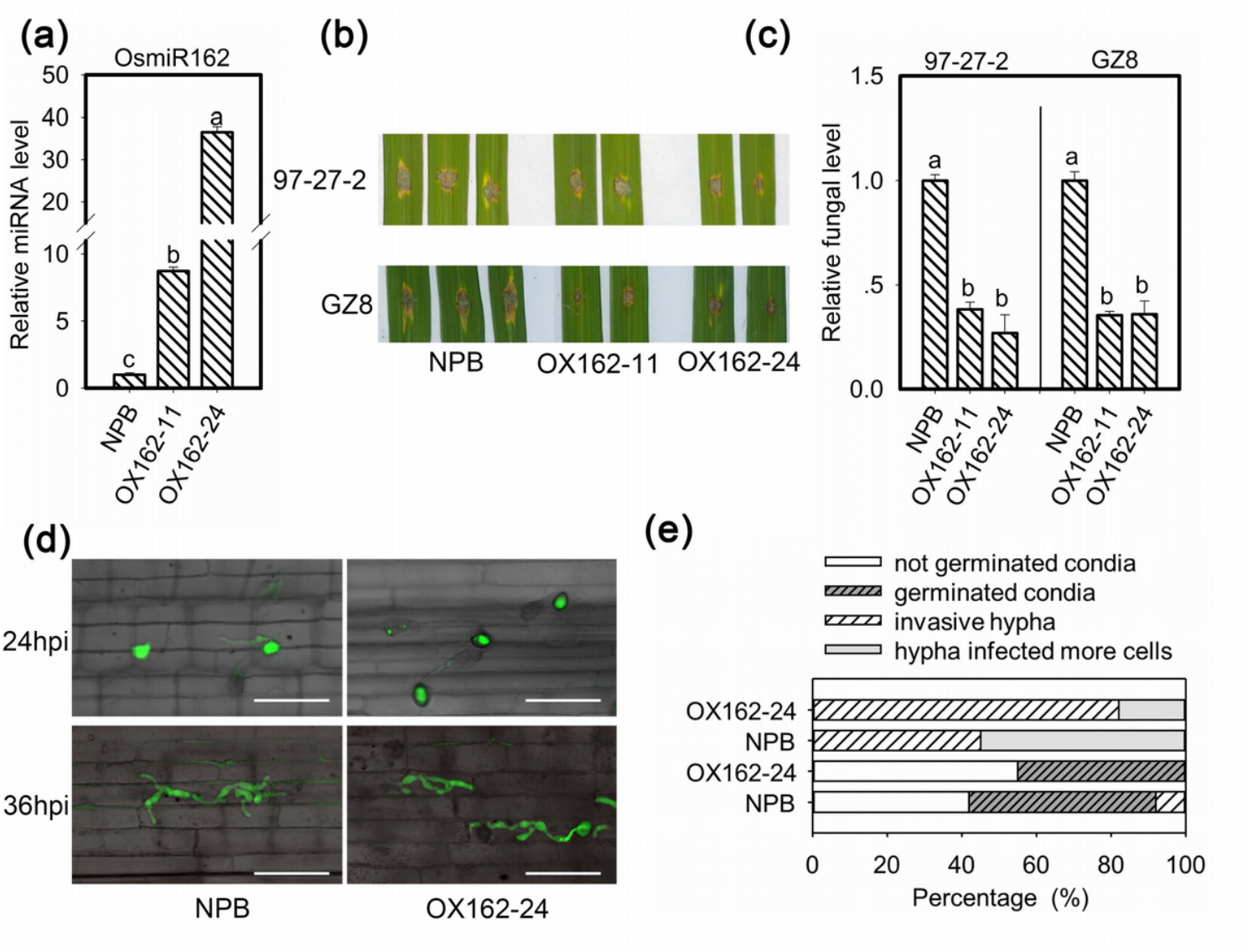

Figure 2

Overexpression of miR162a compromises rice susceptibility to Magnaporthe oryzae. (a) Accumulation of miR162a in transgenic lines harboring 35S:miR162a (OX162). (b) Blast disease phenotypes on leaves at 5 days post-inoculation of M.oryzae strain GZ8 and 97-27-2, respectively. (c) Relative fungal biomass was measured by using the ratio of DNA level of M.oryzae Pot2 genes against rice genomic ubiquitin DNA level. (d) Invasion process of M. oryzae strain GZ8 at 24 and 36 hours post-inoculation (hpi) on sheath cells of indicated lines. Bars=25μm. (e) Quantification analysis on infection process. Over 200 conidia in each line were analyzed. For (a) and (c), Error bars indicate SD (n=3). Different letters above the bars indicate significant differences ($P<0.05$) as determined by One-way ANOVA analysis. Error bars indicate SD. Similar results were obtained in at least two independent experiments.

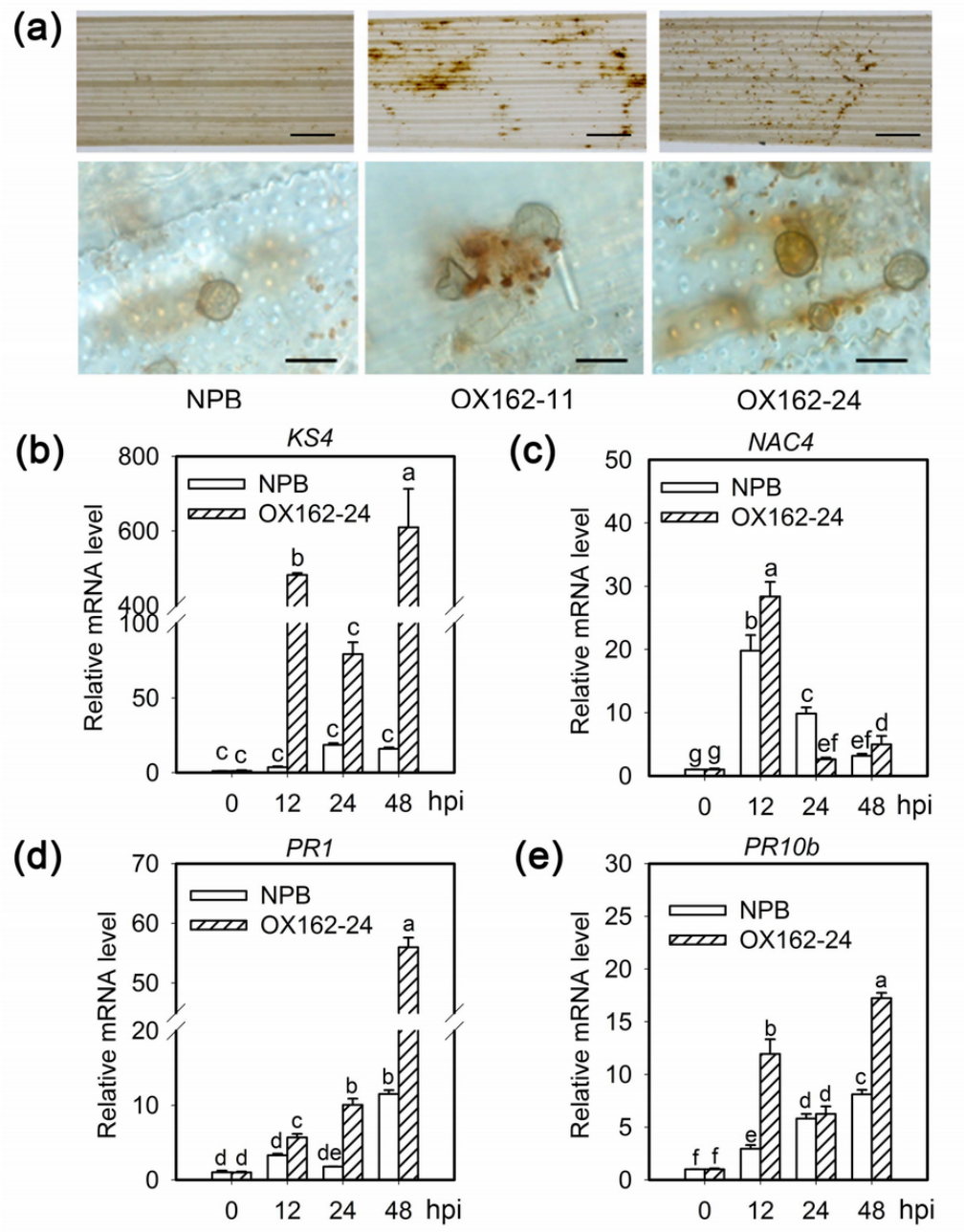

Figure 3

miR162a enhances the induction of disease-related defence responses. (a) 3,3'-diaminobenzidine (DAB) staining showing hydrogen peroxide (H_2O_2) accumulation at 24 hours post-inoculation (hpi) of M. oryzae strain GZ8. The upper photos were taken with a stereo-microscope, scale bars=1mm. The photos at down portion were taken with a microscope (Zeiss imager A2), scale bars=40μm. (b-e), Expression of the defence-related genes (OsKS4, OsNAC4, OsPR1 and OsPR10b) in wild type (Nipponbare, NPB) and OX162 following M. oryzae GZ8 inoculation. The mRNA levels were normalized to that in NPB at 0 hpi. Error bars indicate SD (n=3). Different letters above the bars indicate significant differences ($P<0.05$) as determined by One-way ANOVA analysis. Similar results were obtained in at least two independent experiments.

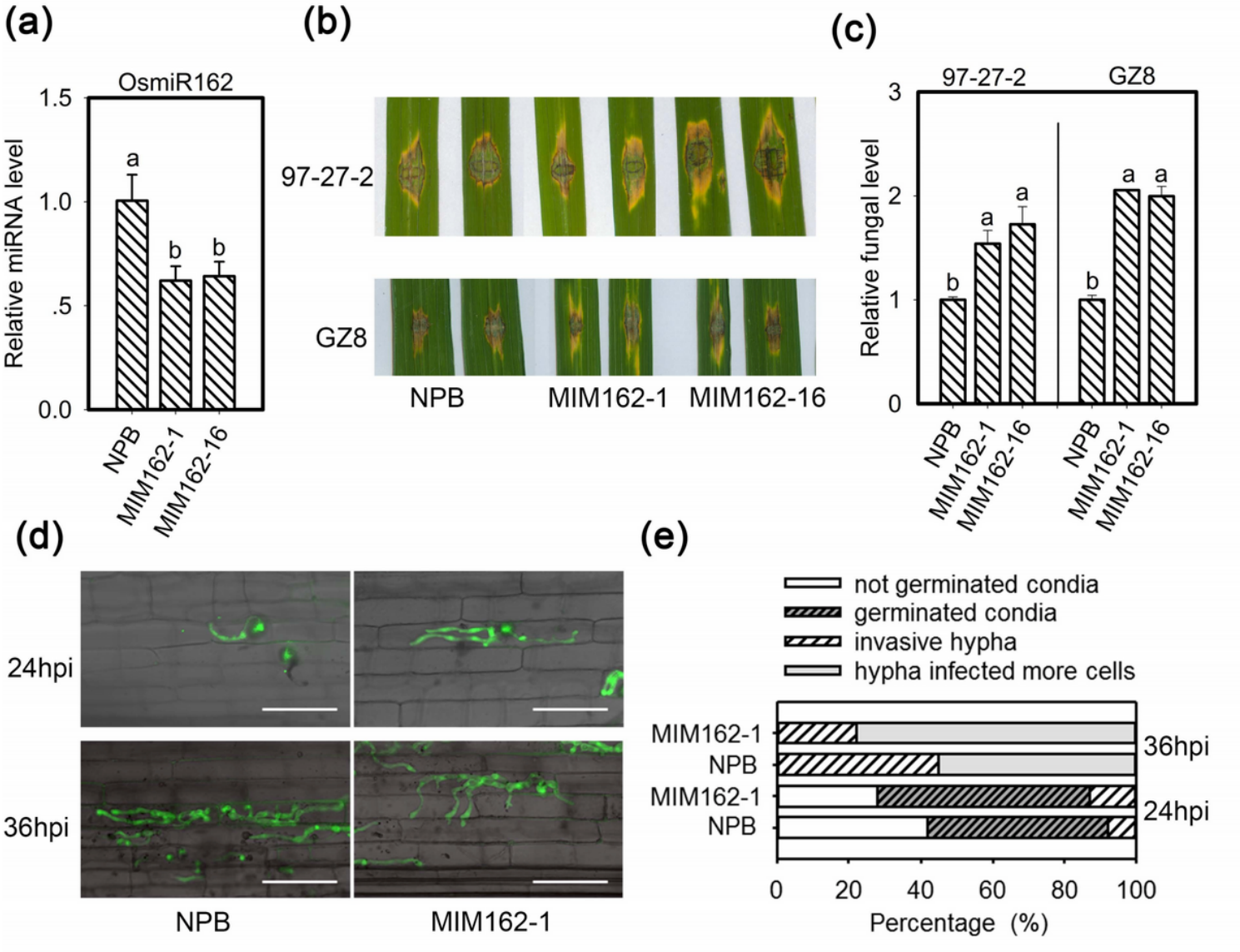

Figure 4

Expression of a target mimic of miR162 (MIM162) enhances rice susceptibility to Magnaporthe oryzae. (a) Accumulation of miR162a in wild type (Nipponbare, NPB) and MIM162. (b) Blast disease phenotypes on leaves of NPB and MIM162 at 5 days post-inoculation (dpi) of M.oryzae strain GZ8 and 97-27-2. (c) Quantification analysis of fungal biomass in (b). Relative fungal biomass was determined by detecting the mRNA level of M.oryzae Pot2 gene against rice Ubiquitin DNA level. (d) Invasion process of GZ8 at 24 and 36 hours post-inoculation (hpi) on sheath cells of the indicated lines. Bars=25μm. (e) Quantification analysis on infection process. Over 200 conidia in each line were analyzed. For (a) and (c), Error bars indicate SD (n=3). Different letters above the bars indicate significant differences (P<0.05) as determined by One-way ANOVA analysis. Similar results were obtained in at least two independent experiments.

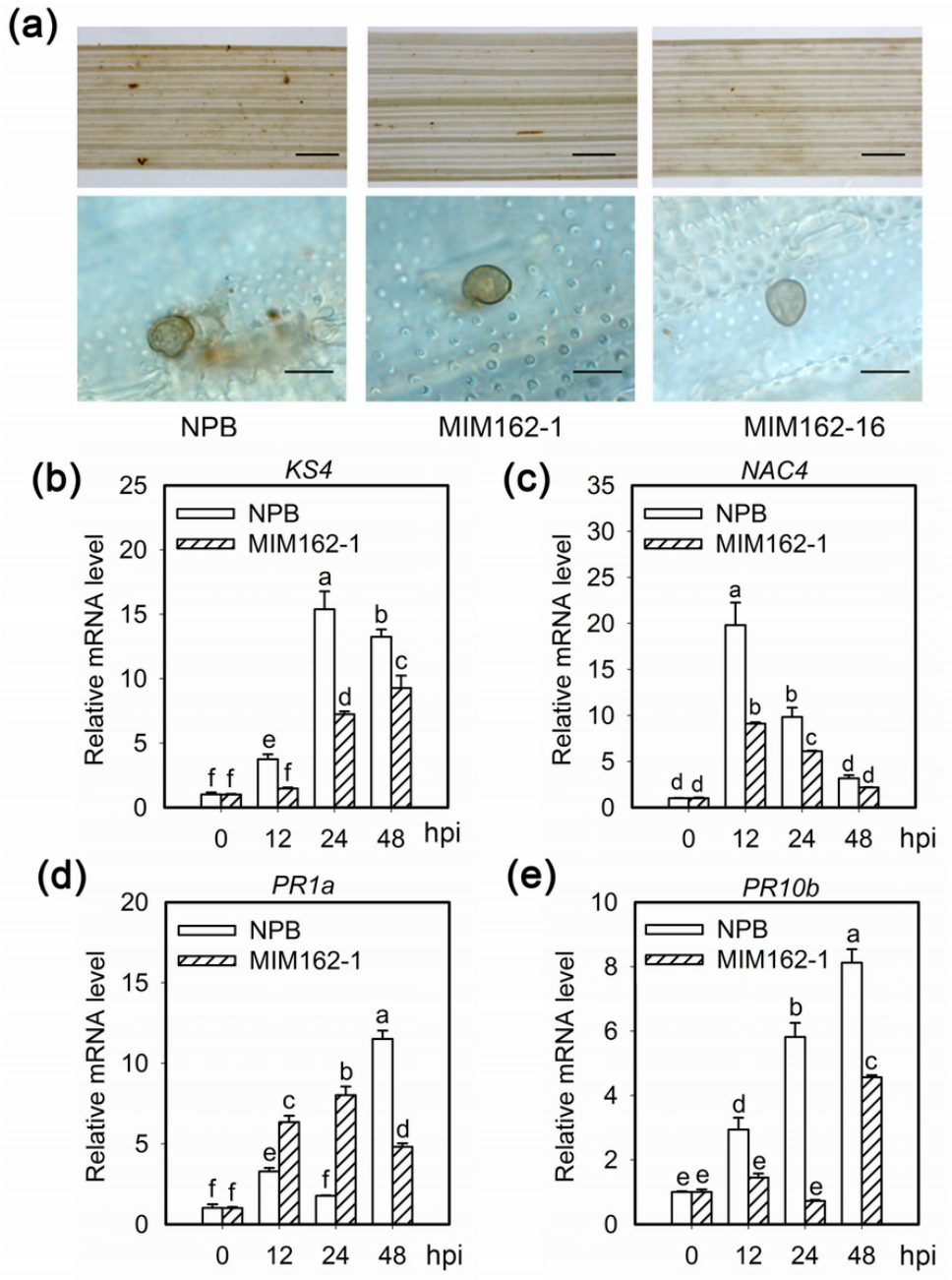

Figure 5

Blocking miR162a compromises induction of disease-related defence responses. (a) 3,3'-diaminobenzidine (DAB) staining showing hydrogen peroxide (H_2O_2) accumulation at 24 hours post-inoculation (hpi) of M. oryzae strain GZ8. The upper photos were taken with a stereo-microscope, scale bars=1mm. The photos at down portion were taken with a microscope (Zeiss imager A2), scale bars=40μm. AP represents appressorium. (b-e) Expression of the defence-related genes (OsKS4, OsNAC4, OsPR1 and OsPR10b) in wild type (Nipponbare, NPB) and MIM162 following GZ8 inoculation. The mRNA levels were normalized to that in NPB at 0 hpi. Error bars indicate SD (n=3). Different letters above the bars indicate significant differences ($P<0.05$) as determined by One-way ANOVA analysis. Similar results were obtained in at least two independent experiments.

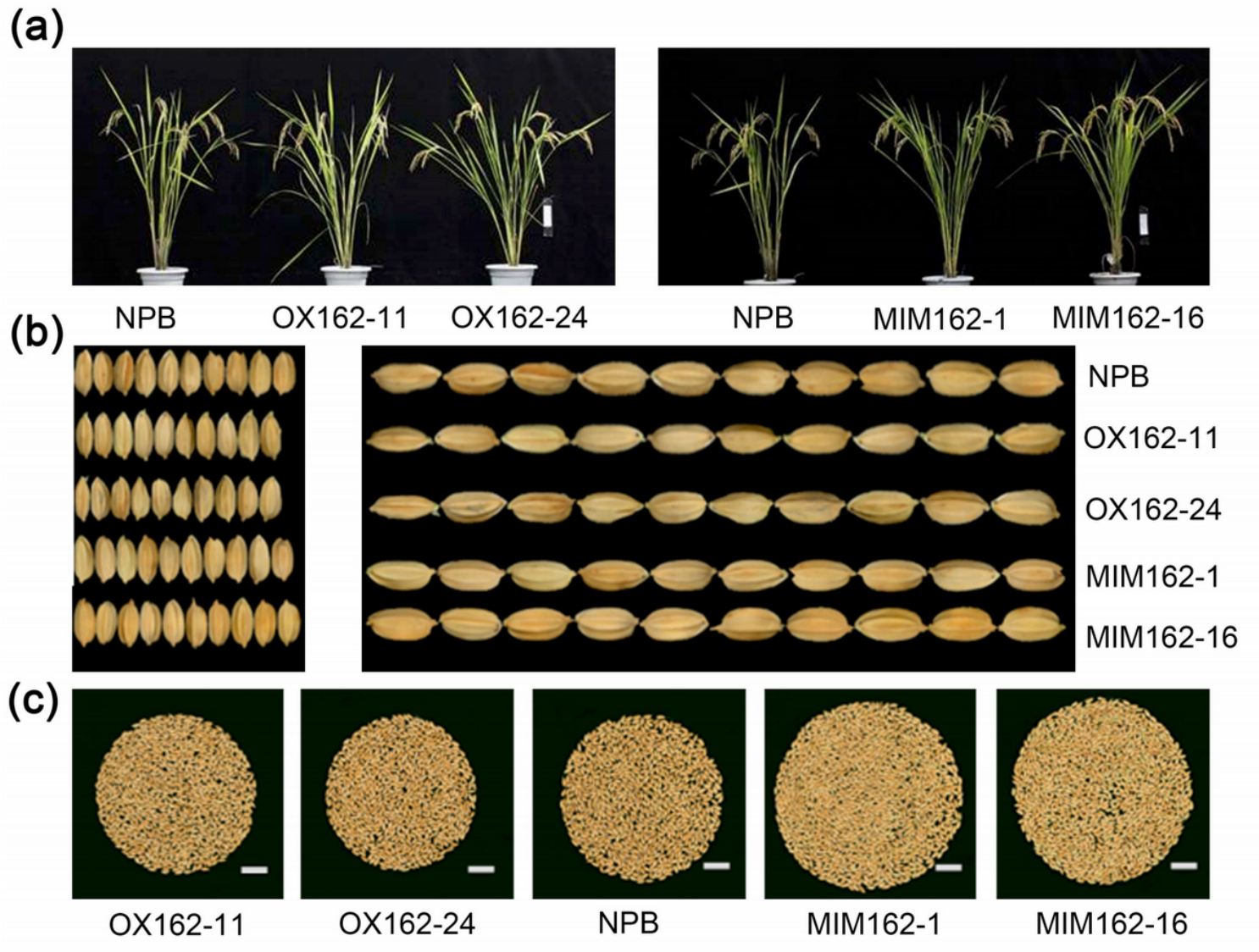

Figure 6

miR162a regulates rice yield straits. (a) Gross morphology of the indicated transgenic lines. Scale bars, 10cm. (b) Photo of grains to show grain length and grain width of indicated lines. (c) Photo of grains per plant of indicated lines. Scale bars, 2cm.

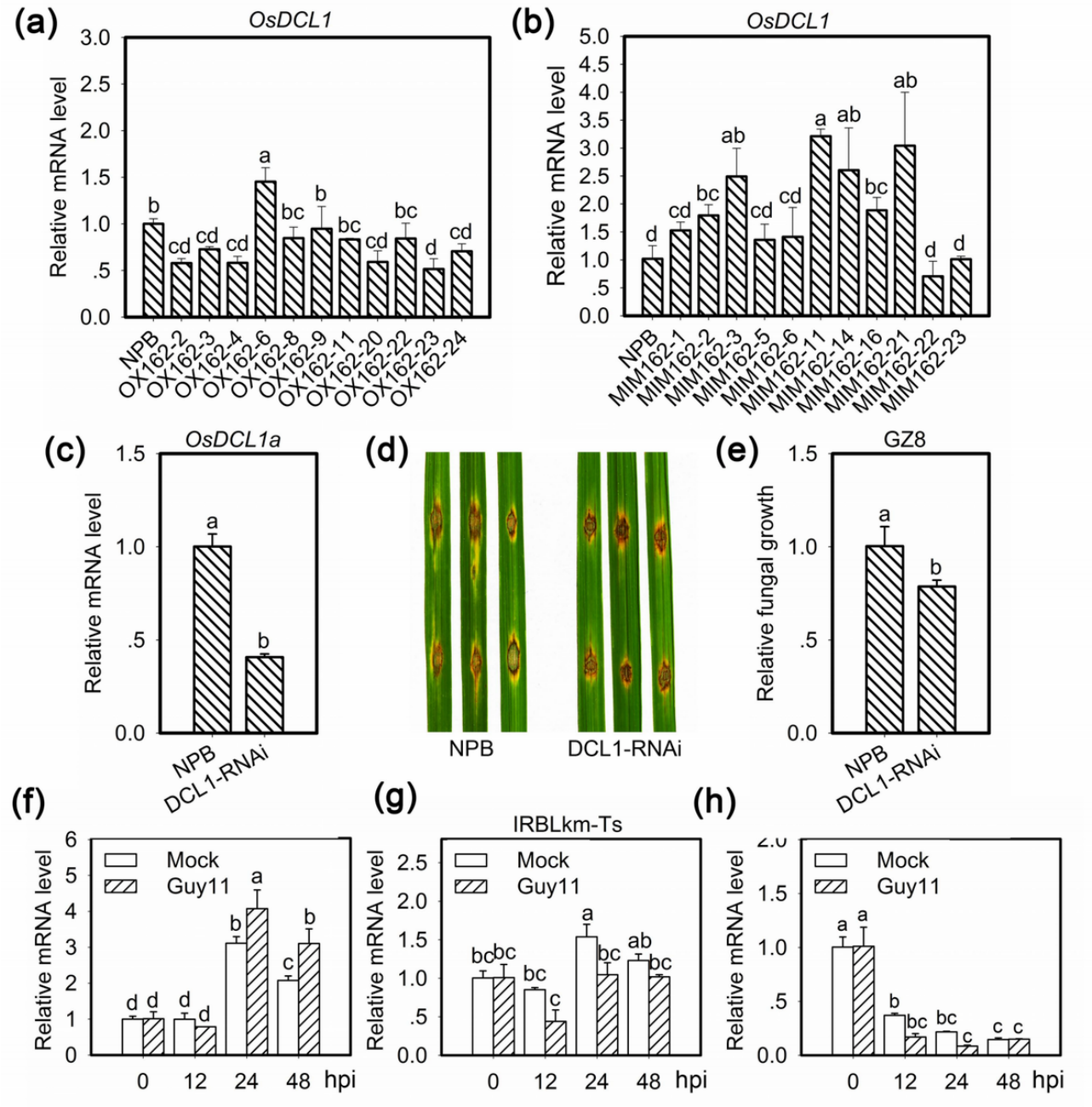

Figure 7

OsDCL1 negatively regulates rice blast resistance. (a and b) Quantitative reverse transcription polymerase chain reaction (RT-qPCR) data indicate amounts of miR162a and mRNA levels of OsDCL1 in OX162 (a) and MIM162 lines (b) in comparison with Nipponbare (NPB). (c) mRNA levels of OsDCL1 in OsDCL1 RNA inference transgenic line (DCL1i) and wild type plants. (d) Blast disease phenotypes on leaves of DCL1i and wild type plants at 5 days post-inoculation (dpi) of M.oryzae strain GZ8. (e) Quantification analysis of the fungal biomass in (c). Relative fungal biomass was measured by using the ratio of DNA level of M.oryzae Pot2 genes against rice genomic ubiquitin DNA level. (f-h) Expression of OsDCL1 in susceptible accession LTH and resistance accessions (IRBLKm-Ts and Yahui2115) with or without M.oryzae strain Guy11 infection. RNA was extracted at the indicated time points for Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis. The relative mRNA levels were normalized with the mRNA level of mock sample at 0 hpi. For (a) and (e-h), error bars indicate SD (n=3). Different letters above the bars indicate significant difference ($P<0.05$) as determined by a one-way ANOVA analysis. All the experiments were repeated two times with similar results.

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