Rice miR1432 Fine-Tunes The Balance of Yield and Blast Disease Resistance Via Different Modules

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Abstract

microRNAs act as fine-tuners in the regulation of plant growth and resistance against biotic and abiotic stress. Here we demonstrate that rice miR1432 fine-tunes yield and blast disease resistance via different modules. The expression of miR1432 is differentially regulated in the susceptible and resistance accessions by the infection of the blast fungus *Magnaporthe oryzae*. Overexpression of miR1432 leads to compromised resistance and decreased yield, whereas blocking miR1432 using a target mimic of miR1432 results in enhanced resistance and yield. Moreover, miR1432 suppresses the expression of *LOC_Os03g59790*, which encodes an EF-hand family protein 1 (*OsEFH1*). Overexpression of *OsEFH1* leads to enhanced rice resistance but decreased grain yield. Consistently, blocking miR1432 or overexpression of *OsEFH1* improves pathogen/microbe-associated molecular pattern- triggered immunity. In contrast, overexpression of *ACOT*, a previously identified target gene of miR1432 involved in the regulation of rice yield traits, has no significant effects on rice blast disease resistance. Altogether, these results indicate that miR1432 balances yield and resistance via different target genes, and blocking miR1432 can simultaneously improve yield and resistance.

Background

In plant-pathogen co-evolution, plants employ two-layered immunity to counterattack the invasion of pathogens, namely pathogen/microbe-associated molecular pattern- (PAMP/MAMP-) triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI is the first layer of plant immunity activated by the recognition of the PAMPs/MAMPs and pattern recognition receptors (PRRs), such as bacterium-derived flg22 and fungus-derived chitin, to effectively protect plants from the invasion of potential pathogens (Boller and Felix, 2009). The typical PTI responses include the activities of MAPK cascades, the influx of $[Ca^{2+}]_{cyt}$, the burst of reactive oxygen species (ROS), the induction of basal defense-related genes and the callose deposition at the infected sites, and so on (Boller and Felix, 2009). However, adapted pathogens can subvert PTI by delivering effectors in host cells (Dou and Zhou, 2012). In turn, plants have involved resistance (R) proteins to recognize these specific effectors resulting in ETI, which offers strong resistance and is often associated with the hypersensitive response (HR) (Cui et al, 2015).

MiRNAs are a category of 20-24-nucleotide (nt) non-coding RNAs expressed from *MIR* genes that regulate target gene expression by sequence-complementary DNA methylation or mRNA cleavage, or translational inhibition (Yu et al, 2017). Based on their roles in the regulation of gene expression, miRNAs act as fine-tuners of various biological processes controlling growth and stress-induced responses (Tang and Chu, 2017). Growing evidence shows that microRNAs (miRNAs) are involved in plant immunity (Padmanabhan et al, 2009, Katiyar-Agarwal and Jin, 2010, Baldrich and San Segundo, 2016, Huang et al, 2016, Tang and Chu, 2017). In Arabidopsis, the PAMP molecule flg22 induces the expression of miR160a and miR393, whereas suppresses the accumulation of other nine miRNAs following the inoculation of the virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) (Navarro et al, 2006, Li et al, 2010). miRNAs are also involved in plant ETI. In Arabidopsis, the amounts of miR863-3p are increased during

ETI triggered by *Pseudomonas syringae* carrying effector avrRpt2 (*Pst* DC3000(*avrRpt2*)). miR863-3p fine-tunes the amplitude and timing of defense responses by suppressing the target genes that play reverse functions in rice immunity. At the earlier infection stage, miR863-3p promotes immunity by suppressing the expression of typical receptor-like pseudokinase1 (*ARLPK1*) and *ARLPK2*, which negatively regulate plant defense (Niu et al, 2016). At a later infection stage, miR863-3p limits immunity amplitude by silencing *SERRATE*, which is required for miRNA accumulation and positively regulates plant defense (Niu et al, 2016).

Rice blast disease caused by *Magnaporthe oryzae* (*M. oryzae*) ranks the first fungal disease threatening food production worldwide. The utilization of disease resistance genes in cultivars generates an economically and environment-friendly strategy for disease control. Intriguingly, miRNAs play important roles in rice resistance against *M. oryzae* (Li et al, 2014, Li et al, 2016). Nowadays, more than 15 miRNAs have been characterized as the regulators of rice blast disease resistance. miR159(Chen et al, 2021), miR160 (Li et al, 2014), miR162 (Salvador-Guirao et al, 2019, Li et al, 2020), miR166 (Salvador-Guirao et al, 2018), miR398(Li et al, 2019), miR7695(Campo et al, 2013), and miR812w (Campo et al, 2021) positively regulate rice resistance against *M. oryzae*, whereas miR156 (Zhang et al, 2020), miR164 (Wang et al, 2018b), miR167(Zhao et al, 2019b), miR169 (Li et al, 2017), miR319 (Zhang et al, 2018), miR396 (Chandran et al, 2019), miR439 (Lu et al, 2021), miR444b.2 (Xiao et al, 2017), and miR1873 (Zhou et al, 2020) negatively regulate rice disease resistance. Among these miRNAs, some and their target genes are involved in both rice immunity and growth. For example, miR162 balances immunity and grain yield via *Dicer-like 1* (*DCL1*). Overexpression of miR162 enhances rice blast resistance whereas compromises yield accompanied by the suppressed expression of *DCL1*; in contrast, blocking miR162a improves yield whereas penalizes immunity associated with enhanced expression of *DCL1* (Salvador-Guirao et al, 2019, Li et al, 2020).

miR1432 is a conserved miRNA family in plants involving in development and defense responses against biotic or abiotic stresses. In barley, the amounts of miR1432-5p increase during barley development (Pacak et al, 2016). In maize, miR1432 is down-regulated in meristem under chilling stress (Aydinoglu, 2020). In wheat, miR1432 in leaves is down-regulated by water deficit in presence of mycorrhizal treatment (Fileccia et al, 2019). In wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*), the expression of miR1432 is induced in root under drought stress (Kantar et al, 2011). In rice, miR1432 is predicted as a key regulator for rice grain-filling and targets *Acyl-CoA thioesterase* (*OsACOT*) (Hu et al, 2018). Blocking miR1432 significantly enhances grain weight resulting in overall grain yield up more than 17% in a field trial via expressing a Short Tandem Target Mimic of miR1432 (STTM1432). Moreover, overexpression of *OsACOT* resembled the yield traits of STTM1432 plants (Zhao et al, 2019a), indicating miR1432 is involved in grain-filling via OsACOT. The expression of rice miR1432 is also responsive to the infection of *M. oryzae* (Li et al. 2014). However, it remains largely unknown how miR1432 coordinates rice immunity and yield traits.

In this study, we constructed the transgenic lines overexpressing miR1432, the lines blocking miR1432 by expressing a target mimic of miR1432, and the lines overexpressing the target genes of miR1432,

OsACOT, and *OsEFH1* (EF-hand family protein 1), respectively. We explored the blast disease resistance and yield traits of these lines. We found that miR1432 negatively regulates rice immunity and yield, whereas blocking miR1432 leads to enhanced blast disease resistance and increased yield. We revealed that *OsEFH1* was targeted by miR1432 and acted as a positive regulator of rice blast disease resistance but a negative regulator of rice yield. Further study revealed that the miR1432-*OsEFH1* module regulated rice PTI responses, whereas *OsACOT* had no obvious effect on rice immunity. Altogether, our results revealed that a miRNA coordinates rice yield and immunity via different target genes that play differential roles, and indicated the capacity of the miR1432-targets module in the improvement of both immunity and yield in rice.

Results

Overexpression of miR1432 compromises rice resistance against blast fungus

In rice, only one *MIR1432* gene was identified locating on chromosome 7. We examined the expression pattern of miR1432 upon *M. oryzae* treatment in a susceptible rice variety Lijiangxin Tuan Heigu (LTH) and a resistant variety International Rice Blast Line Pyricularia-Kanto51-m-Tsuyuake (IRBLkm-Ts). LTH is a *japonica* accession highly sensitive to over 1,300 isolates of *M. oryzae* worldwide, and no major *R* genes are ever identified in it (Lin et al, 2001). IRBLkm-Ts contains an *R* gene locus *Pikm* that mediates ETI against *M. oryzae* strains expressing the avirulence gene alleles *AVR-PikA/ D/ E* (Tsumematsu et al, 2000, Kanzaki et al, 2012). LTH exhibited serious disease lesions following the spray-inoculation of Guy11 (Additional file 1: Figure S1a). In contrast, IRBLkm-Ts only showed a few and small resistance lesions (Additional file 1: Figure S1a). The amounts of miR1432 in LTH were unchanged at 12 hours post-inoculation (hpi) of *M. oryzae* but decreased at 24 hpi, then significantly increased at 48 hpi. Different from that in LTH, miR1432 was slightly fluctuated in IRBLkm-Ts (Additional file 1: Figure S1b). These results indicated that miR1432 is involved in the response of rice to *M. oryzae*.

To investigate the roles of miR1432 in rice immunity, we constructed the transgenic lines overexpressing *MIR1432* (OX1432). We got more than 25 transgenic lines and selected two lines, namely OX1432-22 and OX1432-23, for further study. The two OX1432 lines showed significantly higher miR1432 accumulation than the control Nipponbare (NPB) plants (Fig. 1a). We selected three *M. oryzae* strains for disease assays on OX1432 lines. The strain DZ96 is derived from a paddy yard in the Sichuan Basin, the Southwest of China. RB22 is derived from a paddy yard in North China. GZ8 is GFP-tagged Zhong8-10-14 derived from North China. The OX1432 lines were more susceptible to these strains with significantly larger disease lesions and supported more fungal biomass at punch-inoculation (Fig. 1b-d). These results indicate that miR1432 compromises rice resistance against *M. oryzae*.

Blocking miR1432 results in enhanced rice blast disease resistance

To further investigate the roles of miR1432 in rice immunity, we made transgenic lines expressing a target mimic of miR1432 (MIM1432). MIM1432 blocks miR1432 from suppressing its target genes by forming a double-strand complex with miR1432 (Additional file 2: Figure S2). MIM1432 showed significantly lower miR1432 accumulation than the Nipponbare control (Fig. 2a). We selected three *M. oryzae* strains for disease assays on MIM1432 lines, namely GZ8, 97-27-2, and DZ96. The strain 97-27-2 is a virulence strain derived from a paddy yard in North China. With the treatment of these strains, MIM1432 exhibited enhanced resistance with smaller disease lesions and supported less fungal biomass than that of the Nipponbare control (Fig. 2b-c). Moreover, when inoculated with GZ8, MIM1432 displayed delayed infection progress. At 36 hpi, the invasive hyphae filled the whole primary cells in the Nipponbare control whereas just invaded into the primary cells in MIM1432 (Fig. 2e). At 48 hpi, the invasive hyphae were vigorously grown in NPB, whereas only fewer hyphae grew into the second cell in MIM1432 (Fig. 2d). Quantification analysis revealed that GZ8 became less aggressive in MIM1432 than in NPB control (Fig. 2e). We also conducted disease assays on MIM1432 by spray-inoculation of the virulence strain RB22. MIM1432 exhibited fewer and smaller disease lesions than the Nipponbare control (Additional file 3: Figure S3a). Consistent with the disease phenotype, MIM1432 supported less fungal growth than the Nipponbare control (Additional file 3: Figure S3b). In a summary, blocking miR1432 enhances rice blast resistance and delays the colonization of *M. oryzae*.

Then, we examined the expression of defense-related genes in OX1432 and MIM1432 following inoculation of RB22. The strain RB22 induced the expression of these genes, namely *NAC DOMAIN-CONTAINING PROTEIN 4* (*NAC4*), *ENT-KAURENE SYNTHASE 4* (KS4), and *PATHOGENESIS-RELATED GENE 1a* (*PR1a*) (Li et al, 2020). The expression of these genes was constitutively higher in MIM1432 than that in the Nipponbare control, whereas was unchanged in OX1432 (Additional file 3: Figure S3c), indicating that blocking miR1432 enhanced rice defense responses to fight against *M. oryzae*.

OsEFH1 is targeted by miR1432

We next explored how blocking miR1432 improved rice immunity. miRNAs regulate plant development and defense responses via their target genes. *LOC_Os03g59790* is predicted as a target gene (http://plantgrn.noble.org/psRNATarget/) of miR1432 in rice and encodes an EF-hand family protein (http://rice.plantbiology.msu.edu). We named it *OsEFH1* (Additional file 3: Figure S3). The mRNA levels of *OsEFH1* were decreased in OX1432 whereas increased in MIM1432 in comparison with that in the Nipponbare control (Fig. 3a), suggesting the suppression by miR1432. To confirm the suppression of miR1432 on *OsEFH1*, we made constructs expressing yellow fluorescence protein (YFP)-fused *OsEFH1* (*35S:OsEFH1-YFP*). The YFP intensity and protein level expressed from *35S:OsEFH1-YFP* was decreased following the co-expression of miR1432 in *N. benthamiana*, but recovered when MIM1432 was co-expressed (Fig. 3b-c). These results indicate that miR1432 represses the expression of *OsEFH1*.

OsEFH1 positively regulates rice resistance against M. oryzae

We first examined the expression pattern of *OsEFH1* following *M. oryzae* inoculation. The treatment of *M. oryzae* enhanced the expression of *OsEFH1* in both LTH and IRBLkm-Ts (Additional file 4: Figure S4a),

suggesting the participation in rice blast disease resistance. To further explore the role of *OsEFH1* in rice immunity, we constructed the transgenic lines overexpressing *OsEFH1* (OXEFH1). We selected two transgenic lines displaying a significantly higher expression of *OsEFH1* for disease assay (Fig. 4a). OXEFH1 showed enhanced resistance with smaller disease lesions and supported less fungal growth than the Nipponbare control by punch-inoculation or spray-inoculation of *M. oryzae* strains (Fig. 4b-c; Additional file 4: Figure S4b-c). When inoculated by the virulence strain GZ8, OXEFH1 displayed the delayed infection progress of GZ8 in comparison with that of the Nipponbare control (Fig. 4d; Additional file 4: Figure S4d). Moreover, GZ8 induced more H_2O_2 accumulation in OXEFH1 than that in the Nipponbare control 48 hpi (Additional file 4: Figure S4d). These results indicate that *OsEFH1* improves rice blast disease resistance and defense responses.

miR1432-OsEFH1 module regulates rice PTI responses

EFH proteins are a sort of proteins binding $[Ca^{2+}]_{cyt}$ in cells, the influx of which is an important signaling molecule in PTI (Dodd et al, 2010, Boudsocq et al, 2010) and required for a series of responses downstream such as ROS burst (Ranf et al, 2011). To explore how the miR1432-*OsEFH1* module regulated rice blast resistance, we first examined its subcellular localization and found that the YFP-fused EFH1 (EFH1-YFP) protein is localized in the cytoplasm and around the nucleus when transiently expressed in *N. benthamiana* (Additional file 5: Figure S5). We next tested whether miR1432 and *OsEFH1* were involved in PTI. The accumulation of miR1432 in LTH was decreased at one hpi of chitin, a fungus-derived PAMP, whereas increased at three hpi and recovered at six hpi (Fig. 5a). In contrast, miR1432 was decreased significantly in IRBLkm-Ts at all three detected time points (Fig. 5a). Different from the expression pattern of miR1432, *OsEFH1* was increased in LTH at all detected time points, whereas increased in IRBLkm-Ts at one hpi and decreased subsequently (Fig. 5b). These results indicate that both miR1432 and *OsEFH1* are involved in rice PTI.

We then examined PAMP-triggered ROS burst and callose deposition in these transgenic lines. MIM1432 and OXEFH1 exhibited higher, whereas OX1432 displayed a lower burst of ROS than the Nipponbare control following the treatment of flg22, a well-known bacteria-derived PAMP (Fig. 5c-d). However, we were failed in the detection of chitin-induced ROS in rice. We then examined flg22- and chitin-induced ROS by transient expressing miR1432, MIM1432, and OsEFH1 in *N. benthamiana*, respectively. We obtained a similar result as those in rice triggered by flg22. Both flg22 and chitin triggered higher ROS accumulation in the leaves overexpressing MIM1432 or *OsEFH1* than that in the control samples overexpressing YFP only, whereas triggered lower ROS in the leaves overexpressing miR1432 (Additional file 5: Figure S6a-b). Moreover, MIM1432 and OXEFH1 exhibited more, whereas OX1432 displayed fewer callose deposits triggered by flg22 and chitin than the Nipponbare control (Fig. 5e-f). These results suggest that the miR1432-*OsEFH1* model regulates PTI responses. While overexpression of miR1432 compromises PTI responses, blocking miR1432 or overexpression of *OsEFH1* improves PTI responses.

OsACOT is not involved in miR1432-regulated rice immunity

Except for *OsEFH1*, *LOC_Os04g35590* was identified as another target of miR1432 and encoded an Acyl-CoA thioesterase (*OsACOT*) in rice (Zhao et al, 2019a). Overexpression of *OsACOT* enhanced rice yield by improving grain size (Zhao et al, 2019a). As expected, the expression of *OsACOT* was suppressed in OX1432 whereas enhanced in MIM1432 in comparison with that in the Nipponbare control (Fig. 6a). We then constructed the transgenic lines overexpressing *OsACOT* (OXACOT), which exhibited significantly higher mRNA levels of *OsACOT* (Fig. 6b). We explored the resistance of OXACOT and found that OXACOT lines displayed the unchanged resistance with similar disease lesions and relative fungal biomass in comparison with the Nipponbare control (Fig. 6c-d). These results indicate that *OsACOT* is not involved in miR1432-regulated blast disease resistance.

miR1432-target modules regulate rice yield traits

Except for the regulation of immunity, miR1432 and its target genes also control rice agronomical traits. Blocking miR1432 or overexpressing miR1432-insensitive *OsACOT* significantly boosted grain weight leading to increased grain yield (Zhao et al, 2019a). In this study, we examined the yield traits of OX1432, MIM1432, OXEFH1, and OXACOT. We observed pleiotropic phenotypes in these transgenic lines planted in a paddy field during the normal growing season from 2018 to 2020 (OXEFH1 and OXACOT lines were only detected in 2020). Rice grain yield was determined by three components, including panicle number, panicle size (relying on grain number per panicle and seed setting rate (SSR)), and grain size. All the transgenic lines exhibited a normal plant architecture except that OX1432 showed significantly shorter plants than the Nipponbare control (Fig. 7a, Additional file 7: Table S1). The yield traits of OX1432, MIM1432, and OXACOT in our study were consistent with the phenotype of the transgenic lines in a previous report (Zhao et al, 2019a). OX1432 displayed slightly decreased panicle number, grain number per panicle, and grain weight resulting in decreased yield per plant with a 2.1 % to 23.5 % reduction in normal rice-growing season from 2018 to 2020 than the Nipponbare control (Fig. 7b-f; Additional file 7: Table S1). Conversely, MIM1432 showed slightly more panicles, more grains per panicle, and heaver grains resulting in significantly increased yield with 2.1 % to 31.5 % increase in three years (Fig. 7b-f; Additional file 7: Table S1). OXEFH1 displayed decreased grain number leading to decreased yield with 8.0 % to 10.6 % reduction, whereas OXACOT showed increased grain number and grain weight leading to slightly increased yield with 8.7 % to 9.2 % increase (Fig. 7b-f; Additional file 7: Table S1). These results indicate that miR1432 controls rice yield by suppressing the expression of different target genes that play negative and positive roles in the regulation of rice yield traits. Altogether, miR1432 coordinates blast disease resistance and yield via different target genes that playing different roles in rice immunity and yield traits.

Conclusions

Altogether, these results reveal that miR1432 fine-tunes rice resistance and yield via different target genes. Overexpression of miR1432 results in decreased rice blast disease resistance accompanied with compromised PTI responses and reduced yield, whereas blocking miR1432 leads to enhanced resistance associated with enhanced PTI responses and increased yield. Further study reveals that *OsEFH1*

positively regulates resistance and PTI responses, whereas negatively regulates yield; in contrast, *OsACOT* has no significant effect on rice resistance but positively regulates yield traits. Thus, miR1432-target gene modules can be used to coordinate resistance and yield in rice production.

Discussion

Plant immunity often restricts yield in crops (Nelson et al, 2018). In a review of disease resistance studies, 56% of the studies reported the trade-offs between resistance and biomass or fecundity (Bergelson and Purrington, 1996). However, increasing evidence indicates that resistance and yield can be simultaneously achieved. For example, fitness costless broad-spectrum disease resistance can be engineered via expression of the Arabidopsis *Non-expressor of Pathogenesis-Related genes1* (*NPR1*) in rice controlled by pathogen-inducible upstream open reading frame (uORF) (Xu et al, 2017). The *Pigm*/*Pi50* locus mediates fitness costless rice blast resistance via an epigenetic regulation (Deng et al, 2017, Su et al, 2015). The transcription factor Ideal Plant Architecture 1 (IPA1) promotes both yield and immunity via phosphorylation-mediated switching of binding specificity to the promoter of *Dense and Erect Panicle 1*(*DEP1*) and *WRKY45* with or without infection of *M. oryzae* (Wang et al, 2018a). Here we showed that miR1432 coordinated rice yield and immunity via different miR1432-target modules. Blocking miR1432 enhanced both rice yield and blast disease resistance accompanied by enhancement of the expression of two target genes playing different roles in the regulation of rice yield traits and immunity. *OsACOT* was high-expressed in whole plant, whereas *OsEFH1* was only low-expressed in seedlings and almost not expressed in panicles (http://rice.plantbiology.msu.edu). Consistent with the spatial expression pattern, *OsACOT* improved yield without significant effect on resistance against blast fungus, *OsEFH1* enhanced resistance with a slight penalty in yield. Therefore, the alteration of miR1432 can be exploited to improve rice yield and immunity simultaneously.

In mice, ACOT7 plays roles in long-chain fatty acid elongation and counter-regulates fatty acid metabolism in neurons (Ellis et al, 2013). In rice, acyl-CoA thioesterases participate in the biosynthesis of medium-chain fatty acids and the regulation of lipid metabolism by hydrolyzing Acyl-CoA into free fatty acids and CoA. *OsACOT* was characterized as the 13th member of the acyl-CoA thioesterase superfamily (Zhao et al, 2019a, Ying et al, 2012). Overexpression of a miR1432-insensitive *OsACOT* leads to altered compositions of fatty acids, especially C:16 to C:18 in rice seeds, suggesting the role of *OsACOT* in the regulation of lipid and fatty acid metabolism (Zhao et al, 2019a). The metabolism of lipid and fatty acids is essential for endomembrane system organization, which played a key role in plant growth and development. For example, The Golgi, which belonged to the endomembrane system, was responsible for storage protein trafficking to the protein storage vacuole in rice endosperm cells (Liu et al, 2013). Conversely, the defects of endomembrane resulted in abnormal starch structure in rice endosperm cells and decreased grain filling (Wang et al, 2010). As a result, miR1432 manipulated rice grain filling by controlling the expression of *OsACOT*. However, the increase of grain weight of MIM1432 and OXACOT lines in our study was not as significant as that in the previous report (Zhao et al, 2019a). The possible reasons were that the suppression on miR1432 offered by MIM1432 in our study was not as effective as the suppression offered by STTM1432, and the *ACOT* overexpressed in our study was not a mutant that

could avoid the suppression by miR1432. As a result, although the MIM1432 and OXACOT lines showed increased yield, the increased levels in OXACOT lines were not as remarkable as that in the previous report. Moreover, MIM1432 exhibited significantly increased yield than OXACOT, suggesting that some other target genes of miR1432 were possibly involved in the regulation of yield.

In this study, we showed that miR1432-*OsEFH1* module regulates rice blast disease resistance and PTI responses. EFH family members contain the specific Ca^{2+}-binding motif (helix-loop-helix structure, called EF-hand motifs), and act as Ca^{2+}-sensor proteins to regulate the concentration of Ca^{2+} in plant cells (Zielinski, 1998, Gifford et al, 2007). The Ca^{2+} influx is a core event for PTI (Dodd et al, 2010, Boudsocq et al, 2010) and rice blast disease resistance (Wang et al, 2019). The recognition between PAMPs and plant receptors triggers a series of defense responses, including an increase of cytoplasmic calcium ($[Ca^{2+}]_{cyt}$), a burst of reactive oxygen species (ROS), and the expression of defense-related genes (Boller and He, 2009). The elevation of $[Ca^{2+}]_{cyt}$ is implemented by Ca^{2+} influx from apoplast and intracellular stores (Thor and Peiter, 2014). A recent study revealed that the ZAR1 resistosome is incorporated into planar lipid-bilayers and acted as a Ca^{2+} channel, and the activation of ZAR1 led to Glu11-dependent production of reactive oxygen species (ROS) (Bi et al, 2021). Blocking miR1432, or overexpression of *OsEFH1* enhances PTI responses, indicating that miR1432 regulates rice immunity by controlling PTI responses via *OsEFH1*. However, it is still unclear that whether *OsEFH1* directly binds Ca^{2+} and regulates Ca^{2+} influx. Moreover, overexpression of *OsEFH1* results in decreased grain number resulting in reduced grain yield, indicating that *OsEFH1* plays a negative role in the regulation of rice yield by suppressing the development of panicles (Fig. 6c). However, how *OsEFH1* negatively regulated rice panicle development was elusive and need further study.

Methods

Plant Materials and Growth Conditions.

The rice (*Oryza sativa* L.) accessions Lijiangxin Tuan Heigu (LTH), International Rice Blast Line Pyricularia-Kanto51-m-Tsuyuake (IRBLkm-Ts), and Nipponbare (ssp. *japonica*) (NPB) were used in this study. For resistance assay, the rice plants were grown in a greenhouse with a 28/23 ± 1°C day/night temperature, 70% relative humidity, and a light/dark period of 14 h/10 h. For yield traits assay, the rice plants were grown in a paddy field in Wenjiang District, Chengdu, China (36°N, 103°E) during the normal rice-growing season from mid-April to late-September.

Plasmid construction and genetic transformation

The transgenic lines were generated following previous protocols (Li, 2017 #1206). To construct the transgenic lines overexpressing Osa-miR1432, the sequence of the *MIR1432* gene containing 213 bp upstream and 247 bp downstream sequences was amplified from NPB total genomic DNA with primers Osa-miR1432-F and Osa-miR1432-R (Additional file 8: Table S2). We cloned the amplified fragment in

binary vector 35S-pCAMBIA1300 and got the construct p35S: MIR1432 overexpressing miR1432. To construct the target mimicry of Osa-miR1432, the target mimic sequences of Osa-miR1432 (GTCGGTGTCATAGTCTCTCCTGAT) containing the cutting sites of restrictive enzymes were formed by annealing with primers MIM1432-*Bam*HI-F and MIM1432-*Bgl*II-R (Additional file 8: Table S2). Then the annealing double-strand fragment was inserted into the Arabidopsis *IPS1* gene to substitute the target site of miR399 at *BamH*I and *Bgl*II sites as described previously (Franco-Zorrilla et al. 2007; Li et al. 2017). We cloned the reconstructed *IPS1*-MIM1432 fragment into the binary vector pCAMBIA1300 and got the construct p35S: MIM1432 overexpressing mimicry of miR1432. Then the vectors p35S: MIR1432 and p35S: MIM1432 were transformed into NPB via Agrobacterium strain EHA105 respectively to acquire the transgenic lines OX1432 and MIM1432. The positive transgenic lines were screened with Hygromycin B.

Trait Measurements.

The agronomic traits were measured from five plants growing in the middle of three rows in the paddy yard, including rice height, panicle number per plant, grain number per panicle, seed setting rate, 1,000-grain weight, and yield per plant. The seeds were harvested at the full-mature stage and dried in a 42°C oven for one week. Then the dried seeds were used to detect the yield traits, including grain number per panicle, seed setting rate, and 1,000-grain weight using an SC-A grain analysis system (Wanshen Ltd., Hangzhou, China). These data were analyzed by a one-way ANOVA followed by post hoc Tukey HSD analysis with significant differences ($P < 0.05$).

RNA extraction and gene expression analyses.

Reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analyses were carried out to examine the accumulation of miR1432 and the expression of indicated genes in rice plants. Total RNAs were extracted from rice leaves using TRIzol reagent (Thermo Fisher Scientific, Chengdu, China) following the manufacturer's instruction. To detect the expression of genes, the first-strand cDNA was synthesized from 1 μg of total RNA using Primescript RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instruction. RT-qPCR was performed using specific primers and SYBR Green mix (QuantiNova SYBR Green PCR Kit, QIGEN, Chengdu, China) with BIO-RAD C1000TM Thermal Cycler (Bio-Rad Inc, Chengdu, China). Rice ubiquitin (*UBQ*) gene was used as an internal reference to normalize the relative expression levels of genes. The accumulation of miR1432 was examined in T0 plants. To determine the amounts of miR1432, total RNA was reverse-transcribed using a miRNA-specific stem-loop RT primer (Additional file 8: Table S2) with the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China), and the RT product was subsequently used as a template for RT-qPCR by using miRNA-specific forward primers and the universal reverse primer (Additional file 8: Table S2). snRNA U6 was used as an internal reference to normalize the relative amounts of miR1432. RT-qPCR analyses were performed with three technical replicates. The $2^{-\Delta\Delta CT}$ method was exploited to analyze the relative expression levels of miRNAs.

Chitin treatment. Three-leaf-stage LTH and IRBLKm-Ts seedlings were sprayed with 10 μg ml^{-1} chitin (Sigma, Merck Life Science (Shanghai) Co., Ltd., Shanghai, China), and the leaf samples were collected at 1, 3, and 6 hpi for RT-qPCR assay.

Pathogen Infection and Microscopy Analysis.

Magnaporthe oryzae strain Guy11, DZ96, RB22, 97-27-2, and the eGFP-tagged strain Zhong8-10-14 (GZ8) were used for blast-disease and defense response assays. These *M. oryzae* strains were cultured in plates containing oat-tomato-agar (OTA) medium at 28℃ for two weeks with 12-h/12-h light/dark cycles. After getting rid of the surface mycelia with distilled water, the plates were further incubated for three days to promote sporulation. Then the spores were collected with distilled water and the concentration of which was diluted to $1{\cdot}10^5$ or $5{\cdot}10^5$ conidia mL^{-1} for inoculation.

For the invasive process assay, the diluted spores were inoculated on 5-cm-long leaf sheaths as described previously (Kankanala, 2007 #1048). Then the inoculated epidermal layer was excised and the invasive process, including conidia germination, appressorium development, and invasive hyphae growth, were recorded at 24 to 48 hpi by a Nikon A1 Laser Scanning Confocal Microscopy (Nikon Instruments, Inc., Shanghai, China). The quantitative analysis of the invasive process was conducted following the protocol described previously (Li, 2014 #1068).

For resistance assay, wound- or spray-inoculation was used following a previous report (Kong, 2012 #1469). Briefly, conidia suspension ($5{\cdot}10^5$ conidia mL^{-1}) of indicated strains was wound-inoculated at the wounded sites or spray-inoculated on the three- to five-leaf-stage seedlings. Lesion formation was examined at 4–6 days post-inoculation. The fungal biomass was determined by using the DNA amounts of fungal *Mopot2* against rice DNA amounts of ubiquitin via RT-qPCR (Li et al. 2017).

H_2O_2 accumulation assay

To observe the H_2O_2 accumulation in rice plants, three-leaf-stage seedlings were inoculated with *M. oryzae* strain Guy11 at the concentration of $5{\times}10^5$ conidia mL^{-1}. At 40 hpi, leaves were collected and incubated in 1mg/ ml DAB (Sigma, Merck Life Science Co., Ltd. Shanghai, China) at 22℃ for eight hours at illumination. The DAB-stained leaves were cleaned in 95% ethanol and then observed under a microscope (Zeiss imager A2, Carl Zeiss (Chengdu) Co. Ltd, China).

PTI-related defense responses

The leaves of three to five-leaf-stage rice seedlings were selected to conduct the production of reactive oxygen species (ROS). The leaves of *N. Benthamiana* were used to transiently express miR1432, MIM1432, and *OsEFH1*, respectively. For ROS assay, the leaves were cut with a 5-mm-diameter hole punch and the punched circular leaves were incubated in 200 μL water in a 96-well plate for 12 hours. Then the leaves were treated with or without 1 μM flg22 or 20 μg/ mL chitin in 200 μL buffer containing 20 mM L-012 (Wako, Japan), 10 μg/ mL horseradish peroxidase (Sigma-Aldrich Shanghai Trading Co Ltd, Shanghai, China). The production of ROS was detected using a GLOMAX96 Microplate Luminometer

(Promega Biotech Co., Ltd, Beijing, China) for 30–60 min and determined as relative luminescence units. We examine the PTI-triggered callose deposition in rice following a previous report (Liu et al, 2012). The rice was planted for five days and the first leaves were cut and treated with flg22 or chitin for 12 hours. Then the treated leaves were fixed in ethanol: acetic acid (3:1 [v/v]) solution for five hours and rehydrated in 70% and 50% ethanol for two hours, respectively, and in water overnight. Then the decolored leaves were treated with 10% NaOH for one hour to make the tissues transparent. The transparent leaves were washed three times with water and incubated in the staining buffer containing 150 mM K_2HPO_4, pH 9.5, 0.01% aniline blue (Sigma-Aldrich) for four hours. We used a fluorescence microscope (Zeiss imager A2.0) to capture the images of callose deposition under a UV channel (340 to 380 nm) and calculated the callose deposits using Image J software.

Agrobacterium-mediated transient expression assay in Nicotiana benthamiana

YFP detection and accumulation were conducted following a previous report (Li, 2017 #1206). To generate *EFH1-YFP* reporter fusions, we fused *YFP* with cDNA sequence of *OsEFH1* at its N-terminus. The fused fragments were inserted into *Kpn*I-*Spe*I sites of binary vector 35S-pCAMBIA1300 (*35S: EFH1-YFP*). Then the vector was transformed into Agrobacterium strain GV3101 for agroinfection assay in *N. benthamiana*. In brief, Agrobacterium strain GV3101 harboring the respective expression constructs (*35S: EFH1-YFP*, *35S: miR1432*, *35S: MIM1432*) was incubated at 28°C overnight in liquid LB media containing antibiotics kanamycin (50 mg/ mL) and carbenicillin (50 mg/ mL) on a table shaking at 250 rpm. The Agrobacterium were collected and resuspended in an MMA buffer (10mM MES,10mM MgCl2, 100 mM AS) and infiltrated into leaves of *N. benthamiana* for transient expression assay. Leaves were examined at 48 hpi using a Nikon A1 Confocal Laser Scanning Microscope (Nikon Instruments, Inc., China). The detection of EFH1-YFP fused protein was assayed with BioRad Image soft. The relative protein mass was calculated as the ratio of the mass of *EFH1-YFP* to the mass of HSP.

Abbreviations

LTH
Lijiangxin Tuan Heigu; IRBLkm-Ts:International Rice Blast Line Pyricularia-Kanto51-m-Tsuyuake; NPB:Nipponbare; CRISPR:clustered regularly interspaced short palindromic repeats; OTA:oat-tomato-agar; RT-qPCR:reverse transcription quantitative polymerase chain reaction; LSCM:laser scanning confocal microscopy; OsEFH1:EF-hand family protein 1; OsACOT:Acyl-CoA thioesterase; ROS:reactive oxygen species; PAMP/MAMP:pathogen/microbe-associated molecular pattern; PTI:PAMP/MAMP-triggered immunity; ETI:effector-triggered immunity; PRRs:pattern recognition receptors; HR:hypersensitive response; miRNAs:microRNAs; *M. oryzae*:*Magnaporthe oryzae*; *DCL1*:*Dicer-like 1*; GZ8:GFP-tagged Zhong8-10-14; OX1432:the transgenic lines overexpressing miR1432; MIM1432:the transgenic lines overexpressing a target mimic of miR1432; OXEFH1:the transgenic lines overexpressing *OsEFH1*; OXACOT:the transgenic lines overexpressing *OsACOT*; *NAC4*:*NAC DOMAIN-CONTAINING PROTEIN 4*; *KS4*:*ENT-KAURENE SYNTHASE 4*; *PR1a*:*PATHOGENESIS-RELATED GENE 1a*; SSR:seed setting rate;

NPR1:Non-expressor of Pathogenesis-Related genes1; uORF:upstream open reading frame; IPA1:Ideal Plant Architecture 1; *DEP1*:*Dense and Erect Panicle 1*; WRKY:snRNA:small nuclear RNA.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

All the datasets are included within the article and its additional files.

Competing Interests

All the authors declare no conflict of interests.

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Author contributions

Y. L. and W-M. Wang conceived the experiment, and together with Y-P. Z., X-H. Z., G-B. L., L-L. Z., X-M. Y., X-R. H., Q. F., Y. Z., H. W., J-H. Z., Z-X. Z, and J-W. Zhang carried it out; J. F. and Y-Y. Huang analyzed the data; H.W., M. P., Y-P. Ji and S-X. Zhou carried out the field trial; Y. L. and W-M. Wang wrote the paper.

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Figures

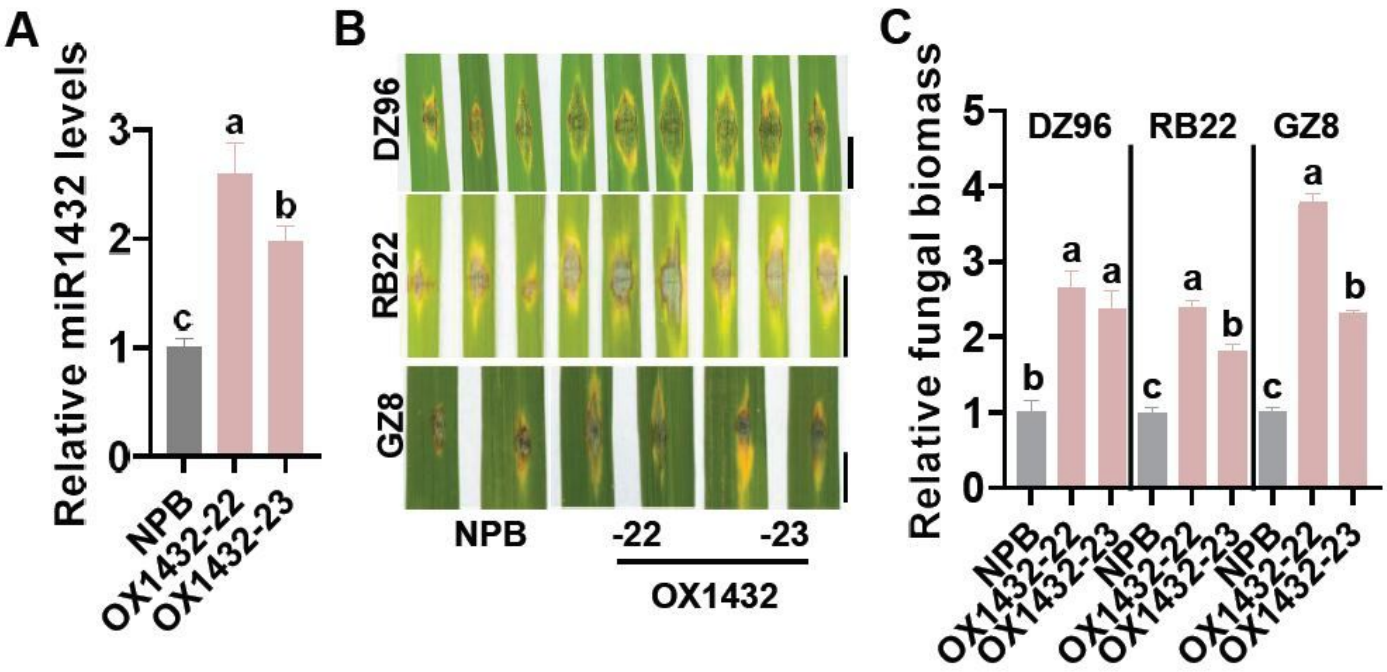

Figure 1

Overexpression of miR1432 enhances rice susceptibility to Magnaporthe oryzae. a The amount of mature miR1432 in the transgenic lines overexpressing MIR1432 gene (OX1432) and the Nipponbare (NPB) control. Reverse-transcription (RT) was carried out with total RNA and an miR1432 specific stem-loop RT primer (Additional file 8: Table S2). The RT product was subsequently used as a template for quantitative polymerase chain reaction (q-PCR) to detect the amounts of miR1432. The amounts of snRNA U6 were examined and used as an internal reference. b The blast disease phenotypes on leaves five days post-inoculation of M. oryzae strains GZ8, RB22, and DZ96, respectively. Bar= 5 mm. c The relative fungal biomass of the indicated strains in OX1432 and the Nipponbare control. The fungal biomass was determined by using the ratio of DNA levels of M. oryzae MoPot2 against the DNA levels of rice ubiquitin. Error bars indicate SD (n= 3 independent samples). Different letters above the bars indicate significant differences ($P < 0.01$) as determined by One-way Tukey-Kramer analysis. Similar results were obtained in at least two independent experiments.

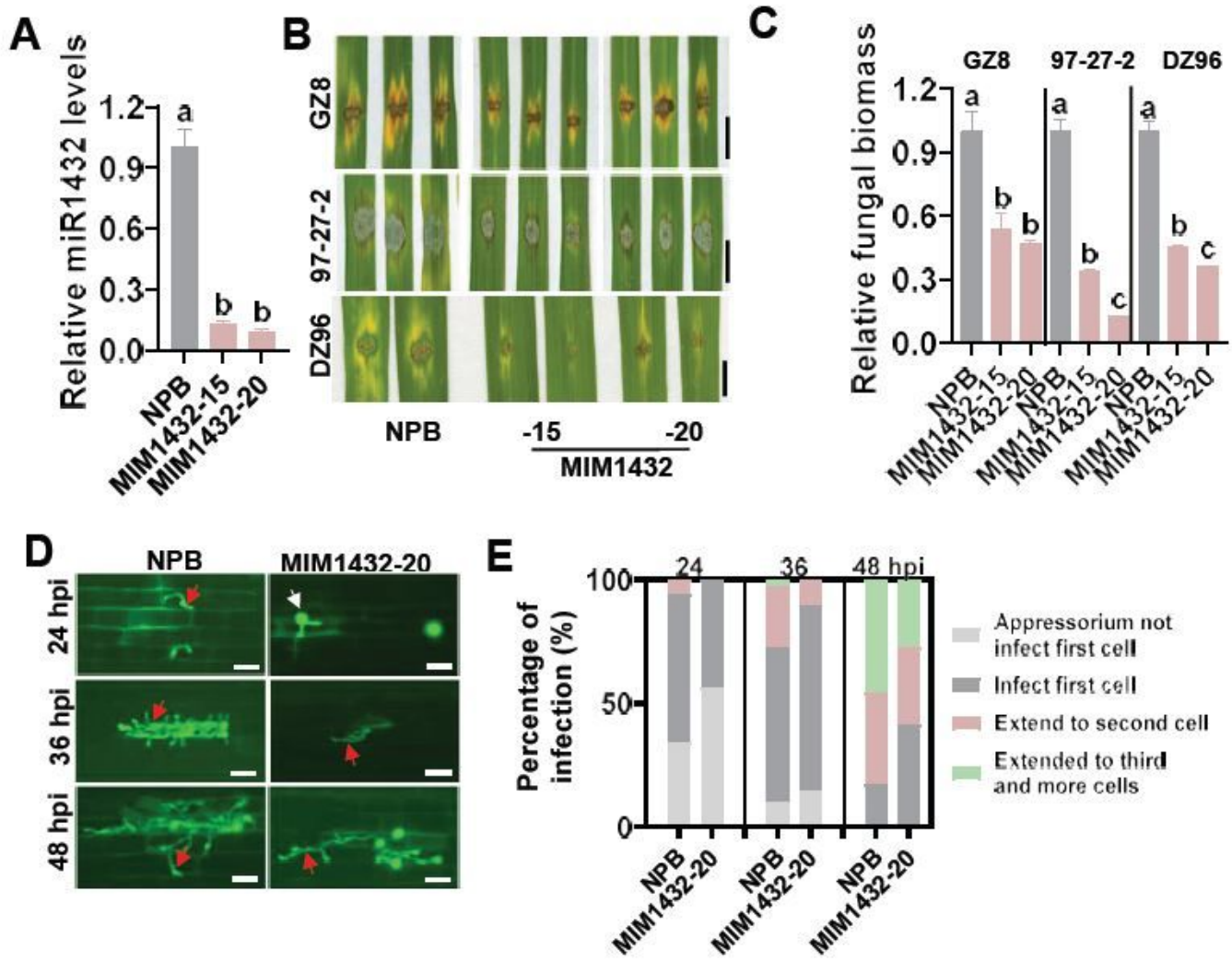

Figure 2

Expression of a target mimic of miR1432 (MIM1432) enhances rice resistance against Magnaporthe oryzae. a The amounts of miR1432 in MIM1432 and the Nipponbare (NPB) control. Reverse-transcription (RT) was conducted with total RNA and an miR1432 specific stem-loop RT primer (Additional file 8: Table S2). The RT product was used as a template for quantitative polymerase chain reaction (q-PCR) to examine the amounts of miR1432. snRNA U6 was served as an internal reference. b The blast disease phenotypes on leaves of MIM1432 and the Nipponbare control at five days post-inoculation of M. oryzae strains GZ8, 97-27-2, and DZ96. Bar= 5 mm. c The relative fungal biomass of the indicated strains on MIM1432 and the Nipponbare control in (B). The relative fungal biomass was determined by using the ratio of DNA levels of the M. oryzae Pot2 against the DNA levels of rice Ubiquitin. d The invasion process of GZ8 at 24, 36, and 48 hours post-inoculation (hpi) in sheath cells of the indicated lines. Bars= 40 μm. The white arrows indicate appressoria formed from conidia, and the red arrowheads indicate invasive hypha in rice sheath cells. e Quantification analysis of the fungal development during the invasive process. Over 200 conidia in each line were analyzed. For a and c, error bars indicate SD (n= 3 independent samples). Different letters above the bars indicate significant differences ($P < 0.01$) as

determined by One-way Tukey-Kramer analysis. Similar results were obtained in at least two independent experiments.

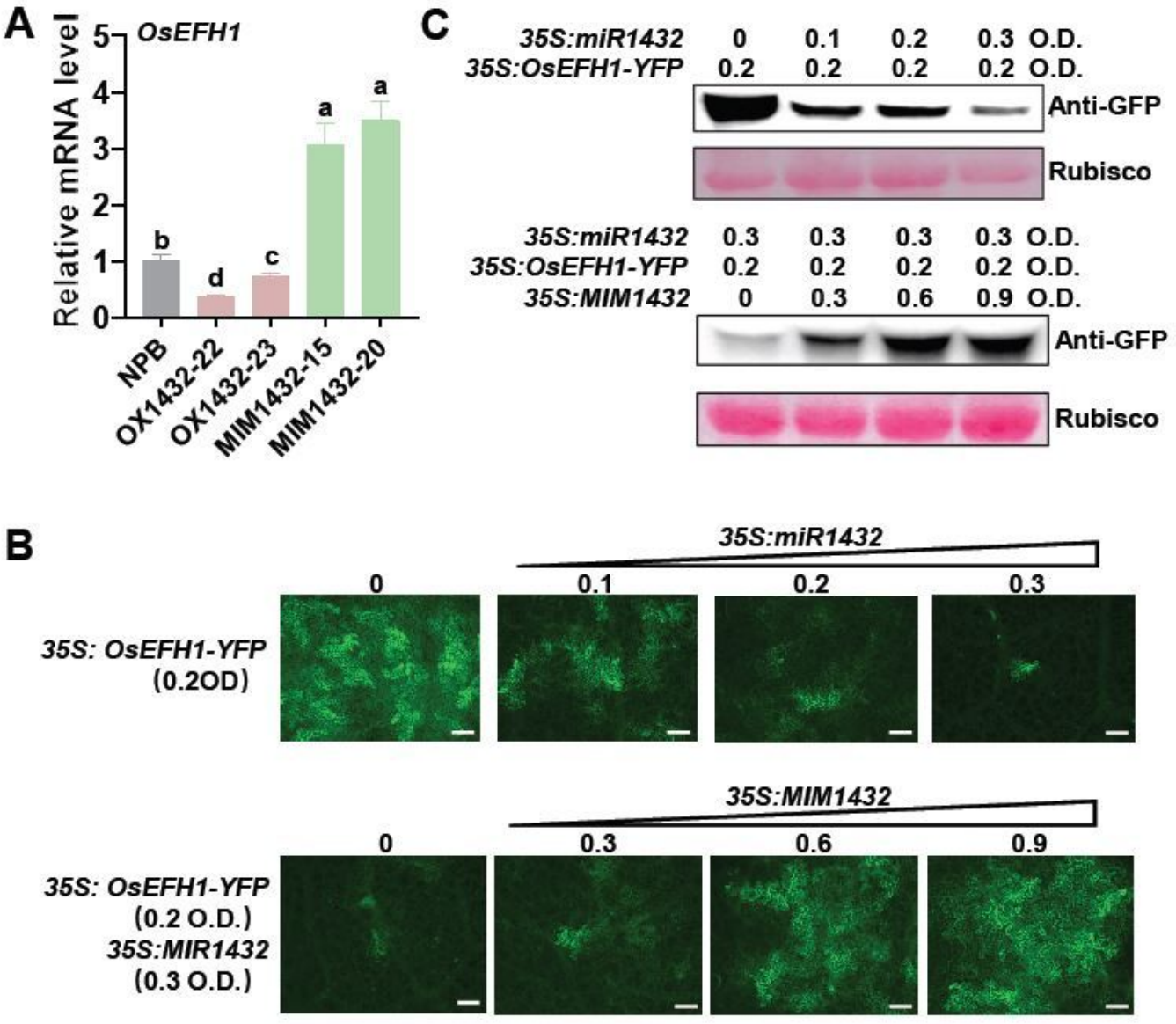

Figure 3

miR1432 suppresses the expression of OsEFH1. a The relative mRNA levels of OsEFH1 in OX1432, MIM1432, and the Nipponbare (NPB) control. Error bars indicate SD (n= 3 independent samples). Different letters above the bars indicate significant differences ($P < 0.01$) as determined by One-way Tukey-Kramer analysis. b The YFP intensity of OsEFH1-YFP fused protein in Nicotiana benthamiana leaves. The OsEFH1-YFP constructs were transiently expressed alone or co-expressed with miR1432 alone or co-expressed with miR1432 and MIM1432 together in Nicotiana benthamiana leaves using Agrobacterium-mediated infiltration at the indicated concentration (Optical Density (O. D.)). Bar= 100 μm.

c Western blotting assay indicates the protein levels of OsEFH1-YFP in (B). All the experiments were repeated two times with similar results.

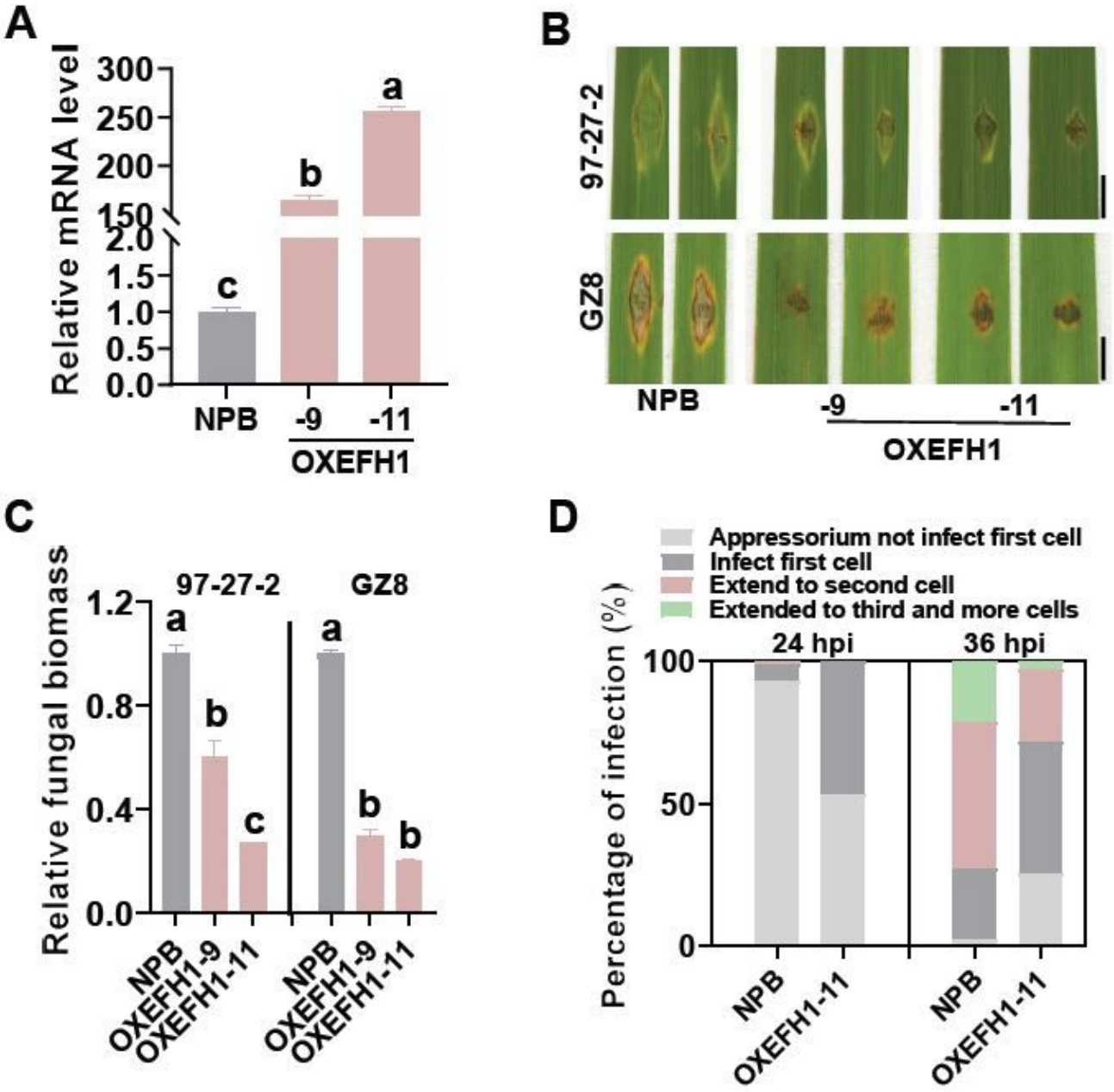

Figure 4

OsEFH1 enhances rice blast disease resistance. a The relative mRNA levels of OsEFH1 in transgenic lines overexpressing OsEFH1 (OXEFH1) and the Nipponbare (NPB) control. Total RNA was extracted from three to five-leaf stage seedlings for reverse-transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The relative mRNA levels of OXEFH1 lines were normalized with that of the Nipponbare control. b The blast disease phenotypes of OXEFH1 lines and the Nipponbare control five days post-inoculation of indicated M. oryzae strains. Bar= 5 mm. c The Quantification analysis of the relative fungal biomass in b.

The fungal biomass was shown as the ratio of DNA level of M. oryzae MoPot2 genes against that of rice ubiquitin. d Quantification analysis of the fungal development during the invasive process. Over 200 conidia in each line were analyzed. For a and c, error bars indicate SD (n = 3 independent samples). Different letters above the bars indicate a significant difference ($P < 0.01$) as determined by a one-way Tukey-Kramer analysis. All the experiments were repeated two times with similar results.

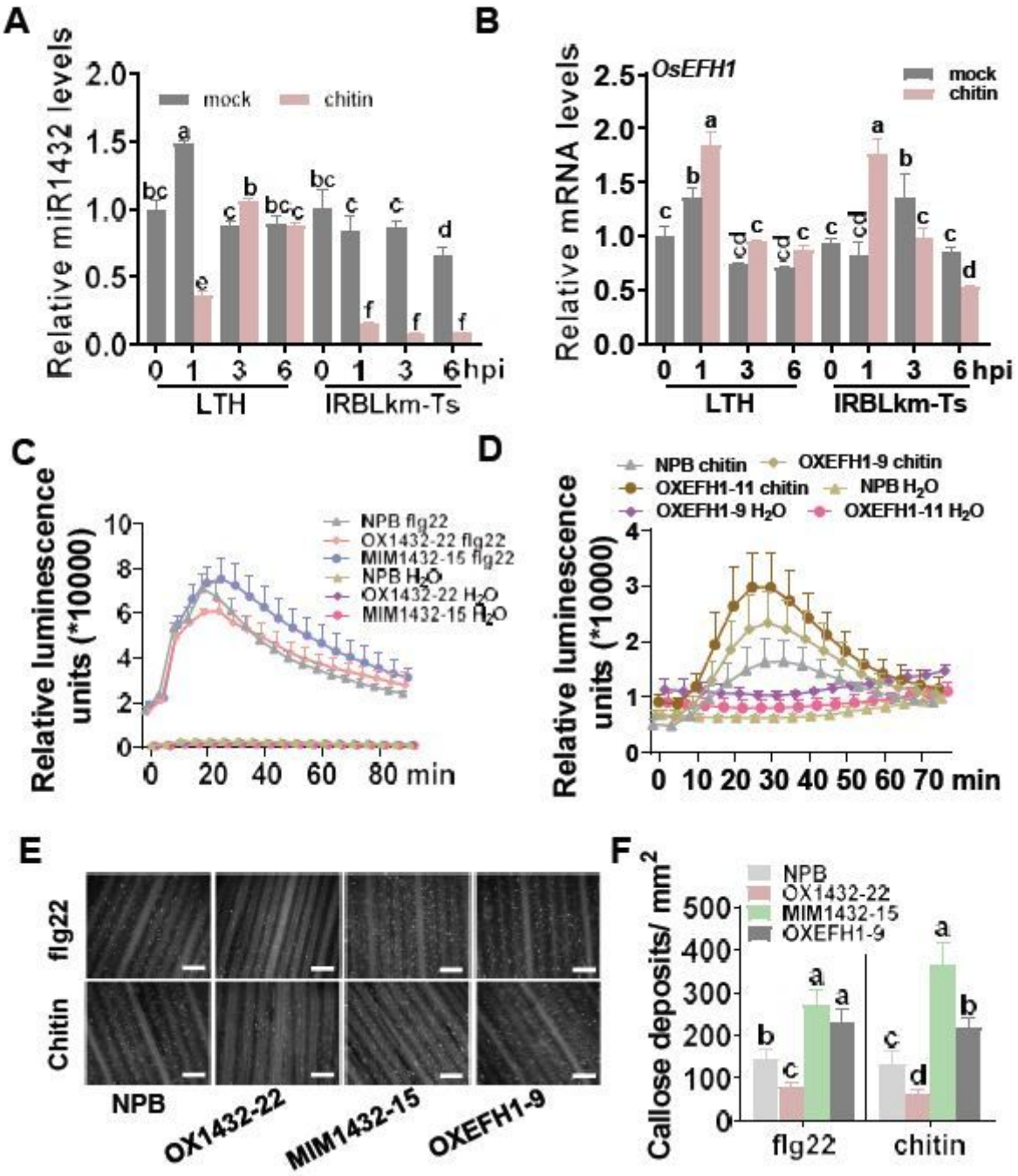

Figure 5

miR1432-OsEFH1 module regulates rice PTI responses. a-b The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) data show the amounts of miR1432 (a) and the mRNA levels of OsEFH1 (b) in LTH and IRBLkm-Ts with or without chitin treatment. Data are shown as mean ± SD (n= 3

independent samples). c-d The burst of reactive oxidative species (ROS) induced by flg22 in the leaves of indicated lines and the Nipponbare control, respectively. Data are shown as mean ± SD (n= 6 independent repeats). e PAMPs (flg22 and chitin) induced callose deposition in the leaves of indicated lines and the Nipponbare control. Bar= 0.5 mm. f Quantitative analysis of PAMPs-induced callose deposition in e. Data are shown as mean ± SD (n= 6 independent repeats). For a, b, and f, different letters above the bars show significant differences (P < 0.01) as determined by the One-way Tukey-Kramer test. These experiments were repeated two times with similar results.

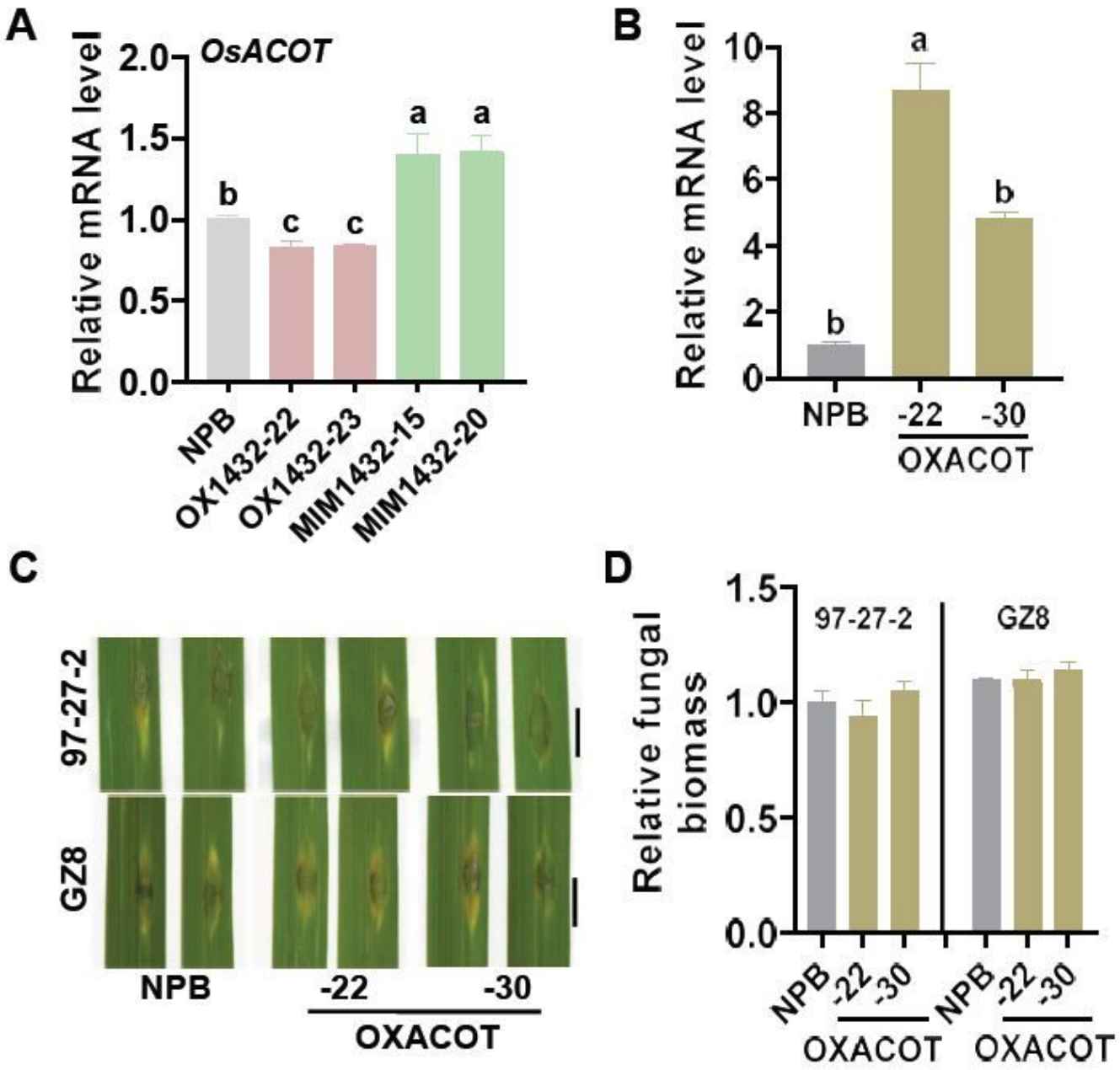

Figure 6

OsACOT is not involved in the regulation of rice blast resistance. a The relative mRNA levels of OsACOT in OX1432, MIM1432, and the Nipponbare control (NPB). b The relative mRNA levels of OsACOT in the

transgenic lines overexpressing OsACOT (OXACOT) and the Nipponbare control. For a and b, total RNA was extracted from three to five-leaf stage seedlings for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. c Blast disease phenotypes of OXACOT and the Nipponbare control five days post-inoculation of indicated M. oryzae strains. Bar= 5 mm. d Quantification analysis of the relative fungal biomass in c. The relative fungal biomass was measured by using the ratio of DNA level of M. oryzae MoPot2 genes against that of rice ubiquitin. For a, b, and d, error bars indicate SD (n= 3 independent samples). N.D. indicate no difference as determined by a one-way ANOVA analysis. All the experiments were repeated two times with similar results.

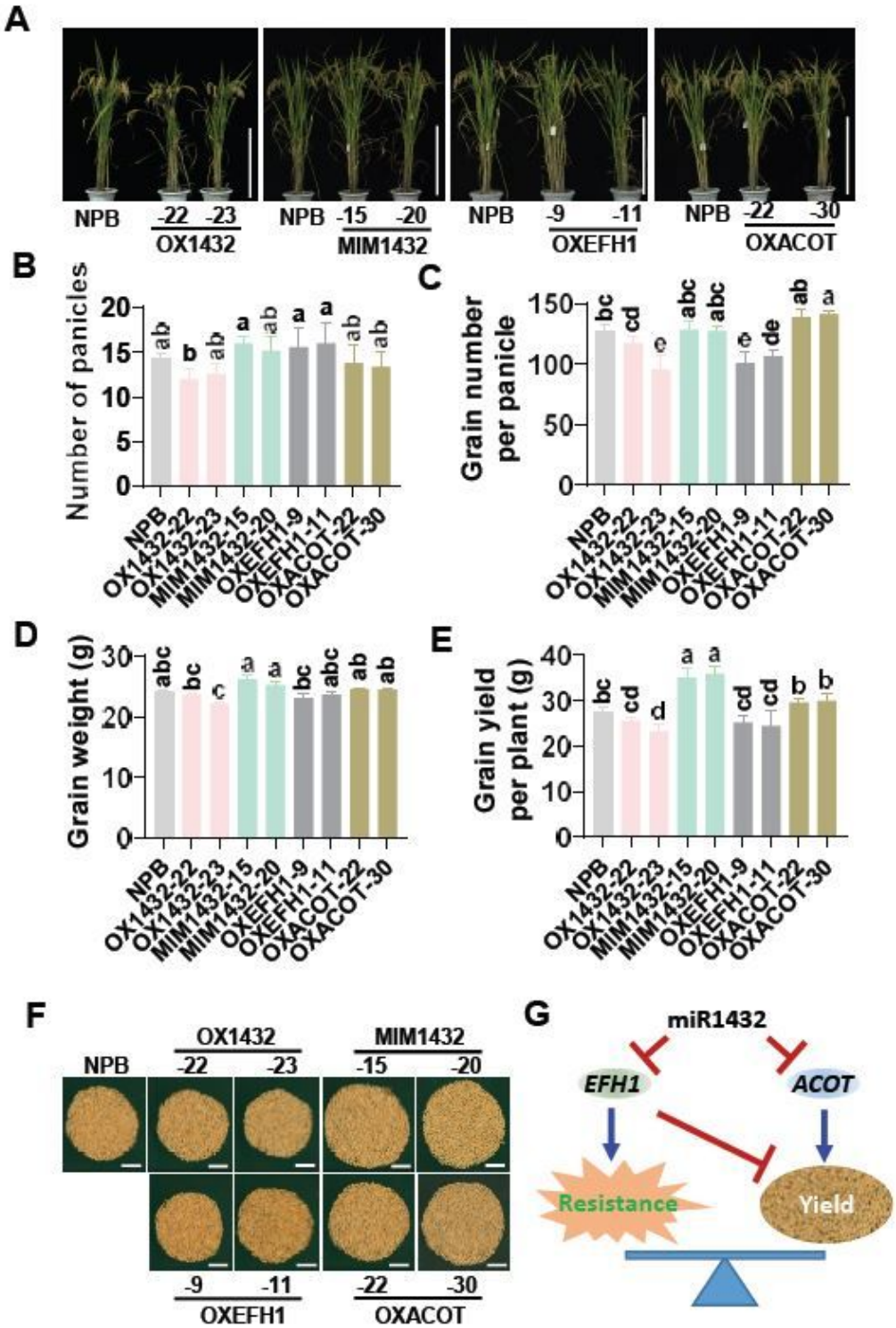

Figure 7

miR1432 and its target genes regulate rice yield traits. a The gross morphology of the OX1432, MIM1432, OXEFH1, and OXACOT lines planted in a paddy yard during the normal growing season in Sichuan province, the Southwest of China in 2020. Scale bars, 50 cm. b-e the panicle number, grain number per panicle, grain weight, and grain yield per plant of the indicated lines. Data are shown as mean ± SD (n= 5 independent samples). Different letters above the bars show significant differences ($P < 0.05$) as

determined by the One-way Tukey-Kramer test. f Photo of grains per plant of the OX1432, MIM1432, OXEFH1, OXACOT lines, and the Nipponbare control. Bars= 5 cm. (G) A model of miR1432 coordinates rice immunity and grain yield via OsEFH1 and OsACOT1 that play different roles in the regulation of yield and resistance.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfiles.pdf
- Supptables12.xlsx