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Overexpression of OsMed16 inhibits rice growth and causes spontaneous cell death

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Abstract

Background

The Mediator complex transduces information from the DNA-bound transcription factors to the RNA polymerase II transcriptional machinery. Research on plant Mediator subunits was mainly performed in Arabidopsis, while very few of them have been functionally characterized in rice.

Results

Here the rice Mediator subunit 16, *OsMed16*, was studied. *OsMed16* encoded a putative protein of 1301 amino acids, which is longer than the reported version. It was expressed in various rice organs, and localized in nucleus. Knockout of *OsMed16* caused rice seedling lethality. Its overexpression led to rice growth retardation, low yield, and spontaneous cell death in leaf blade and leaf sheath. RNA sequencing suggested that overexpression of *OsMed16* altered the expression of a large number of genes. Among them, the up-regulation of some defense-related genes was verified.

Conclusions

Our results demonstrated that *OsMed16* can regulate the expression of a wealth of genes, and alterations in its expression have profound impact on plant growth, development and defense response in rice.

Background

Unlike prokaryotic genes, transcription of eukaryotic genes is orchestrated by RNA polymerase II (Pol II) and multiple regulatory proteins, including general transcription factors (TFs), gene-specific TFs and Mediator (Martinez 2002; Orphanides and Reinberg 2002). Mediator is a highly conserved multi-protein complex consisting of 25–34 subunits depending on the species (Zhai and Li 2019). The structure of the whole Mediator complex can be divided into three main modules (Head, Middle, and Tail) and a transiently associated Kinase module, and every module has different functions in transcription (Zhai and Li 2019). The Head and Middle modules constitute the core Mediator, and contact with Pol II and general TFs, while the tail module interacts with gene-specific TFs (Jeronimo and Robert 2017; Zhai and Li 2019). The Kinase module and Pol II associate with the main modules in a mutually exclusive fashion, so it acts as a transcriptional repressor (Chadick and Asturias 2005; Clark et al. 2015). Generally, during the formation of Pol II pre-initiation complex (PIC), Mediator can pass regulatory information from DNA-bound TFs to the basal transcriptional machinery, thereby regulating the expression of downstream genes (Jeronimo and Robert 2017; Zhai and Li 2019).

Mediator was first biochemically identified in yeast in 1990 (Kelleher et al. 1990), and their counterparts were subsequently isolated from human and other animals (Fondell et al. 1996; Jiang et al. 1998; Kwon

et al. 1999). Biochemical identification of Mediator complex in plants was very late. The first plant Mediator complex was purified from Arabidopsis cell suspension culture in 2007 (Bäckström et al. 2007). In addition to multiple Mediator subunits, Pol II subunits were also isolated in the purified Arabidopsis Mediator fraction, but the Kinase module subunits (Med12, Med13, CDK8, and CycC) were not isolated together with the bulk complex. Furthermore, the Kinase module subunits were identified by bioinformatics approaches (Bäckström et al. 2007). Now it is usually thought that the Arabidopsis Mediator complex comprises 33 subunits, among which 29 subunits are conserved with yeast or animal counterparts, and 4 subunits are unique to plants (Yang et al. 2016; Zhai and Li 2019). To date, Mediator subunits in other plants have not yet been biochemically identified, but were characterized by bioinformatics analysis. Mathur et al. (2011) *in silico* identified Mediator subunits in 16 plant species from algae to higher angiosperms. It is found that at least one homolog for all the animal/fungal Mediator subunits is present in the plant kingdom (Mathur et al. 2011). In addition to *in silico* analysis, the biological functions of some Arabidopsis Mediator subunits have been studied through genetic and molecular analysis. It is found that these Mediator subunits participate in multiple biological processes, including plant growth, development, flowering, pathogen defense and stress tolerance (Elfving et al. 2011; Xu and Li 2011; Iñigo et al. 2012; Knight et al. 2009; Huang et al. 2019).

Rice is an important staple crop, and also used as a monocot model plant. 55 Mediator genes, including paralogs of some main module subunits and Kinase module subunits, have been identified in the whole rice genome by *in silico* approaches (Mathur et al. 2011). However, unlike that in Arabidopsis, very few rice Mediator subunits have been functionally characterized. *OsMed15a* and *OsMed14-1* are the two well-studied Mediator subunits in rice. *OsMED15a* is implicated in rice seed development through linking rice grain size/weight-regulating TFs to their target genes. Reduction in *OsMed15a* expression (RNAi plants) down-regulated the expression of genes associated with grain size/weight, *GW2*, *GW5* and *DR11*, and reduced grain length, weight, and yield (Dwivedi et al. 2019). *OsMed14-1* plays an important role in rice development. RNAi-mediated repression of *OsMed14-1* expression led to growth inhibition and slender organs, which was caused by defective cell-cycle progression and reduced auxin level in *OsMed14-1* knockdown plants (Malik et al. 2020).

OsMed16 (*OsSFR6*) is a homolog of *AtSFR6*, and its function was preliminarily studied in Arabidopsis (Wathugala et al. 2011). The *atsfr6* mutant showed freezing sensitivity, pale cotyledons and leaves. Overexpression of *OsMed16* in *atsfr6* mutant could restore the wild-type phenotype and elevate freezing and osmotic tolerance (Wathugala et al. 2011). Moreover, the expression of *COLD-ON REGULATED* (*COR*) genes could also be restored in *atsfr6* mutant overexpressing *OsMed16*, so *OsMed16* is thought to act as a regulator of COR gene expression, osmotic stress and freezing tolerance in Arabidopsis (Wathugala et al. 2011). However, the biological function of *OsMed16* remains unclear in rice. In this study, the expression pattern and function of *OsMed16* was investigated in rice. The results revealed that the knockout mutant *osmed16* exhibited severe growth inhibition, and were unable to complete the life cycle. Overexpression of *OsMed16* also led to growth inhibition, low yield and spontaneous cell death. RNA-seq data indicated that overexpression of *OsMed16* altered the expression of a large number of genes

involved in multiple biological processes. In particular, alterations of some defense related genes were further examined.

Result

1 Sequence and Phylogenic Analysis of OsMed16

Owing to its high homology to *AtSRF6* (*AtMed16*), the rice gene *LOC_Os10g35560* was previously named *OsSRF6* (Wathugala et al. 2011). However, as a subunit of the Mediator complex, *LOC_Os10g35560* should be named *OsMed16* according to the common unified nomenclature for Mediator subunits (Bourbon et al. 2004). In Wathugala's studies, *OsSFR6* (*OsMed16*) was predicted to encode a protein of 1170 amino acids. When searching in GenBank (National Center for Biotechnology Information, NCBI) and Rice Genome Annotation Project database, we found the ORF of *OsMed16* was 3906 bp in length, and thus encoded a putative protein with 1301 amino acid residues, which is 131-aa longer than OsSRF6 reported by Wathugala et al. (2011). To test this, the full-length ORF of *OsMed16* (3906 bp) was amplified from the model *japonica* rice variety Nipponbare by high-fidelity PCR, and verified by sequencing. Subsequently, the gene structure of *OsMed16* was analyzed, which contains 16 exons and 15 introns (Fig. 1a).

To understand the evolutionary relationship of *OsMed16*, its counterparts were obtained from different plant species, including algae, mosses, ferns, gymnosperms and angiosperms. Then sequence alignment and phylogenetic analysis were performed. On the whole, the phylogenetic tree is organized into two major clades. The Med16 subunits from unicellular algae (*CrMed16*, *VcMed16, GpMed16*) were grouped in one clade and shared less than 15% identity with OsMed16 (Fig. 1b); The Med16 subunits from other plant species were grouped in another clade and shared higher identity with OsMed16 (Fig. 1b). Among the sequences retrieved from NCBI database, OsMed16 displays the highest percentage of identity with ObMed16 from *Oryza brachyantha* (96%), and has 69% identity with AtMed16.

2 OsMed16 mRNA Expression Pattern and Protein Subcellular Localization

Quantitative real-time PCR (qRT-PCR) assays were performed with total RNA isolated from rice root, leaf, stem, leaf sheath and young panicle. The results showed that *OsMed16* mRNA was expressed in all the examined organs, which have similar expression level except a little lower in leaf sheath (Fig. 2a). Furthermore, public microarray databases (eFP browser) indicated that *OsMed16* was also expressed in inflorescence and seed (Additional file 1 Fig. S1) (Winter et al. 2007). The wide expression pattern of *OsMed16* is consistent with its function as a basic transcriptional regulator.

To determine the subcellular localization of OsMed16, a p35S-*OsMed16*-*GFP* construct was generated and transiently expressed in rice protoplasts with a red fluorescent protein (RFP) fused to OsGhd7, a nucleus-localized protein (Xue et al. 2008). The p35S-GFP empty vector was used as a control. As a result, the green fluorescence signal in the control was observed in cytoplasm, while OsMed16-GFP fluorescence was present in the nucleus, co-localized with the OsGhd7-RFP protein (Fig. 2b). These

results indicated that OsMed16 is localized in the nucleus, which was in agreement with its role as a Mediator subunit.

3 Overexpression of OsMed16 Caused Rice Growth Inhibition and Spontaneous Cell Death

To investigate the function of *OsMed16 in planta*, it was disrupted using CRISPR/Cas9 genome-editing technology (Additional file 1 Fig. S2a). The *osmed16* mutants exhibited a stunted growth phenotype, failed to head, and died prematurely (Additional file 1 Fig. S2b), indicating that disruption of *OsMed16* caused rice seedling lethality.

We further employed gain-of-function approach to investigate the roles of *OsMed16*. *OsMed16* overexpression vector driven by CaMV 35S promoter was constructed and transformed into Nipponbare via an *Agrobacterium*-mediated method. The expression level of *OsMed16* in transgenic plants was detected using qRT-PCR assay, and two representative homozygous transgenic lines with high expression level of *OsMed16* (named *OsMed16*-OE) were used for further investigation (Additional file 1 Fig. S3). Unexpectedly, overexpression of *OsMed16* also inhibited rice growth. Compared with the wild-type, *OsMed16*-OE lines had a dwarf phenotype with fewer tillers (Fig. 3d). Another distinct visible phenotype observed was spontaneous cell death in *OsMed16*-OE lines. At three leaves stage, small necrotic spots first appeared on the leaf sheath of *OsMed16*-OE seedlings (Fig. 4a), and then were also observed on leaves (spotted leaf, Fig. 4b-c). As plants grew, the brown spots gradually became large irregular lesions (Fig. 4b-c). The cell death was further confirmed by Trypan Blue staining. The *OsMed16*-OE leaves showed increased staining intensity compared to wild-type leaves (Fig. 4d-f). Accumulation of reactive oxygen species may cause cell damage and even death (Khanna-Chopra. 2012). By DAB (3,3-diaminobenzidine) staining, the over accumulation of H_2O_2 was observed in the leaves of *OsMed16*-OE plants (Fig. 4g-i). We also used NBT (nitroblue tetrazolium) staining and observed the increase of superoxide anion in *OsMed16*-OE plants (Fig. 4j-l). With the increasing of number and size of lesions, the old leaves of *OsMed16*-OE lines withered prematurely, and the whole plants exhibited early senescence (Fig. 3b).

4 Overexpression of OsMed16 Reduced Rice Grain Yields

In addition to the growth inhibition, plants overexpressing *OsMed16* exhibited significant yield reduction. Compared to wild-type plants, grain yield per plant was reduced by 91.8% and 91.3% in the two overexpression lines (Fig. 5a-b). The yield components were further analyzed. The panicle number per plant, panicle length, and 1,000-grain weight of *OsMed16*-OE plants decreased significantly compared to the wild-type (Fig. 5c-e). Additionally, the seed length and width were also compared between the *OsMed16*-OE lines and the wild-type. The results showed that the seed length was unchanged (Additional file 1 Fig. S4a-b), but seed width decreased slightly in *OsMed16*-OE lines (Additional file 1 Fig. S4c-d).

5 Transcriptome Changes in OsMed16-OE Plants

To assess the influence of *OsMed16* overexpression on gene expression, *OsMed16*-OE plants exhibiting necrotic lesions were harvested, and RNA sequencing (RNA-Seq) was performed on wild-type and *OsMed16*-OE plants. Overall, we obtained 6 transcriptome data sets, each of which contains an average of about 50 million paired-end (PE) reads (Additional file 1 Fig. S5). The raw sequencing reads were first trimmed and mapped to the rice reference genome using HISAT2. More than 96% reads could map to unique loci per sample (Additional file 1 Fig. S5). Differentially expressed genes (DEGs) were determined with stringent criteria: $\log_2$ fold change ≥ 1 and *P*-value (false discovery rate, FDR) ≤ 0.05. Compared with the wild-type, 2402 DEGs were detected in *OsMed16*-OE plant leaves, of which 1419 were up-regulated (Additional file 2 Table.S1), whereas 983 were down-regulated (Additional file 2 Table.S2). Gene ontology (GO) enrichment analysis indicated that the up-regulated genes in *OsMed16-OE* plants were involved in multiple biological processes, including heme binding (66), tetrapyrrole binding (66), oxidoreductase activity (57) and iron ion binding (56). Among these DEGs, *CYP71Z2* (*LOC_Os07g11739*) is the rice cytochrome P450 gene, which participates in plant defense by regulating the secondary metabolism of plant phytoalexin (Li et al. 2013;Li et al. 2015). Rice *D3* gene (*LOC_Os06g06050*), a multi-tiller dwarf gene, encodes an F-box protein rich in leucine repeat sequence, which is not only necessary for the signal transduction of strigolactone (SL), but also involved in leaf senescence and cell death (Ishikawa et al. 2005). *OsCAld5H1* (*LOC_Os10g36848*) encodes a ferulic acid 5-hydroxylase, whose biological function is mainly involved in the synthesis of rice lignin, and its expression affects the composition of S/G lignin in the main nutritional tissues of rice without affecting the structure of vascular bundles (Takeda et al. 2017). *HAN1* (*LOC_Os11g29290*) encodes an oxidase which can catalyze the conversion of biologically active Jasmonate-L-isoleucine (JA-Ile) into the inactive 12-hydroxy-jasmony-L-isoleucine (12Oh-Ja-ILE) and regulate JA-mediated low temperature reaction and cold tolerance as a negative regulator of cold tolerance (Mao et al. 2019). Whereas the down-regulated genes mapped to categories including tetrapyrrole binding (40), heme binding (39), and oxidoreductase activity (34) (Fig. 6a, b). Among these genes, *OsAPX2* (*LOC_Os07g49400*) is an ascorbic acid peroxidase gene that plays an important role in the growth and development of rice by clearing reactive oxygen species to protect seedlings from abiotic stress (Zhang et al. 2013). *CYP93G2* (*LOC_Os06g01250*) encodes the flavanone 2-hydroxylase, which is not only a member of the cytochrome P450 gene but also the first enzyme in its biosynthetic pathway (Du et al. 2010). Our results confirmed that the up-regulated genes and the down-regulated genes were indeed associated with multiple biological pathways in rice.

Overexpression of *OsMed16* led to spontaneous cell death in rice, which resembled the hypersensitive response (HR) caused by pathogenic infection. This led us to speculate that overexpression of *OsMed16* might trigger the expression of defense-related genes. Thus, we examined these genes in the RNA-seq data. Indeed, some defense-related genes, including *PR1a* and *PR1b*, were up-regulated in *OsMed16*-OE compared with the wild-type. To confirm these results, we further performed qRT-PCR to check the expression levels of eight defense-related genes in the *OsMed16*-OE and wild-type plants. The transcript levels of all these genes were elevated in *OsMed16*-OE plants (Fig. 7), suggesting that overexpression of *OsMed16* did activate the expression of defense-related genes.

Discussion

So far, the research on plant Mediator complex subunit Med16 has mainly focused on *Arabidopsis thaliana*, while less research has been done on rice. Wathugala et al. (2011) reported that the *OsMed16*/ *OsSFR6* gene, when ectopically expressed in the *Arabidopsis* mutant *atsrf16*, restored the wild-type phenotype of the *atsrf16* mutant. The main function of *OsMed16* gene in rice has not been reported. In this study, the *OsMed16* was identified in rice, and the *OsMed16* gene knockout mutants and overexpression lines were constructed for the first time. The function of the Mediator complex subunit Med16 in rice was studied through reverse genetics. The results showed that the leaves of the *OsMed16* overexpressing plants showed brown spots, and the spots continued to expand with the growth of the plant till it filled the entire leaf, resembling spontaneous lesions.

Spontaneous lesions refer to lesion-like spots that are spontaneously produced on the leaf surface without pathogen infections. It is very similar to the lesions associated with hypersensitive responses triggered by incompatible pathogens (Johal et al. 1995). Spontaneous lesions are widely present in various plants, such as *Arabidopsis*, rice, corn, wheat, barley, and soybeans (Dietrich et al. 1994; Büschges et al. 1997; Gray et al. 1997; Badigannavar et al. 2002; Malamy et al. 1990; Takahashi et al.1999; Matin et al. 2010). In this study, the pathological changes of *OsMed16*-overexpressing plants were mainly caused by the abnormal expression of *OsMed16*. The *OsMed16*-overexpressing plants spontaneously presented necrotic spots without pathogen infection. At the same time, the expression level of defense-related genes *PR1* and *PR10a* (Takahashi et al.1999) was up-regulated in overexpressor plants, suggesting that *OsMed16* may exist in a signaling pathway which was usually activated in the absence of pathogen infection, leading to hypersensitivity in *OsMed16*-overexpressing plants. The cell death phenotype and the activation of defense-related genes in *OsMed16*-overexpressing plants indicated that *OsMed16* may play a positive regulatory role in programmed cell death (PCD) and resistance-related signaling pathways in plants. However, the mechanism of *OsMed16* gene's positive regulation of PCD and defense signals is still unclear. The results of this study will provide a new perspective for the molecular regulation mechanism of Mediator complex in plant cell death and disease resistance signaling, especially in monocotyledons. The results of agronomic traits showed that the overexpression of *OsMed16* seriously affected the growth and development of plants. Therefore, it is speculated that *OsMed16* may be related to the overall physiology and morphology of plants. Further elucidating the mechanism of *OsMed16* and its downstream genes will contribute to the understanding of the role of *OsMed16* in regulating the plant cell death and defense mechanisms as well as the rice plant growth and development.

As part of general transcriptional regulation, the Mediator complex subunits connect specific transcriptional activators to the RNA polymerase II complex, and some of the individual Mediator complex subunits receive signals from specific pathways and transfer them to general transcriptional mechanisms. Some Mediator complex subunits have been shown to be related to defense, such as Med21 and Med25 (Dhawan et al. 2009; Elfving et al. 2011; Ou et al. 2011; Cevik et al. 2012; Chen et al. 2012). Meanwhile, in *Arabidopsis thaliana*, Med16 was not only proved to regulate the immune response, but also proved to be an important subunit in the tail module, because the whole tail module was missing

after extraction of the mediator complex in *atsrf6* mutants. Our experimental results showed that the *OsMed16*-overexpressing plants affected the various periods of rice growth and development, that is to say *OsMed16* plays a crucial role in different developmental stages. However, the mechanisms by which *OsMed16* regulates rice growth and development are still largely unknown. Future studies will be required to dissect these regulatory mechanisms.

Conclusion

In the present study, the rice Mediator subunit 16, *OsMed16*, was functionally characterized. It was expressed in various rice organs, and localized in nucleus. Loss of function of *OsMed16* causes rice seedling lethality. Its overexpression led to rice growth inhibition, low yield and spontaneous cell death in leaf blade and leaf sheath. RNA-seq data suggested that overexpression of *OsMed16* altered the expression of a large number of genes, including the defense-related genes. These results demonstrated that *OsMed16* regulates not only rice growth and development, but also defense response.

Materials And Methods

Plant Materials and Growth Conditions

The WT rice (*Oryza sativa* cv. Nipponbare), the two knockout lines of *OsMed16*, and the two *OsMed16* overexpression lines were used in this study. The seeds were soaked in deionized water for 2 days in an incubator at 28°C under dark conditions. After germination, the seeds were grown either hydroponically or in paddy field. For hydroponic culture, the seeds were first grown in 0.5 mM $CaCl_2$ solution for 5–7 days. Then the seedlings were transferred to a 4 L plastic pot containing 1/2 Kimura B solution (pH 5.6) (Yamaji and Ma. 2007). The nutrient solution was changed with fresh solution every two days. The plants were grown in a greenhouse under natural light at 25°C-30°C. The paddy field is located in the rice planting base of Guangxi University, Nanning city, Guangxi Province. Each experiment had at least three biological replicates.

Generation of Transgenic Plants

To create the knockout lines of *OsMed16*, the CRISPR/Cas9 genome targeting system was used. The pCRISPR-OsMed16 plasmids with *OsMed16* specific target sites were constructed as described previously (Ma et al. 2015). Briefly, specific target sequences (ATGCCCTCGTGCATTACTGG and GTTGCTTTTGATCCCACTCG) within the *OsMed16* gene were selected by a Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the rice genome sequence. Then the two specific sequences of *OsMed16* gene were respectively introduced into the sgRNA expression box by overlapping PCR to produce pU6a-*OsMed16*-SgRNA and pU6b-*OsMed16*-SgRNA.

These fragments were cloned into pYLCRISPR/Cas9 Pubi to construct pCRISPR-*OsMed16* by using the restricted connection reactions containing BsaI and T4 DNA ligase. The constructed plasmids were introduced into *Agrobacterium tumefaciens* EHA101 and transformed into wild-type Nipponbare rice. The

mutants were screened by PCR using primer pairs flanking the *OsMed16*-specific target site, and the homozygous mutants were selected for further study and analysis.

Transgenic plants overexpressing *OsMed16* gene (named *OsMed16*-OE) was obtained by *Agrobacterium*-mediated transformation. Total RNA was extracted from Nipponbare using the TRIzol reagent kit (Life Technologies) and reverse transcribed with a Hiscript II Q RT SuperMix Kit (Vazyme). The resulting cDNA was used as template for PCR-amplification of the *OsMed16* full length cDNA with 5'-AATTGGTACCATGACCTCTTCCTCCGCCCC-3' and 5'- AATTACGCGTTCAAACGACTTTCACCCATG-3' as primers. The full-length cDNA of *OsMed16* was inserted into the pCAMBIA1300-Ubi vector carrying the maize Ubiquitin promoter and the terminator of the nopaline synthase gene. *OsMed16* gene specific primers (5'-CGATGGCAATTACACTGTGC-3' and 5'- TAGAAGGCCAGCAGCATCA-3') and were used to identify the positive transgenic plants. The relative expression levels of *OsMed16* in transgenic plant leaves were determined by quantitative reverse transcription-PCR (qRT-PCR) as described below.

RNA Isolation and Gene Expression Analysis

To examine the expression pattern of *OsMed16* gene, the root, leaf blade, leaf sheath and spike were sampled at the heading stage for extraction of total RNA. Total RNA (1 μg) was used for first-strand cDNA synthesis using a PrimeScript II 1st Strand cDNA Synthesis Kit (Takara). qRT–PCR was performed with ChanQTM SYBR Color qPCR Master Mix (Vazyme) on a StepOnePlus Real-Time PCR System (Analytik Jena AG). The primers for gene expression analysis of *OsMed16* were 5'-CGATGGCAATTACACTGTGC-3' and 5'-TAGAAGGCCAGCAGCATCA-3'. *Histone H3* was used as an internal standard with the primers 5'-GGTCAACTTGTTGATTCCCCTCT-3' and 5'-AACCGCAAAATCCAAAGAACG-3'. The relative expression levels of the genes were calculated by the $2^{-\Delta\Delta CT}$ method. The primers for defense-related genes are shown in (Additional file 1 Table 1).

Subcellular Localization of OsMed16

To detect the subcellular localization of OsMed16, plasmid to express the OsMed16-GFP fusion protein was constructed. *OsMed16* cDNA was amplified from the Nipponbare cDNA by PCR using the *OsMed16* specific primers 5'-CCG*GAATTC*ATGACCTCTTCCTCCGCCCC-3' (EcoRI site in italic text) and 5'-CGG*GGTACC*CAACGACTTTCACCCATGTCC-3' (KpnI site in italic text). The amplified cDNA was cloned downstream of the green fluorescent protein coding region in PYL322-GFP vector (Ma et al. 2018) to produce *OsMed16-GFP* vector.

The vectors expressing the nuclear marker OsGhd7-mcherry, the OsMed16-GFP fusion protein and GFP alone were all transduced into the protoplast of rice. Preparation of rice protoplasts and plasmid transformation have been described previously (Chang et al. 2015). After transformation, the cells were incubated at 28 °C in dark for 12-15 h, and images were taken using a confocal laser scanning microscope (TCS SP8; Leica Microsystems).

Histochemical Stain

Leaves from the *OsMed16* overexpressing plants with obvious lesions mimics and the WT at the same growth stage were harvested for histochemical analysis. Dead cells were detected by trypan blue staining (Yin et al. 2000). H_2O_2 accumulation was determined using DAB staining (Thordal-Christensen et al. 1997). The amount of reactive oxygen species in cells were determined using NBT staining (Qiao et al. 2010).

RNA-seq Data Analysis

Leaves from *OsMed16*-overexpressing plants showing spontaneous lesions and WT plants at the same developmental stage were collected for RNA-seq analysis. Purification and construction of the cDNA library were as described (Shen et al. 2014). The library was sequenced using the Illumina NovaSeq platform to generate raw reads, then low quality and adaptor reads were filtered to obtain clean reads for further research.

To identify statistically significant differentially expressed genes, the standard of log2 fold change ≥1 and false discovery rate (FDR) ≤0.05 were adopted. In order to obtain the GO term with significant gene enrichment, GO gene function annotation analysis was performed to obtain functional annotations, biological functions, and metabolic pathways of screened differential genes. GO (Gene Ontology; http://geneontology.org/) analysis of DEGs was conducted by hypergeometric tests, and each p-value indicates the enrichment of the corresponding category.

Phenotypic Analysis of *osmed16* Mutants and *OsMed16*-overexpressing Plants

Plants growing in hydroponic medium and soil were used for phenotypic observation. Hydroponic culture was as described above with 1/2 Kimura B solution (pH 5.6), changing the nutrient solution every 2 days. Soil culture was performed in the rice planting base of Guangxi University. The seedlings growing in field were covered with a plastic film dome for 30 days for heat preservation. The lesion mimic phenotype was documented when the plants were 48-day old. Agronomic traits such as effective tillers, seed setting rate, 1000-grain weight, grain width, grain length, grain number per panicle were analyzed at mature stage. Each measurement had at least three replicates per sample.

Declarations

Authors' contributions

XT and JX conceived the study and designed the experiments. JJ performed most of the experiments. YX, ZW, WY, and ZC participated in the research. GY, XT, and JX wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data supporting the conclusions of this article are provided within the article (and its additional 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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Figures

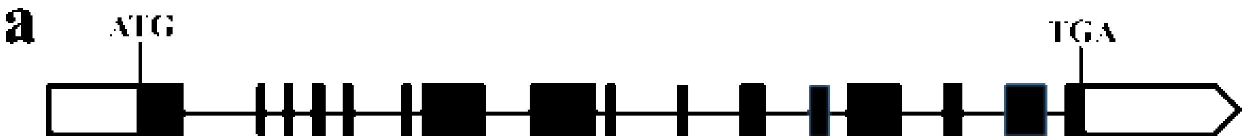

Figure 1

Gene structure and phylogenetic analysis of OsMed16. a Gene structure of OsMed16. The boxes (filled and unfilled) represent the exons; The lines between the boxes indicate introns; The unfilled boxes represent the UTR regions. b Phylogenetic analysis of OsMed16 and its counterparts in other plant species. The phylogenetic tree was constructed using MEGA6 program with the neighbor-joining method. The percentage of replicate in the bootstrap test (1000 replicates) are shown at the branch points of the

tree. The first two letters of each protein represent the abbreviated species name. Ob, Oryza brachyantha; Os, Oryza sativa; Sb, Sorghum bicolor; Si, Setaria italic; Hv, Hordeum vulgare; At, Arabidopsis thaliana; Sl, Solanum lycopersicum; Cs, Cucumis sativus; Pt, Populus trichocarpa; Pa, Picea abies; Sm, Selaginella moellendorffii; Mp, Marchantia polymorpha; Af, Azolla filiculoides; Sc, Salvinia cucullata; Pp, Physcomitrella patens; Sf, Sphagnum fallax; Gp, Gonium pectoral; Cr, Chlamydomonas reinhardtii; Vc, Volvox carteri.

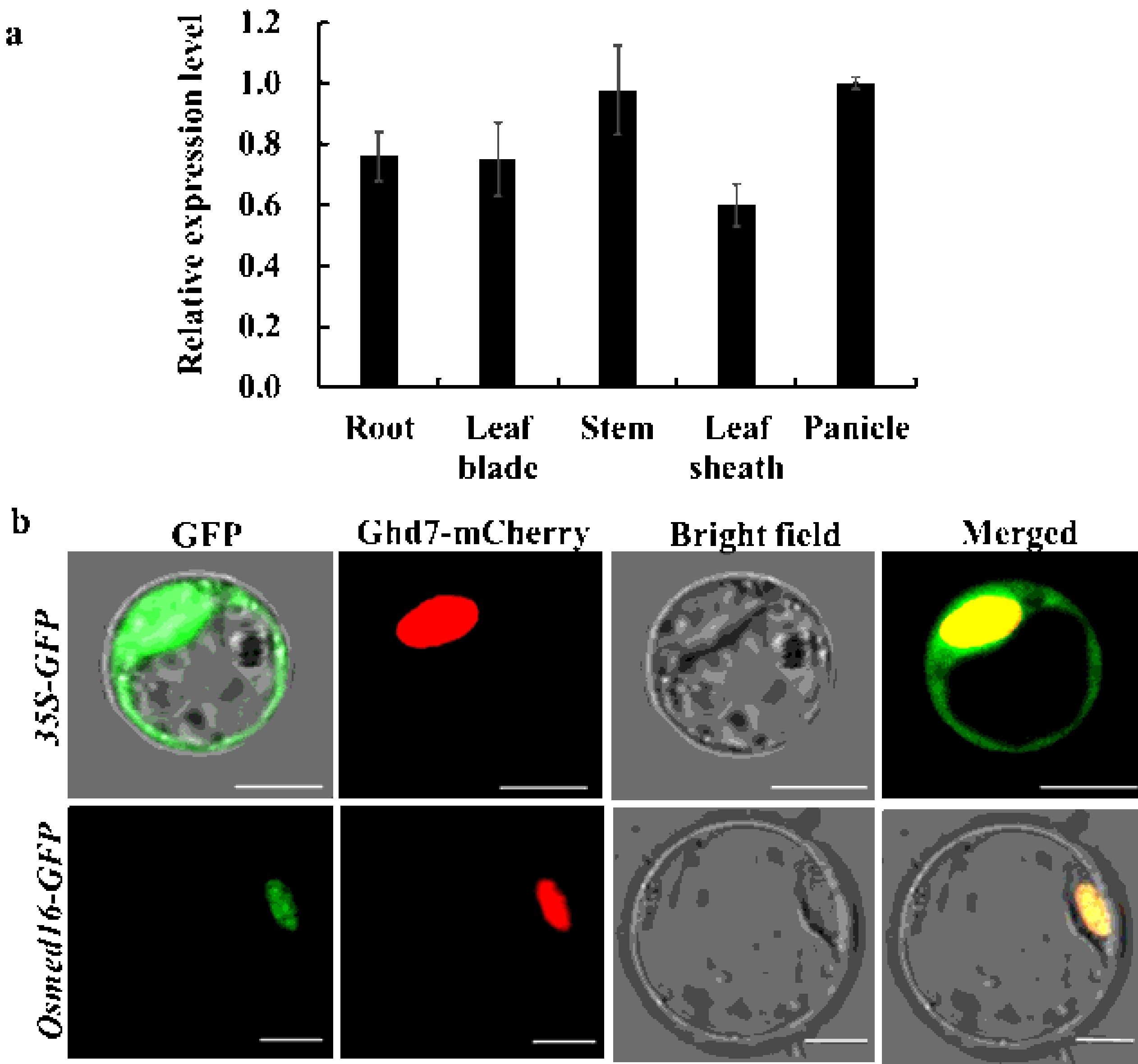

Figure 2

Organ-specific expression and subcellular localization of OsMed16. a Expression of OsMed16 in different rice organs analyzed by qRT-PCR. Data are means ±SD of three biological replicates. b Subcellular localization of OsMed16. GFP: OsMed16 or GFP was transiently expressed in rice protoplast along with Ghd7-mCherry. Fluorescence signals from GFP, mCherry, and the merged images are shown. Free GFP was used as a control. Bars =10 μm.

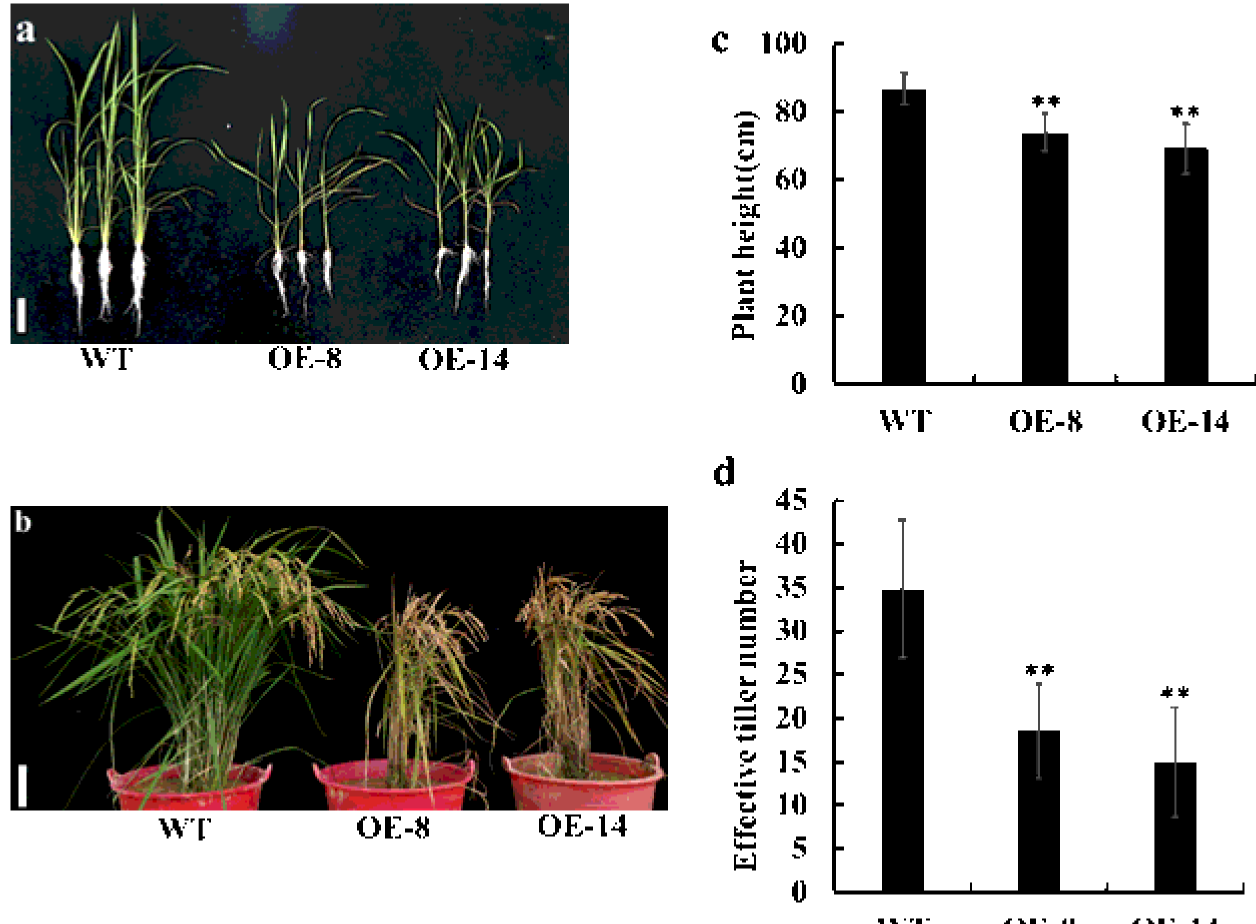

Figure 3

Overexpression of OsMed16 inhibited rice growth. a Phenotypes of the OsMed16-overexpressing (OE) and wild-type rice seedlings. Scale bars = 10 cm. b Phenotypes of the OsMed16-overexpressing (OE) and wild-type (WT) rice plants at the mature stage. Scale bars = 10 cm. c and d Comparison of plant height (c) and tiller number (d) of the WT and OE plants at mature stage. Values are the mean ± SD (n = 10). Asterisks indicate significant differences from the wild type (*P < 0.05; **P < 0.01 by Student's t-test).

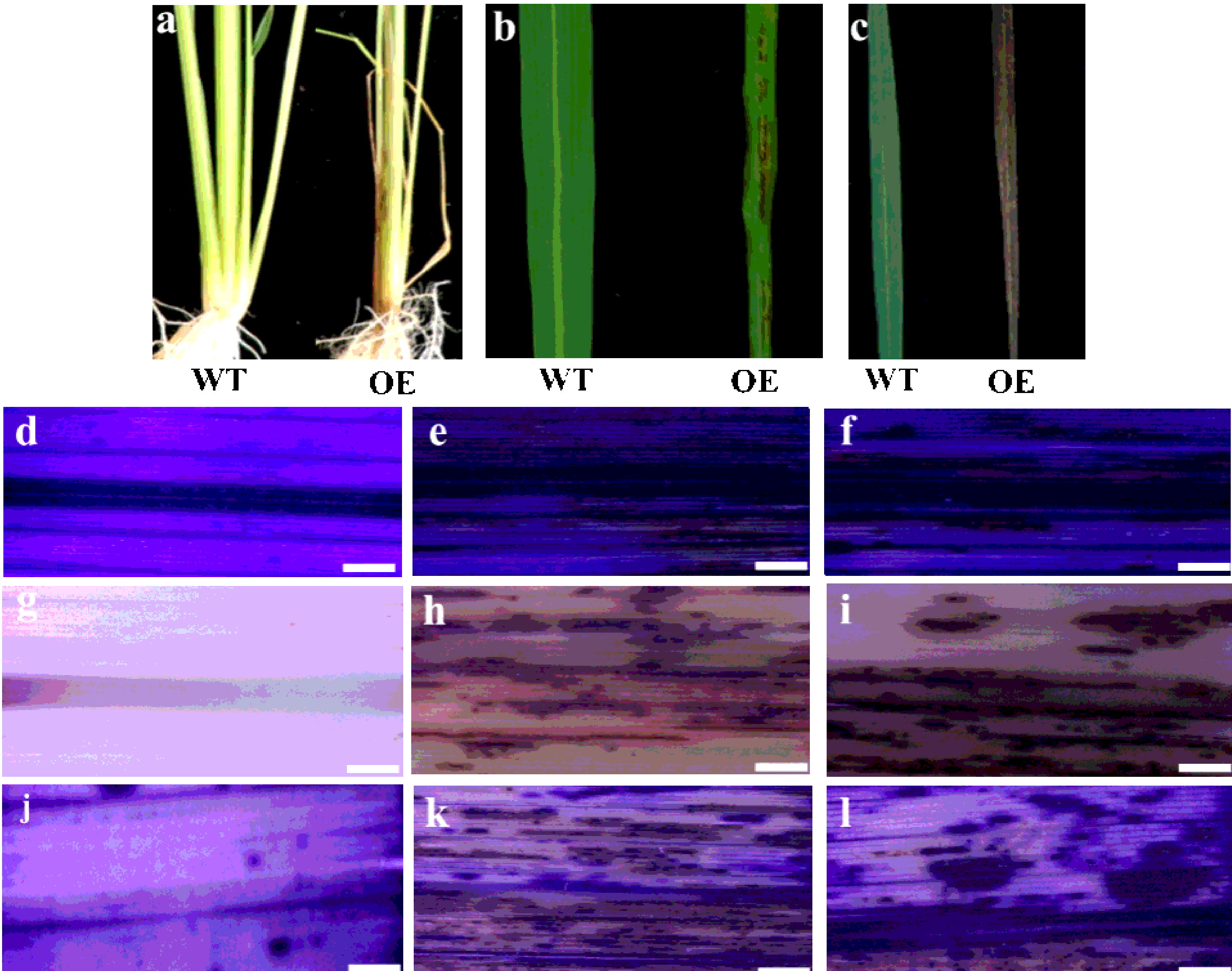

Figure 4

Overexpression of OsMed16 causes spontaneous cell death. a-c Lesion phenotype of OsMed16-OE plants in leaf sheath and flag leaf grown for 30 d (b) and 60 d (c). Scale bars = 10 cm. Trypan Blue staining (d-f), DAB staining (g-i) and NBT staining (j-l) of the leaves of the wild-type and OsMed16-OE plants grown in a nutrient solution for 48 d. WT (d, g, j), OE-8 (e, h, k), OE-14 (f. i, l).

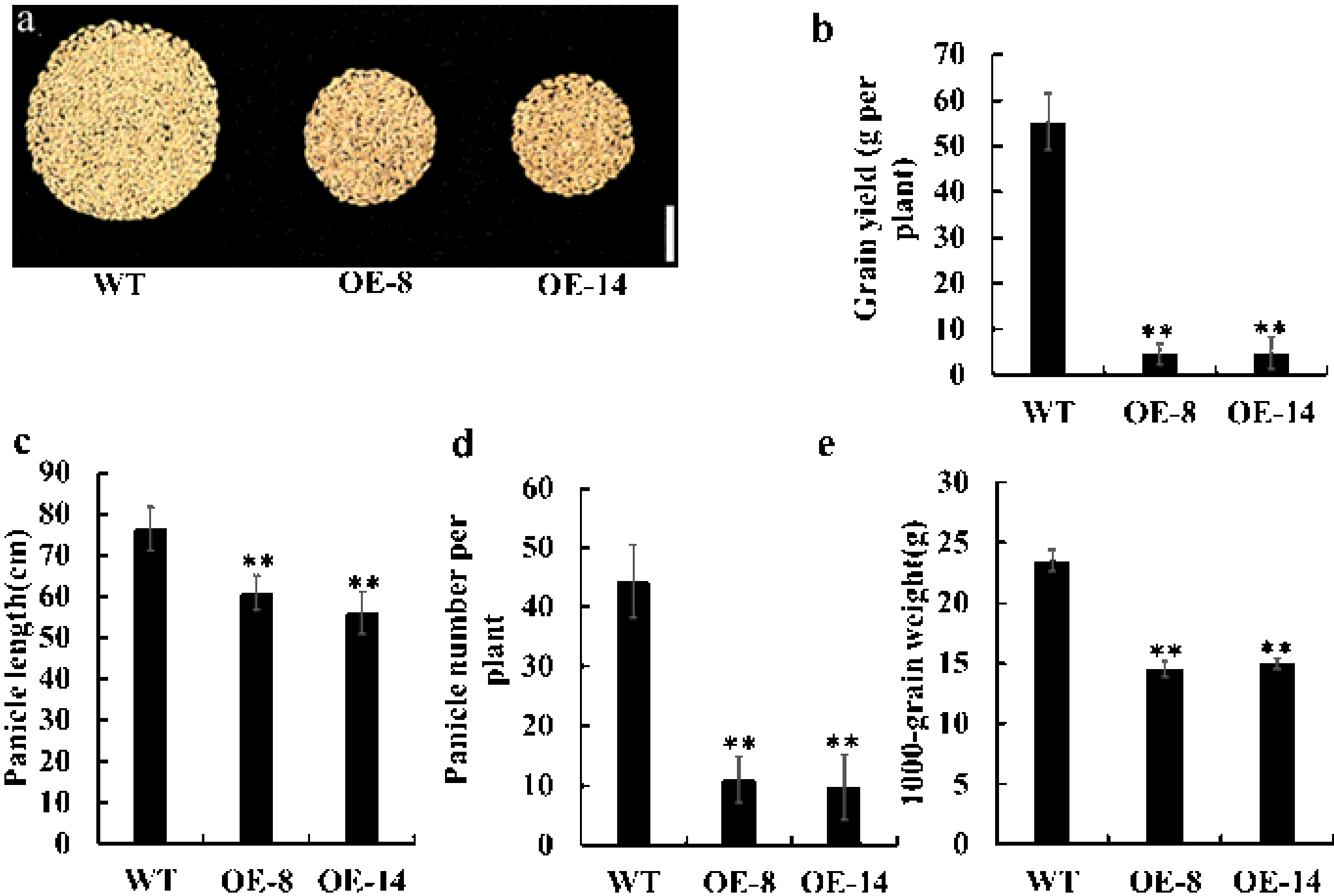

Figure 5

Overexpression of OsMed16 reduced rice grain yield. a Total grains per plant of WT and OsMed16-OE plants grown in field. Scale bars = 10 cm. b-e Comparison of grain yield per plant (b), panicle length (c), panicle number per plant (d) and 1000-grain weight (e) of the WT and OsMed16-OE plants. Data (b-e) are means ±SD of three biological replicates. * represents $P < 0.05$, * * represents $P < 0.01$.

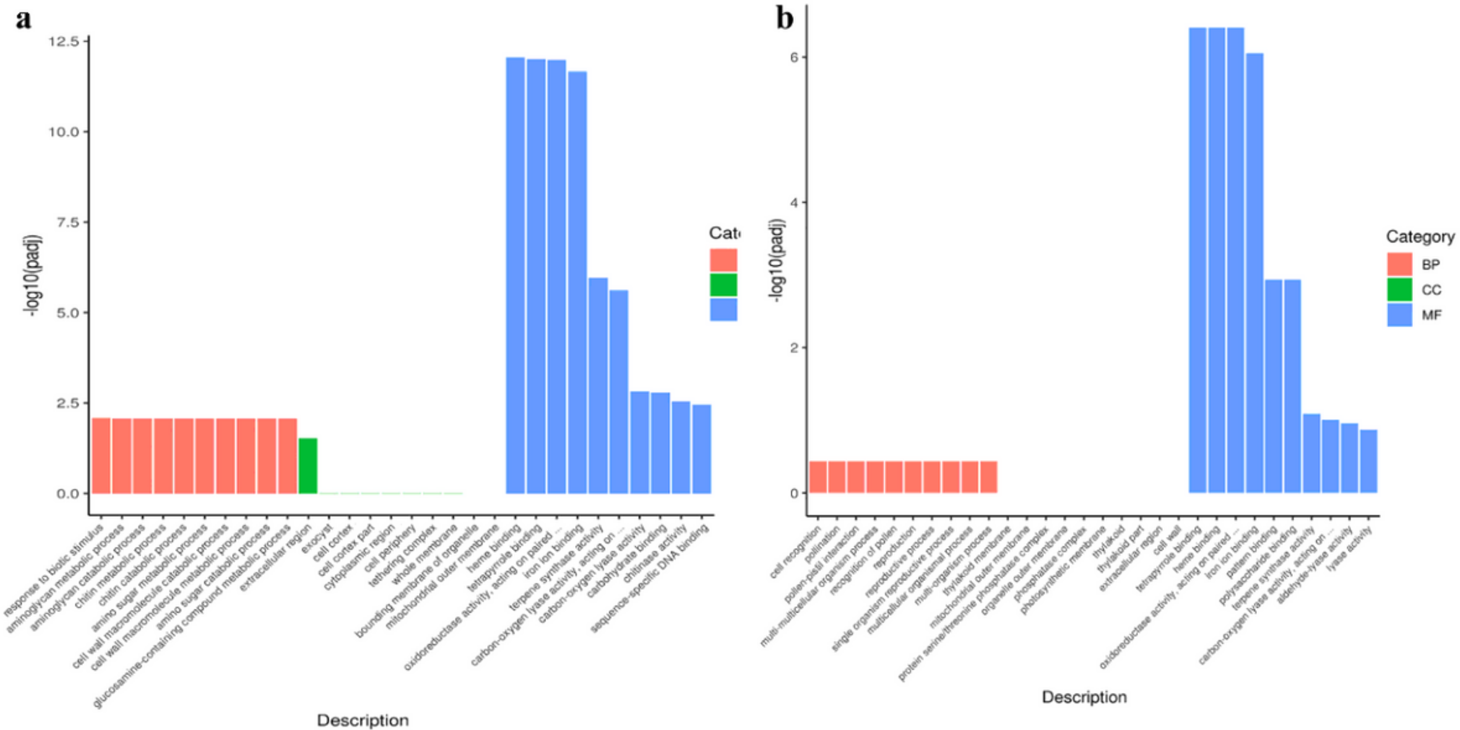

Figure 6

GO enrichment analysis of DEGs between OsMed16-OE and wild-type plants. (a) Differentially up-regulated gene GO rich map in WT and OE leaves with lesions. (b) Differentially down-regulated gene GO rich map in WT and OE leaves with lesions.

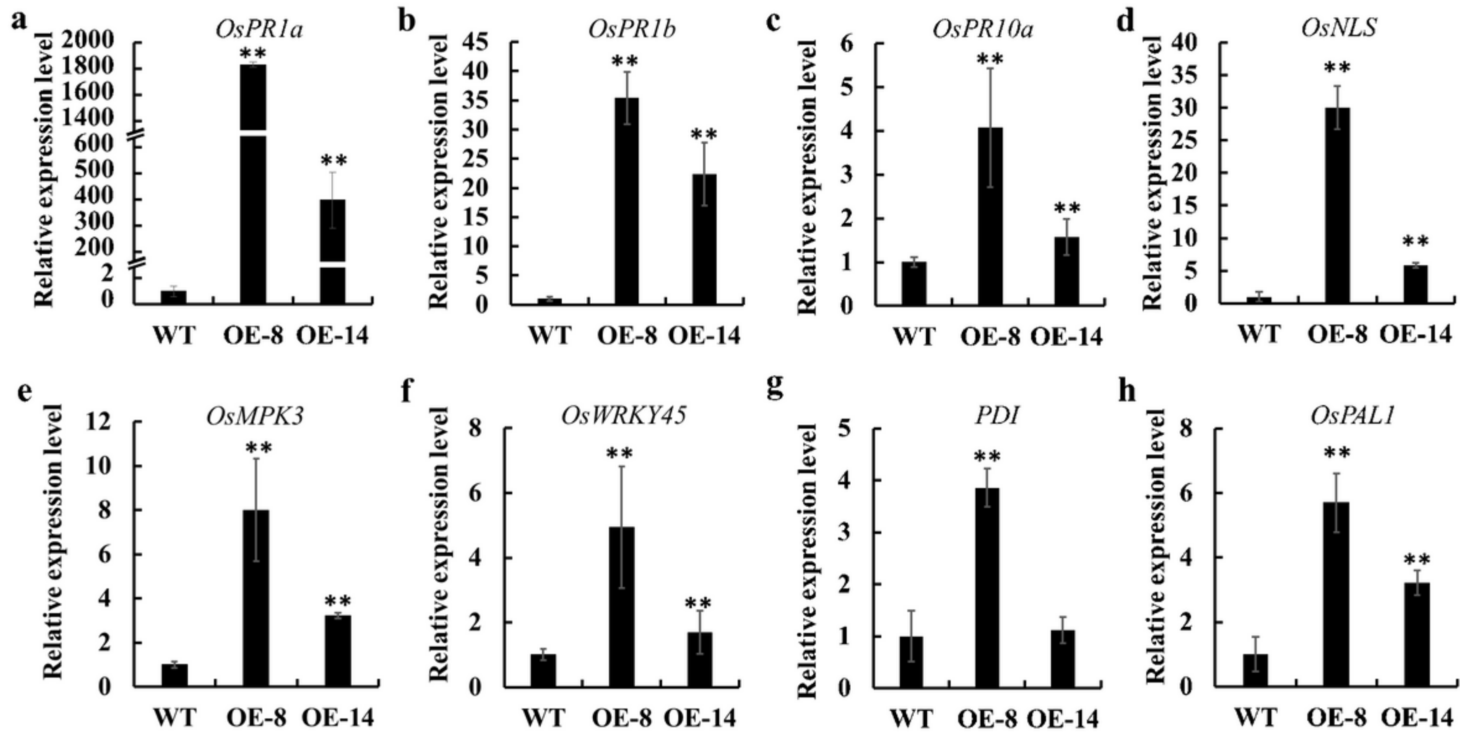

Figure 7

Elevated expression of some defense-related genes in OsMed16-OE plants. The expression patterns of OsPR1a (a), OsPR1b (b), OsPR10a (c), OsNLS (d), OsMPK3 (e), OsWRKY45 (f), PDI (g), and OsPAL1 (h) genes in OE and WT; Data are means ±SD of three biological replicates

Supplementary Files

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