The rice terpene synthase gene OsTPS19 functions as an (S)-limonene synthase in planta, and its overexpression leads to enhanced resistance to the blast fungus Magnaporthe oryzae

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Summary

Rice blast disease, caused by the fungus Magnaporthe oryzae, is the most devastating disease of rice. In our ongoing characterization of the defence mechanisms of rice plants against M. oryzae, a terpene synthase gene OsTPS19 was identified as a candidate defence gene. Here, we report the functional characterization of OsTPS19, which is up-regulated by M. oryzae infection. Overexpression of OsTPS19 in rice plants enhanced resistance against M. oryzae, while OsTPS19 RNAi lines were more susceptible to the pathogen. Metabolic analysis revealed that the production of a monoterpenic (S)-limonene was increased and decreased in OsTPS19 overexpression and RNAi lines, respectively, suggesting that OsTPS19 functions as a limonene synthase in planta. This notion was further supported by in vitro enzyme assays with recombinant OsTPS19, in which OsTPS19 had both sesquiterpene activity and monoterpenoid synthase activity, with limonene as a major product. Furthermore, in a subcellular localization experiment, OsTPS19 was localized in plastids. OsTPS19 has a highly homologous paralog, OsTPS20, which likely resulted from a recent gene duplication event. We found that the variation in OsTPS19 and OsTPS20 enzyme activities was determined by a single amino acid in the active site cavity. The expression of OsTPS20 was not affected by M. oryzae infection. This indicates functional divergence of OsTPS19 and OsTPS20. Lastly, (S)-limonene inhibited the germination of M. oryzae spores in vitro. OsTPS19 was determined to function as an (S)-limonene synthase in rice and plays a role in defence against M. oryzae, at least partly, by inhibiting spore germination.

Keywords: Oryza sativa, limonene synthase, disease resistance.

Introduction

Rice blast disease caused by Magnaporthe oryzae, one of the top 10 fungal pathogens (Dean et al., 2012), is the most devastating rice disease, causing severe loss of production. As a model system for molecular studies, rice and M. oryzae have been investigated for pathogenicity, host resistance and their interactions. The basal resistance triggered by pathogen-associated molecular patterns and specific effector-triggered immunity is associated with a similar network and activate innate immune responses, including global transcriptional reprogramming (Zhang and Zhou, 2010). Rice defences, which are activated by blast resistance (called R) genes, often break down in practical applications because the pathogen effectors evolve rapidly to evade recognition by the corresponding R genes. Therefore, efforts have been undertaken to dissect the molecular processes as well as metabolites related to plant defences and provide useful resources for breeding of durable disease resistance. Recently, a natural allele of a C2H2-type transcription factor in the rice cultivar Digu was found to confer nonrace-specific resistance against blast fungi (Li et al., 2017). In addition to innate immune responses, plants also established an inducible immune response system, including systemic acquired resistance (SAR). Plant-synthesized metabolites, such as salicylic acid (SA), ppecolic acid, azelaic acid and glycerol-3-phosphate, function as signal molecules in SAR (Fu and Dong, 2013). It was recently reported that volatile monoterpenes, particularly pinenes, promote SAR within and between Arabidopsis plants (Riedmeier et al., 2017).

Upon pathogen infection, plants produce low-molecular weight compounds with antimicrobial activities, such as phenolics, terpenoids and glucosinolates. Most of them are categorized as secondary metabolites. In the case of blast fungal attack, rice plants accumulate several types of diterpenoids and the flavonoid sakuranetin (methylated naringenin) as phytoalexins (Ahuja et al., 2012). To develop strategies to protect plants from biotic stresses,
efforts have also been devoted to regulate the accumulation of defence chemicals, especially by identifying the genes responsible for metabolite biosynthesis. In rice, many such genes have been identified, such as the terpene synthase genes and SA methyltransferase genes involved in the production of insect-induced volatiles (Yuan et al., 2008; Zhao et al., 2010). An increase in neomenthol and menthol contents confers pepper and Arabidopsis resistance against bacterial and fungal pathogens (Choi et al., 2008).

A number of terpene synthases (TPSs) from a range of plant species have been characterized, and some of them have been used for metabolic manipulation (Aharoni et al., 2006; Yu and Utsumi, 2009). Ectopic expression of lemon ω-zingiberene synthase, a sesquiterpene synthase gene, increased the accumulation of ω-zingiberene and other sesquiterpenes and monoterpenes in tomato (Davidovich-Rikanati et al., 2008). (E)-β-caryophyllene in rice and maize has been shown to play a role in the attraction of parasitoid wasps of Anagrus nilaparvatae or entomopathogenic nematodes (Cheng et al., 2007; Degenhardt et al., 2009). Genetic modification has also been applied to change the composition of essential oils, primarily composed of monoterpenes and sesquiterpenes. The compound mixtures are used as fragrances, flavours and chemopreventive agents to protect plants from pathogen infection (Gershenson and Dudareva, 2007). For example, overexpression of a limonene synthase gene from spearmint (Mentha spicata) altered the monoterpene composition of developing leaves of transgenic spike lavender (Muñoz-Bertomeu et al., 2008). In addition, heterologous expression of monoterpene synthase genes from lemon altered the fragrance of tobacco plants (Lücker et al., 2004). The monocyclic monoterpene limonene has been found to be released from rice plants constitutively, and its emission is enhanced under abiotic and biotic stress conditions (Lee et al., 2015, 2016; Lou et al., 2006; Obara et al., 2002; Yuan et al., 2008).

Two recently duplicated terpene synthase genes, OsTPS19 (Os04g27190) and OsTPS20 (Os04g27340), were ascribed different functions as reported by Taniguchi et al. (2014) and Lee et al. (2015, 2016), respectively. OsTPS20 was found to be induced by oxidative stress or the bacterial pathogen Xanthomonas oryzae pv. oryzae (Xoo) (Lee et al., 2015, 2016). The recombinant OsTPS20 protein has been shown to produce many monoterpene including (S)-limonene (Lee et al., 2015). In another report, OsTPS19 was characterized as a sesquiterpene β-elemene synthase gene (Taniguchi et al., 2014). In our previous studies, OsTPS19 was induced remarkably in rice plants by fall armyworm (Spodoptera frugiperda, FAW) infestation (Yuan et al., 2008). Accumulated data indicate that transcription factors, such as WRKYs family members, play important roles in regulation of primary and secondary metabolic pathways (Akagi et al., 2014; Han et al., 2014; Liang et al., 2017; Xu et al., 2004). In searching for potential target genes of the transcription factor OsWRKY89, OsTPS19 was found to be up-regulated in OsWRKY89 overexpression lines, which enhanced resistance to the rice blast fungus M. oryzae and S. furcifera (Wang et al., 2007). In this article, we are interested in identifying genes of secondary metabolism that are involved in OsWRKY89-mediated rice defence against M. oryzae. Here, we provide biochemical, subcellular localization and transgenic evidence to support that OsTPS19 functions as a limonene synthase in planta. Overexpression of OsTPS19 in rice led to enhanced the resistance against M. oryzae.

## Results

### Induced expression of OsTPS19 in relation to rice defence against M. oryzae infection

Transcription of OsTPS19 was increased in transcription factor OsWRKY89 overexpression plants, according to the microarray analysis data. To confirm this result, specific primers were designed to distinguish OsTPS19 and OsTPS20, two close homologs with an amino acid identity of 95.5% (nucleotide identity of 97.3% in the open reading frame region) on chromosome 4. As shown in Figure 1a, the level of OsTPS19 mRNA accumulation was markedly increased in the OsWRKY89 overexpression plants (OW89-S13, Figure S1), whereas the transcription level of OsTPS20 did not change significantly.

Induction of OsTPS19 and OsTPS20 was analysed in three-week-old rice seedlings inoculated with M. oryzae, a causal agent of rice blast fungus. As shown in Figure 1b, only OsTPS19 transcription was induced by a virulent M. oryzae S2 strain in comparison with the mock treatment. As there was a significant induction of OsTPS19 in the mock treatment (Figure 1b), we speculate that expression of OsTPS19 was related to diurnal rhythms because some volatile terpenoids are released in a fluctuating pattern (Cheng et al., 2007; Yazaki et al., 2017). OsTPS19 transcription showed an oscillating pattern with the shifting of dark/light periods, that is an increase during the dark period, reaching a peak at the switching point of the dark/light periods (Figure S2). The induction pattern of OsTPS19 expression gave an explanation for increase its transcription in the mock treatment of pathogen infection in which the rice seedlings were kept in the dark for 24 h under high humidity (Figure 1b).

### Altered expression of OsTPS19 led to changes in rice resistance against M. oryzae

To verify the in planta function of OsTPS19, transgenic rice plants were generated by means of overexpression or RNAi knock-down of both OsTPS19 and OsTPS20 due to their high similarity. Transgenic rice plants did not exhibit any apparent morphological changes. Three independent overexpression (S4, S5 and S11) and double RNAi (d30, d31 and d51) lines were selected for experimental analyses. First, the expression of OsTPS19 in transgenic and wild-type rice plants was measured using quantitative RT-PCR (qRT-PCR). OsTPS19 transcription levels in all three overexpression lines were increased over 100-fold compared to those in wild-type plants, while the expression of OsTPS19 led to changes in rice resistance against M. oryzae.
possible role of OsTPS19 in defence against the microbial pathogen *M. oryzae*. Six days after the inoculation of *M. oryzae* SZ, the sizes of the lesions on individual leaf blades were quantified. The lesion areas on OsTPS19 overexpression plants were smaller than those on wild-type plants. By contrast, the lesions areas on RNAi lines were larger than those on wild-type plants (Figure 2d and e). Spot inoculation of the pathogen showed similar results (Figure S4), suggesting that OsTPS19 might play a role in the defence reaction of rice. Nevertheless, the contribution of OsTPS20 should not be ignored even though its transcription level is relatively low.

Transgenic rice plants with altered OsTPS19 transcription showed altered levels of limonene emission

To understand the mechanisms underlying the OsTPS19-related defence against the fungus, we performed metabolic profiling of the transgenic plants. As OsTPS19 has been reported as a β-elemene/β-bisabolene synthase *in vitro* (Taniguchi et al., 2014), we measured the headspace of transgenic and wild-type rice plants to determine the metabolic changes. There were low levels of β-elemene, β-bisabolene and sabine emmissions among the wild-type and transgenic lines. Surprisingly, the emission rates of
transgenic lines were analysed using Student’s t test. Limonene emission was measured in three-week-old wild-type (WT) and transgenic seedlings using GC-MS. ND indicates not detectable due to low amounts. Significant differences between WT and transgenic lines were determined by Student’s t test (% in Figure 3).

Limonene is a chiral molecule. Two enantiomers of limonene are known to be produced biologically: (R)-limonene and (S)-limonene. In our previous study, rice plants were shown to release elevated levels of limonene when exposed to fall armyworm larvae (Yuan et al., 2008). In this study, volatiles released from rice plants infested with fall armyworm larvae were collected as previously described. The mixture of volatiles was analysed using chiral gas chromatography mass spectrometry (GC-MS). In comparison with authentic standards, the limonene produced by rice plants was determined to be (S)-limonene (Figure S5).

OsTPS19 has both monoterpene synthase and sesquiterpene synthase activities in vitro

To examine the catalytic activities of the OsTPS19 protein, the cDNA of the gene was expressed in Escherichia coli, and the recombinant protein was used for the determination of terpene synthase activity. Crude extracts from E. coli harbouring only the vector without any OsTPS gene inserted were assayed as a negative control.

OsTPS19 was first assayed with geranyl diphasphate, the substrate for monoterpene synthases. OsTPS19 catalysed the formation of 16 monoterpene, including α-thujene, β-pinene, sabinene, myrcene, α-phellandrene, α-terpinene, (S)-limonene, cis-ocimene, trans-ocimene, γ-terpinene, trans-sabinene hydrate, α-terpinolene, cis-sabinene hydrate, neo alloocimene, terpen-4-ol, and α-terpineol hydrate (Figures 4a and S5). However, (S)-limonene was the predominant compound.

OsTPS19 was then assayed for sesquiterpene synthase activities. Using farnesyl diphasphate as a substrate, OsTPS19 catalysed the formation of 12 sesquiterpenes with β-bisabolene as the most abundant product (Figure 4b).

OsTPS19 is targeted to plastids

OsTPS19 was constructed to the 5’ end of GFP, and the fused gene was controlled by a CaMV35 promoter (CaMV35: OsTPS19-GFP). Fluorescence of GFP was observed in 2-week-old root cells of CaMV35:OsTPS19-GFP transgenic rice plants of T1 progeny (Figure 5) and was colocalized with red autofluorescence of chlorophylls, indicating that the OsTPS19-GFP protein is targeted to plastids. Additionally, expression of OsTPS19-GFP was performed transiently in leaves of Nicotiana benthamiana by agroinfiltration. Co-localization of GFP with red autofluorescence of chlorophylls confirmed the plastidial target of OsTPS19-GFP (Figure S6).

Functional variation of OsTPS19 and OsTPS20 is determined by a single amino acid

The high sequence similarity and the close locations of OsTPS19 and OsTPS20 suggest that these two genes resulted from a recent gene duplication event. Similar to OsTPS19, OsTPS20 also showed monoterpene synthase and sesquiterpene synthase activities in vitro and is targeted to plastids (Figures S7 and S8). In addition to the difference in limonene production, OsTPS19 and OsTPS20 also showed differences in the relative proportions of other minor products. For instance, α-terpinolene was the second most abundant product of OsTPS19, while OsTPS20 formed cis-sabinene hydrate as the second most abundant product (Figures 4a and S7a).

To understand the structural basis underlying the functional divergence of OsTPS19 and OsTPS20, we generated a homology-based structural model of OsTPS19 and identified active site residues that differed between OsTPS19 and OsTPS20. Among all the variations between the two proteins, only one residue (alanine 567 in OsTPS19) was localized in the active site cavity (Figure 6a). This amino acid variation was hypothesized to be responsible for the different product specificities of OsTPS19 and OsTPS20.

To test this hypothesis, two mutants, OsTPS19A567S and OsTPS20A567A, were created, and the mutant proteins were tested for activity using geranyl diphasphate. The product profile of OsTPS19A567S was highly similar to that of the native OsTPS19, while the product profile of OsTPS20A567A was highly similar to that of the native OsTPS19 (Figure 6b). Thus, the functional variation between OsTPS19 and OsTPS20 was determined by a single amino acid change.

To determine whether OsTPS19 and OsTPS20 are differentially regulated in the process of subfunctionalization, we examined their transcription in dsOW62/76-108 plants, which harbour an RNAi construct to knock down both OsWRKY62 and OsWRKY76 and show high resistance to M. oryzae and bacterial blight pathogen Xanthomonas oryzae pv. oryzae (Xoo) (Liu et al., 2016). Interestingly, the transcription of OsTPS19 was down-regulated, whereas the level of OsTPS20 mRNA was remarkably enhanced (Figure 7a). On the other hand, the induction of OsTPS20 was higher in comparison with OsTPS19 in rice leaves treated with MeJA (Figure 7b). Additionally, we noticed that the basal transcription level of OsTPS19 was several fold higher than that of OsTPS20 in wild-type plants. These results suggest that OsTPS19 and OsTPS20, two recently duplicated genes, have evolved to respond differently to environmental stimuli.

(S)-Limonene has an inhibitory effect on the germination of M. oryzae spores

To assess the effect of (S)-limonene on the pathogen, the germination of M. oryzae spores was examined at different concentrations of (S)-limonene. Spore germination was decreased by treatment with (S)-limonene and was even completely inhibited under increased (S)-limonene concentrations (Figure 8). The results imply that (S)-limonene might have a direct role in the suppression of fungal infection.
Discussion

OsTPS19 is a rice (S)-limonene synthase gene in planta

Among numerous metabolites that may prevent pathogen invasion by forming chemical barriers, terpenoids are the major contributors to the chemical arsenal of plants and play roles in pollinator attraction and signalling to other plants (Dudareva et al., 2006). A number of TPSs from a variety of plant species have been well studied, and some of them have been applied for metabolic manipulation (Aharoni et al., 2006; Yu and Utsumi, 2009). Sequence comparison indicates that OsTPS19 is identical to AK071447, which has recently been reported as a sesquiterpene synthase in rice (Taniguchi et al., 2014). Jasmonic acid treatment of rice plants resulted in a significant up-regulation of AK071447 expression, correlating with enhanced levels of \( \beta \)-elemene, one of the in vitro sesquiterpene products of recombinant AK071447 (Taniguchi et al., 2014). Here, we found that...
the OsTPS20 gene was induced more than OsTPS19 by MeJA treatment using gene-specific primers (Figure 7b). In addition, OsTPS19 acts as a monoterpene synthase in planta (Figure 3). The emission level of limonene was increased less than 10-fold in transgenic rice plants, likely due to photosynthetic limitations (Wang et al., 2016). OsTPS19 was reported to be localized in the cytosol or other organelles other than plastids (Taniguchi et al., 2014). However, in our study, for OsTPS19 with almost identical N-terminal sequences, the subcellular localization was confirmed to be plastidial based on the root cells of stable transformants (Figure 5). Further, the level of limonene, not β-elemene, increased in overexpression rice plants. The fact that β-elemene was induced after JA treatment (Taniguchi et al., 2014) could be explained by the presence and up-regulation of a distinct β-elemene synthase gene. Additional support that OsTPS19 functions as a monoterpene synthase is based on the functional characterization of OsTPS20, which has been shown to also function as an (S)-limonene synthase (Lee et al., 2015). OsTPS20 and OsTPS19 are close homologs and are hypothesized to be derived from a relatively recent gene duplication event. It is interesting that OsTPS20 was previously shown to have no sesquiterpene synthase activity (Lee et al., 2015). By contrast, our analysis demonstrated that OsTPS20 can catalyse the biosynthesis of sesquiterpenes in in vitro assays, similar to OsTPS19.

The biological functions of OsTPS19

The list of plant terpene synthases with characterized biochemical functions has grown rapidly. Many terpenoids have antimicrobial activities (Ahuja et al., 2012). For example, limonene has both antifungal (Tao et al., 2014) and bactericidal (Diao et al., 2013; Lee et al., 2016) activities. OsTPS19, identified as a limonene synthase, was characterized to play a role in the defence of rice plants against the fungal pathogen M. oryzae. The significant differences in the lesion sizes (Figures 2 and S4) indicate that the OsTPS19 overexpressors had a stronger resistance to M. oryzae than wild-type rice plants, and the OsTPSs-RNAi lines had a
The transcription factor OsWRKY62/76 is responsible for the functional divergence of OsTPS19 and OsTPS20. It remains to be determined whether the lack of formation of sesquiterpenes from farnesyl diphosphate (Figures 4b and S7b) is the cause or the outcome of the acquired functional divergence.

Evolutionary implications of limonene synthase genes

As described previously, most limonene synthases from angiosperms belong to the TPS-b subfamily (Chen et al., 2011). The identification of two members of the TPS-a subfamily in rice encoding limonene synthase raises an interesting question about the evolution of limonene synthase genes in angiosperms. OsTPS19 and OsTPS20 were reported as different types of terpene synthases by Taniguchi et al. (2014) and Lee et al. (2015, 2016), respectively. OsTPS20 was found to be induced by oxidative stress or the bacterial pathogen *Xoo* (Lee et al., 2016) and produces limonene (Figure 8). Additionally, limonene might be oxidized to limonene hydroperoxide, which acts as an active oxygen species to initiate wide defence responses (Ben-Yehoshua et al., 2008).

Some nontarget pathways and products of metabolites (Pasoreck et al., 2016) may also be involved in the increased resistance against rice blast fungus in OsTPS19 overexpression rice plants.

Experimental procedures

Plant growth and treatments

Rice seeds of wild-type (*Oryza sativa* L. Zhonghua 17, ZH17 or Xiushui 11, XS11) and transgenic progenies were germinated, and the seedlings were grown in vermiculite at approximately 28°C under a 14/10-h (light/dark) photoperiod in a greenhouse. For circadian rhythm treatment, a short day condition of 10/14-h (light/dark) cycle was used (Cai et al., 2014). For MeJA treatment, three-week-old rice seedlings were treated with 100 μM MeJA (dissolved in 10 mL 4-morpholine ethanosulphonic acid buffer, pH 5.6) by foliar spray. Treated leaves were sampled at 1, 3, 6, 12 and 24 h after treatment for RNA isolation.

Terpene synthase enzyme assays

Full-length cDNAs for OsTPS19 and OsTPS20 were cloned using RT-PCR. The primers used were 5'-atgtcaacttccatccctctc-3' and 5'-ctaaaggtgacaggattcac-3' for OsTPS19 and 5'-ctaaaggtgacaggattcac-3' (reverse, TPS19r) for OsTPS19 and

Figure 8 Inhibitory effect of (S)-limonene on the spore germination of *M. oryzae*. An aliquot of 0.1 mL of *M. oryzae* spore suspension (5 × 10⁵ spores/mL) was placed on separate water agar plates that contained various concentrations of (S)-(−)-limonene (0, 20, 50, 80 and 100 mmol/L). Each concentration had three repeats. Approximately 200 spores were counted, and the spore germination percentage was calculated (a) and photographed (b) after 5 h of incubation at 28°C. ND indicates not detectable due to no spore germination. S-50 represents the treatment, in the presence of 50 mmol/L (S)-(−)-limonene. Asterisks show statistically differences between the mock and treatment (Student’s *t* test; *P* < 0.05 and ***P* < 0.001).
5'-atgtcacttccatccctc-3' and 5'-tagatggggacaggattc-3' (reverse, TP520r) for OsTPS20. After cloning the full-length cDNAs, forward primers of 5'-atgcgacaagcgctgccatc-3' and 5'-atgcgacagcgctgccatc-3' paired with TP519r and TP520r, respectively, were used to amplify the truncated forms of OsTPS19 and OsTPS20. Expression of OsTPS19 and OsTPS20 in E. coli and in vitro terpene synthase enzyme assays using farnesyl diposphophate and geranyl diposphophate as substrates were performed as previously described (Yuan et al., 2008).

**Generation of transgenic plants**

OsTPS19 was amplified using the forward primer 5'-tattggatccgcaagggaaatatactagtatg-3' (referred to as OsTPS19) and the reverse primer 5'-gctagatggggacaggattc-3' (reverse). The RNAi construct was placed under the control of the cauliflower mosaic virus promoter (CaMV35S).

For the determination of OsTPS19 subcellular localization, the chimeric gene of OsTPS19 and the green fluorescent protein (GFP) gene were used to generate the CaMV35S:OsTPS19-GFP construct.

Transgenic plants were obtained by the Agrobacterium-mediated transformation method using Zhonghua 17 as the donor. For each construct, more than 10 independent transgenic lines were screened for hygromycin resistance, and the antibiotic resistant lines were used for experiments.

To detect the subcellular localization of OsTPS20, the cDNA of OsTPS20 was amplified using PCR with two primers: 5'-gctagcattcacttccatc-3' (forward), 5'-ccgcgtcatcattccag-3' (reverse). The verified PCR product was cloned into the vector pANS81 to generate a C-terminal fusion of OsTPS20 to YFP.

**Headspace collection of rice plants and volatile analysis using GC-MS**

The collection of volatiles emitted from 3-week-old rice plants was performed using a previously described open headspace system (Sun et al., 2017). In brief, volatiles were collected for 24 h on 50 mg of 60/80 mesh Tenax-TA (Shanghai ANPEL Scientific Instrument Company, Shanghai, China). The collected volatiles were extracted with 300 μL of HPLC-grade hexane (Fisher Scientific, New Jersey), to which 25.95 ng ethyl decanoate (Sigma-Aldrich, Oakville, ON) was added as an internal standard. One microlitre of each sample was analysed using a Shimadzu GC-MS (GC-MS-QP2010 SE, Japan) on an Rxi-5Sil MS column (30 m × 0.250 mm × 0.25 μm, Restek, Bad Homburg, Germany). The GC oven temperature program was 40°C for 1 min followed by an increase to 130°C at a rate of 4°C/min (5-min hold) and then to 250°C at a rate of 10°C/min (5-min hold). Chiral GC-MS analysis of limonene was performed using an RT®-βDEXsrm column (Restek, Bad Homburg, Germany) and a temperature program of 40°C (1-min hold) followed by an increase to 100°C at a rate of 5°C/min and then to 240°C at a rate of 10°C/min (2-min hold). The flow rate of the carrier gas (helium) was 1 mL/min. Limonene standards were obtained from Sigma.

**Subcellular localization of OsTPS19 and OsTPS20**

Localization of OsTPS19 was analysed using CaMV35S:OsTPS19-GFP transgenic rice seedlings. The seeds of the transgenic progenies were germinated on solid half-strength MS medium for 2 weeks at 28°C under a 14/10-h light/dark cycle. The cells of 2-week-old rice roots were examined using a confocal laser scanning microscope (Eclipse TE2000, Nikon, Beijing, China). For transient expression, the CaMV35S:OsTPS19-GFP plasmid was introduced into the leaves of 4-week-old N. benthamiana by agroinfiltration. Fluorescence was visualized using the confocal laser scanning microscope.

The plasmid coding for the OsTPS20-YFP fusion was co-introduced together with the plastid-targeted plasmid (pt-r), which codes for a plastid-targeted variant of mCherry, into onion epidermal cells by PDS-1000 biolistic particle delivery system (Bio-Rad, Hercules, CA). Fluorescence in the epidermal cells was examined 16 h postbombardment using fluorescence microscopy on a Zeiss Axiovert 200M (www.zeiss.com) with a 20 × objective and appropriate filters (Chroma set 69308; www.croma.com).

**Real-time quantitative RT-PCR**

DNase-treated RNA (2 μg) was reverse-transcribed for analysis of gene expression. Quantitative RT-PCR (qRT-PCR) was performed in a 15-μL reaction volume using SYBR Green dye. The level of the rice ubiquitin gene (UBQ) expression was used to normalize the expression of other genes. Primer sequences used for the qRT-PCR analysis were 5'-gtgcgtcctctgtaaagttttcttc3' (forward) and 5'-aatggtgggtctgtttaggtctc-3' (reverse) for OsWRKY89, 5'-gactgctatatactgagc3 (forward) and 5'-acagattcacaatctccc3'-3' (reverse) for OsTPS19, 5'-cactgttagtgctatag3'-3' (forward) and 5'-caacagatctcccaacctc3' (reverse) for OsTPS20, and 5'-gtgtgggcctcaaatgctc-3' (forward) and 5'-ggcacaatgtaggat cat-3' (reverse) for UBQ.

**Pathogen inoculation**

To test disease resistance capability, the wild-type plants (ZH17) and three-week-old transgenic lines were inoculated with the spore solution (105 conidia per millilitre containing 0.02% silwet-L77) by spraying or spot inoculation as described previously (Fujisaki et al., 2015; Wang et al., 2007). The area of lesions formed 6 days postinoculation was measured using an image analysis method. For spot inoculation, the vertical length of disease lesions was measured 6 days after inoculation.

**Spore germination assay**

An aliquot of 0.1 mL of M. oryzae spore suspension (5 × 105 spores/mL) was placed on separate water agar plates containing various concentrations of (S)-(-)-limonene. Each concentration had three repeats. Approximately 200 spores were counted, and the spore germination percentage was calculated after 5 h of incubation at 28°C.

**Homology-based structural modelling**

The Swiss-Model Server (http://www.expasy.org; Schwede et al., 2003) was used to generate three-dimensional structural models of OsTPS19 and OsTPS20. For modelling purposes, the protein sequences of OsTPS19 and OsTPS20 were fitted to the crystal structure of the tobacco 5-epi-aristolochene synthase mutant C440W (Starks et al., 1997; PDB ID, 1HXCA). The quality of the models was assessed using the program ProSA-web (Wiederstein et al., 2000).
and Sippl, 2007) as well as the assessment tools provided by the Swiss-Model Server. The program PyMOL (http://www.pymol.org) was used for visualization of the resulting structural models.

Site-directed mutagenesis

The primers used for generating OsTPS19 A567S and OsTPS20 S567A were 5’-ttgtaatggcctagctggctctttctgcatc-3’ and 5’-tgcctaaaatgccgctgctcagccttcactcttgcc-3’, respectively. After confirmation by sequencing, the mutant proteins were expressed in E. coli. Terpene synthase activity assays were performed as described in the previous subsection.

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Author contributions

X.C., H.C. J.S.Y, Z.G. and F.C. designed experiments; X.C., H.C. J.S.Y, Z.G. and F.C. conducted experiments and analyzed the data; X.C., H.C., Z.G. and F.C. wrote the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Transcription level of OsWRKY89.

Figure S2 Expression of OsTPS19 was related to diurnal rhythms.

Figure S3 Suppression of OsTPS20 transcription in the double RNAi plants.

Figure S4 Overexpression of OsTPS19 enhanced resistance against rice blast fungus through spot inoculation.

Figure S5 Chiral GC-MS analysis of limonene emitted from rice seedlings.

Figure S6 Plastid localization of OsTPS19 in tobacco leaf.

Figure S7 Recombinant OsTPS20 exhibited monoterpene and sesquiterpene synthase activities.

Figure S8 Plastid localization of OsTPS20.