Overexpression of the homoterpenoid synthase gene, OsCYP92C21, increases emissions of volatiles mediating tritrophic interactions in rice

Enhancing Homoterpenes Emissions in Rice

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Abstract

Plant defense homoterpenes can be used to attract pest natural enemies. However, the biosynthetic pathway of homoterpenes is still unknown in rice, and the practical application of such indirect defense systems suffers from inherent limitations due to their low emissions from plants. Here, we demonstrated that the protein OsCYP92C21 is responsible for homoterpene biosynthesis in rice. We also revealed that the ability of rice to produce homoterpenes is dependent on the subcellular precursor pools. By increasing the precursor pools through specifically subcellular targeting expression, genetic transformation and genetic introgression, we significantly enhanced homoterpene biosynthesis in rice. The final introgressed GM rice plants exhibited higher homoterpene emissions than the wild type rice and the highest homoterpene emission reported so far for such GM plants even without the induction of herbivore attack. As a result, these GM rice plants demonstrated strong attractiveness to the parasitic wasp Cotesia chilonis. This study discovered the homoterpene biosynthesis pathway in rice, and lays the foundation for the utilization of plant indirect defense mechanism in the “push-pull” strategy of integrated pest management through increasing precursor pools in the subcellular compartments and overexpressing homoterpene synthase by genetic transformation.
Keywords: homoterpene, subcellular location, transgenic rice, GM, rice pest, *Cotesia chilonis* parasitic wasp, sustainable agriculture.

Introduction

Plants defend themselves from the attack of herbivorous insects directly and indirectly by emitting herbivore-induced plant volatiles (HIPV) to repel herbivorous insects and/or to attract their natural enemies (Baldwin & Preston, 1999; Sabelis, Janssen, & Kant, 2001). For example, linalool emission repels the rice brown planthopper (*Nilaparvata lugens*) and attracts predatory mites and parasitic wasps on rice (Xiao et al., 2012) and maize (Tamiru et al., 2015). Similarly, (*E*)-caryophyllene can attract parasitoid wasps of *Anagrus nilaparvatae* (Cheng et al., 2007) and (*E*)-β-farnesene can repel aphids (Gao, Zhang, Zhou, Chen, & Lin, 2015; Sun et al., 2017).

Research on plant indirect defense mechanisms can provide opportunities for enhancing plant defense against pests by using plant volatiles, thereby replacing pesticides in pest management. The homoterpenes (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) play important roles in recruiting natural enemies of herbivores in plant indirect defense (Brillada et
al., 2013; Herde et al., 2008; Liu et al., 2017; Tholl, Sohrabi, Huh, & Lee, 2011). These homoterpenes are plant volatiles with irregular terpene structures, and are widely produced and specifically emitted by higher plants upon herbivore feeding.

The metabolic and biosynthetic pathways of the homoterpenes in plants seem to be specific between dicot and monocot plants as well as among different plant tissues. The biosynthesis of DMNT and TMTT involves the formation of tertiary alcohol precursors (E)-nerolidol and (E,E)-geranyllinalool by terpene synthases and the oxidation to DMNT and TMTT in the presence of cytochrome P450 (CYP) enzymes (Wilhelm Boland & Gäbler, 1989; Wilhelm Boland, Gaebler, Gilbert, & Feng, 1999). This pathway has been confirmed in Arabidopsis thaliana and a homoterpene synthesis gene AtCYP82G1 has been identified, which is only present in dicots (Wilhelm Boland & Gäbler, 1989; Lee et al., 2010). An alternative root specific pathway in A. thaliana involving CYP705A1 (a member of CYP705 gene family specific to the Brassicaceae family) was shown to degrade triterpene arabidiol into DMNT (Sohrabi et al., 2015).

DMNT and TMTT were also detected in monocot maize (Zea mays) after herbivore attack (Turlings, Scheepmaker, Vet, Tumlinson, & Lewis, 1990), where two specific P450 monooxygenases (ZmCYP92C5 and ZmCYP92C5) with low identities to other published homoterpene synthases were found to be involved in the biosynthesis
of homoterpenes (Richter et al., 2016). This is the first molecular study on the biosynthesis of homoterpenes in monocot plants. DMNT and TMTT were also found to be emitted from rice plants under jasmonic acid (JA) treatment (Lou, Du, Turlings, Cheng, & Shan, 2005; Lou et al., 2006), and from the transgenic rice plants overexpressing the terpene synthase genes \textit{PlTPS3} and \textit{PlTPS4} of the lima bean \textit{Phaseolus lunatus} (Li et al., 2018), suggesting the presence of related homoterpene synthase genes in rice.

In this study, the biosynthesis and associated molecular genetics of DMNT and TMTT in rice were investigated with the wild type and transgenic plants. The importance of their precursors in individual subcellular structures (endoplasmic reticulum, chloroplast and mitochondria) was examined with the aim to enhance DMNT and TMTT emissions. The biological function of the GM and introgression transgenic rice plants in attracting adult females of \textit{Costesia chilonis}, a natural enemy of the rice pest striped stemborer \textit{Chilo suppressalis}, was also measured.

**Materials and Methods**

**Rice plants and insect materials.**

The rice cultivar Zhonghua 11 (\textit{Oryza sativa L. ssp. japonica}) (ZH11) was used as the
The striped rice stemborer *C. suppressalis* was raised with an artificial diet (W. Ma et al., 2012). The natural enemy of *C. suppressalis* the parasitic wasps *C. chilonis* were obtained from the parasitized *C. suppressalis* larvae, and fed with 10% honey water after cocoon hatching. *C. suppressalis* and *C. chilonis* were maintained in climatic chambers (27 ± 1°C, 70%–80% RH, 16L:8D photoperiod) (Teng et al., 2017).

**Plant treatment.**

Alamethicin was diluted to 10 mg/mL stock solution with methanol in advance, and then 10 μL alamethicin stock solution was mixed with 9.99 mL sterilization water to 10 μg/mL. Similarly, jasmonic acid (JA) was dissolved with a small amount of absolute ethanol, and then diluted to 100 μmol/mL with sterile ultrapure water. One 20-day-old ZH11 plants was washed and placed in a 20 mL glass container. Then, either 10 mL of 10 μg/mL alamethicin solution or 10 mL of 100 μmol/mL JA solution or 10 BPH female adult were added into to the container per plant for alamethicin treatment (Li et al., 2018), JA treatment and BPH treatment, respectively. Each treatment was repeated with 5 plants. All alamethicin-treated, JA-treated, BPH-treated and untreated control plants were maintained in a biochemical incubator (27 ± 1°C, 50–70% RH, 14L:10D photoperiod) for 24 h. The volatiles from these plants were collected by solid phase
microextraction (SPME).

**Collection and identification of rice plant volatiles.**

After root removal, 20-day-old rice plants were inserted into a 20-mL glass container with a septum on the lid. Then, the SPME fibre (50/30 μm DVB/CAR/PDMS, Supelco; Sigma-Aldrich) was inserted into the glass container through the septum. The volatiles emitted from rice plants were collected by SPME at 40 °C for 1 h.

For the wild-type plants and transgenic plants at the tillering stage (45–50 days), the headspace volatiles were collected as previously reported (Gao et al., 2015). In brief, the plants were placed in a large sealed glass chamber (35 cm in diameter and 90 cm in height). The air was purified by activated charcoal and blown into the glass chamber from the above to the bottom at a flow rate of 750 mL min⁻¹. The plant volatiles carried by the airflow were collected by 50 mg adsorbent (Tenax TA 60/80 Mesh, Supelco, Bellefonte, PA, USA) for 24 h. The volatiles on the adsorbent were eluted into the injection bottle with 700 μL of hexane (Sigma-Aldrich), and nonyl acetate (Sigma-Aldrich) was added as an internal standard at the final concentration of 1 ng μL⁻¹. After being sealed in glass vials, the volatiles were stored in a refrigerator at −20 °C.

For the collection of volatile samples by SPME, the SPME fiber was directly
inserted into the GC-MS injection port after the volatile collection. For the collection of samples by Tenax, 1 μL portion of the eluted sample was injected into the GC-MS injection port. The GC-MS analysis was conducted with GCMS-QP2010 (Shimadzu, Japan). Helium was used as the carrier gas at 1 mL min$^{-1}$. The injector, transfer line and ion source temperatures were set at 250°C, 280°C and 250°C, respectively. Volatile samples were separated using the SH-Rsi-5sil-MS column with low-polarity phase of 100% dimethyl polysiloxane (30 m × 0.25 mm × 0.25 μm, Shimadzu, Japan). The oven temperature was initially maintained at 50°C for 1 min and then increased to 60°C at a rate of 5°C min$^{-1}$, held for 5 min and finally increased to 250°C at a rate of 10°C min$^{-1}$ and held for another 5 min. The MS scanning range was 40–550 m/z. Hewlett-Packard Chemstation software was used for system control and data analysis. Volatiles were identified by comparing the retention time and mass spectra with those of the authentic standards and data from the literature, and the quantitative analysis was carried out by internal standard method. DMNT and TMTT were synthesized from geraniol and (E,E)-farnesol, respectively (Leopold, 1990). (E)-nerolidol and (E,E)-geranyllinalool were purchased from Sigma-Aldrich (MDL number: MFCD00008911, MFCD00059363), and used in the enzymatic activity assays with the recombinant proteins. The (3S)- and (3R)-enantiomeric composition for compounds with asymmetrically substituted carbons from rice plants studied here were not determined, e.g. linalool, (E)-nerolidol
and \((E,E)\)-geranyllinalool, and the compound names are given without such designations unless otherwise specified.

**Yeast expression and CYP activity assays.**

Gene-specific primers (Table S1) were used to amplify the full-length cDNA sequences of candidate CYP genes from the cDNA of rice leaves treated with alamethicin for 24 h. The PCR products were then cloned with the one step cloning kit (Vazyme Biotech) into the yeast expression vector pYES2 under the control of GAL1 promoter (galactose-induced expression). The recombinant plasmid and empty pYES2 vector were separately transformed into the *Saccharomyces cerevisiae* strain WAT11 (Urban, Mignotte, Kazmaier, Delorme, & Pompon, 1996), and the transformed cells were screened on SC-U selective medium containing 2% raffinose. Briefly, a single colony containing the recombinant plasmid was inoculated in the 10 mL SC-U liquid medium containing 2% raffinose and cultured at 30°C, shaken at 280 rpm until the OD<sub>600</sub> reached 1.0. Then, the cultured cells were induced to express the recombinant protein in the medium containing 2% galactose for 48 h (Liu et al., 2017). To measure the enzyme activity, 50 mL of the induced cells was precipitated and re-suspended with 5 mL SC-U selective medium into a 20 mL PTFE/Silicon Septa screw cap glass vial (Agilent Technologies). Then, either \((E)\)-nerolidol or \((E,E)\)-geranyllinalool was added to the vial
at a final concentration of 10 μM and incubated at 30 °C for 4 h. The enzymatic reaction was terminated by the addition of HCl to a final concentration of 0.05 N (Lee et al., 2010). Then, the headspace volatiles from the vial were collected by SPME as described above at 30 °C for 1 h. The yeast cells transformed with the empty pYES2 vector were used as the control.

**Real-time quantitative PCR.**

Total RNA was extracted from the leaves of the wild type and transgenic rice plants by TransZol (TransGen Biotech, Beijing, China). Then, 2 μg of total RNA was digested with DNaseI (Invitrogen, Carlsbad, CA) to remove DNA contamination, and the first-strand cDNA was synthesized with M-ML V reverse transcriptase (Invitrogen, Carlsbad, CA). The rice *GAPDH* gene (*LOC_Os04g40950.1*) was used as the reference gene for normalizing gene expression. The gene-specific RT-qPCR specific primers (Table S1) were designed using Beacon Designer 7 software. The RT-qPCR analysis was performed with Fast Start Universal SYBR Green Master (Roche Applied Science) with the ABI 7500 system (Applied Biosystems, Carlsbad, CA). The program of RT-qPCR was performed as follows: 94 °C for 10 min, followed by 40 cycles of 94 °C for 5 s and 60 °C for 40 s, and the experiments were performed in triplicate. The expression of target genes in transgenic plants was calculated as the fold change relative that in the
wild type plants and presented as means ± standard deviation.

**Agrobacterium-mediated genetic transformation.**

Calli induced from the mature seeds of *Japonica* rice cultivar Zhonghua11 were used for *Agrobacterium*-mediated transformation. The procedure for inducing calli and *Agrobacterium*-mediated transformation was based on the previously reported method (Lin & Zhang, 2005), except that the resistant calli were selected with 200 mg/L glyphosate (Cui et al., 2016).

**Molecular cloning and selection of single copy homozygous transgenic lines.**

To constitutively overexpress *OsCYP92C21* in rice for functional characterization, its full-length coding region (CDS) was cloned into the plant expression vector pTGH-3 modified from pU130 at multiple cloning sites between BamHI and SacI under the control of the ubiquitin promoter (Cui et al., 2016), and then transformed into the rice variety ZH11. Southern-blot was performed on 36 T₀ generation transgenic plants to determine the copy number of T-DNA for the selection of single copy homozygous transgenic lines, and RT-qPCR was carried out to detect the gene expression. In detail, about 4 g of leaves from T₀ generation transgenic plants was collected from rice plants at the tillering stage, and after grinding with liquid nitrogen, the genomic DNA of the
rice leaves was extracted by CTAB method (Murray & Thompson, 1980). PCR analysis with specific primers (Table S1) was performed to determine the presence of the glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) from *Isoptericola variabilis* (*I. variabilis*-EPSPS*) (Cui et al., 2016) or hygromycin B phosphotransferase (hpt) in T0 transgenic plants. The PCR products of 576-bp *I. variabilis*-EPSPS* fragment and 720-bp hpt fragment were detected by 0.8% agarose gel electrophoresis. For Southern blot analysis, 8 μg genomic DNA was completely digested by *Hind*III and separated by 1% agarose electrophoresis, then transferred to a nylon membrane after alkali denaturation (Lin & Zhang, 2005), and hybridized with the PCR product of *I. variabilis*-EPSPS* gene (labeled with 0.01 μM DIG-dUTP) following the Roche Digoxin Application Manual.

Mature seeds of T1 generation transgenic rice were harvested from individual plants, treated with 75% alcohol for 1 min and disinfected by 0.15% HgCl2 for 15 min, and then washed with sterile water for several times. The sterilized seeds were placed on fresh 1/2 MS medium containing 200 mg/mL glyphosate for seed germination assay. The germination rate was recorded after 5 d of 2000 Lux light culture (26 ± 1 °C, 16/8 photoperiod). The line was considered as a homozygous positive line if all the seeds were germinated; the line was regarded as heterozygous if part of the seeds were germinated, and as a homozygous negative line if all the seeds were not germinated.
The homozygous rice lines were identified from the T₀ generation transgenic plants by Southern blot as above (Fig. 2). In T₂ generation plants, two single copy number homozygous lines, OsCYP92C21-4 and OsCYP92C21-5, with high expression of OsCYP92C21 were selected. The volatiles from these lines and the wild type line ZH11 were collected and detected by the air entrainment with Tenax.

**Construction of CRISPR/Cas9 knockout vector and detection of knockout mutants.**

The construction of CRISPR/Cas9-OsCYP92C21 knockout constructs was performed according to the method described previously (X. Ma & Liu, 2016). Two knockout target sites 5′-gggatcgtgcacaccccgtacggg-3′ and 5′-acttctgcaactctccggcgagg-3′ were designed through the website (http://crispr.hzau.edu.cn/cgi-bin/CRISPR/CRISPR) based on the OsCYP92C21 sequence. Ten lines were randomly selected from the T₀ generation transgenic plants using specific primers (Table S1) by PCR amplification and sequencing, and compared with wild-type sequences of OsCYP92C21 to determine the mutation sites and identify homozygous mutant rice lines. Two deletion mutant lines CYP92C21Δ1 and CYP92C21Δ16 were obtained (Fig. 3).


**Subcellular targeting expression of the terpene synthase.**

The terpene synthase PlTPS4 (Li et al., 2018) was introduced into different subcellular compartments to boost the precursor pool. The codon optimized chloroplast targeting signal peptide (ctp*) (Cui et al., 2016) and the mitochondrial targeting signal peptide (mtp) (Luo et al., 2013) derived from rice *Rf1* gene (Chen & Liu, 2014) were tagged to the 5’ terminus of the coding sequence of the terpene synthase gene *PlTPS4* to obtain the *ctp*-*PlTPS4* and *mtp-PlTPS4* fusion gene for subcellular targeted expression, respectively. The full-length CDS of *OsCYP92C21, PlTPS4, ctp*-PlTPS4* and *mtp-PlTPS4* was amplified using the gene-specific primers (Table S1) and cloned into the GATEWAY destination binary vector pXCSG-YFP (Feys et al., 2005), and then fused to the N terminus of the YFP gene.

The fusion vectors (35S-*OsCYP92C21-YFP, 35S-*PlTPS4-YFP, 35S-ctp*-*PlTPS4-YFP and 35S-mtp-*PlTPS4-YFP) and either the corresponding marker vector of F1-ATPase-RFP (mitochondrial marker) (Jin et al., 2003) or the 35S-Bip-RFP vector (endoplasmic reticulum marker) (Jin et al., 2001) were co-transformed into rice protoplasts with polyethylene glycol (PEG). The isolation and transformation of rice protoplasts were performed according to the method described previously (Shen et al., 2017). After 12 h to 16 h of the transformation, the fluorescence signal was observed.
with a confocal laser scanning microscope (Leica TCS SP2, Leica FV1200).

As for OsCYP92C21, the PITPS4, ctp*-PITPS4 and mtp-PITPS4 fusion genes were cloned into overexpression vector pTGH-3 respectively targeting the endoplasmic reticulum, chloroplast and mitochondria of rice variety ZH11. Then, the T0 generation plants were screened for copy number by Southern blot and mRNA expression levels of PITPS4 by RT-qPCR. From each T2 homozygous transgenic plant with PITPS4 expression in a specific subcellular compartment, two single-copy lines (PITPS4-9 and PITPS4-22; mtp-PITPS4-5 and mtp-PITPS4-9; ctp*-PITPS4-10 and ctp*-PITPS4-20) with higher expression of PITPS4 were selected for subsequent experiments.

**Acquisition of double-gene introgression transgenic lines.**

OsCYP92C21-4 homozygous transgenic lines and PITPS4-9, PITPS4-22, mtp-PITPS4-5, mtp-PITPS4-9, ctp*-PITPS4-10 and ctp*-PITPS4-20 homozygous transgenic lines were sown and transplanted at the same time. At the heading and flowering stage, the anthers of the PITPS4-9, PITPS4-22, mtp-PITPS4-5, mtp-PITPS4-9, ctp*-PITPS4-10 and ctp*-PITPS4-20 lines were removed, and the plants were pollinated with the pollen of OsCYP92C21-4 line. After maturation, the seeds of the hybridized plants were harvested and sown in a rice field. Genomic DNA was extracted at the seedling stage and PCR amplified for positive detection of OsCYP92C21 and PITPS4 genes to
eliminate false hybrids with specific detection primers (Table S1). The volatiles from these lines were collected and detected by the air entrainment with Tenax.

**Y-tube olfactory assays.**

The olfactory responses of female *C. chilonis* wasps to the transgenic rice plants were examined using a Y-tube olfactometer (10 cm in length and 1.5 cm in diameter). The air was sucked from the central arm of Y-tube into the system by an air pump at 300 mL/min flow rate. The air was purified and humidified by passing through activated charcoal and distilled water before entering the two side arms of the Y-tube. During the test, the volatiles from the transgenic rice plant in a glass jar were fed into one side arm of the Y-tube, and the volatiles from the wild-type plant of the same size were fed into the other side arm of the Y-tube as a control.

In each experiment, a female wasp was gently placed at the base of the Y-tube central arm and observed for 10 min. The judgment criteria were as follows: when a wasp did not make a choice during this period, it was recorded as no response and removed from the Y-tube; when a wasp reached into one of the arms for more than 5 cm and stayed there for more than 10 s, it was recorded that the wasp made a choice to the arm. In order to eliminate the error caused by the instrument, the directions of the arms of the olfactometer were exchanged after testing every five wasps. The
olfactometer was washed with acetone and subsequently by ethanol, and then air-dried after each treatment. A total of 60 female parasitic wasps were tested in each treatment. Olfactory response experiments were performed between 10:00–16:00, and the test temperature was $25 \pm 2^\circ$C. Chi-square ($\chi^2$) analysis was used to evaluate the results of olfactory responses of the female parasitic wasps (Li et al., 2018).

**Results**

**Emissions of DMNT and TMTT from rice upon ALA, JA and BPH treatment.**

The monoterpenoid linalool, the homoterpene precursor nerolidol, and the homoterpenes DMNT and TMTT significantly increased in the headspace volatiles of alamethicin-treated and JA-treated wild type rice plants (ZH11, *Oryza sativa* L. ssp. *Japonica*) compared with those in the control un-treated ZH11 plants (Fig. 1). Interestingly, the emissions of linalool, nerolidol and DMNT also increased in brown planthopper-treated ZH11 plants, but the emission of TMTT showed no significant change. These results indicated the presence of the biosynthetic pathway of DMNT and TMTT in rice and that their emissions are induced by alamethicin, JA and brown planthopper.

**OsCYP92C21 was responsible for DMNT and TMTT production *in vitro*.**
Six CYP genes (OsCYP71AK2, OsCYP71C16, OsCYP89B1, OsCYP92A9, OsCYP92A11, OsCYP92C21), which were selected as candidate biosynthesis genes from the comparative microarray analysis between the resistant rice variety Rathu Heenati (Oryza sativa L. ssp. indica) (RH) and the susceptible rice variety Taichung Native 1 (Oryza sativa L. ssp. indica) (TN1) infested by the brown planthopper (BPH) (Li et al., 2017) and the comparative transcriptome analysis of the rice cultivar ZH11 (Oryza sativa L. ssp. Japonica) before and after alamethicin-treatment (unpublished data), showed significantly up-regulated expression after alamethicin treatment (Fig. S1a).

The in vitro biochemical functional characterization showed that only the recombinant protein OsCYP92C21 (LOC_Os03g44740) expressed in yeast could convert \((E)\)-nerolidol and \((E,E)\)-geranyllinalool into DMNT and TMTT, respectively (Fig. 2). Phylogenetic analysis with other known homoterpene synthases showed that OsCYP92C21 was closely clustered with the maize homoterpene synthases ZmCYP92C5 and ZmCYP92C6 with identities of 74% and 72%, and only shared 34%, 35% and 35% identities with the Arabidopsis homoterpene synthase AtCYP82G1 and the cotton homoterpene synthases GhCYP82L1 and GhCYP82L2, respectively (Fig. S1b).
Abolishment of DMNT and TMTT emissions by knockout of OsCYP92C21 gene.

Two deletion genes were generated with the CRISPR/Cas9 system: \(CYP92C21\triangle1\), which had a one-base deletion at the 4th base before the PAM site, and \(CYP92C21\triangle16\), which had a deletion of 16 bases before the PAM site (Fig. 3a). DMNT and TMTT were detected in the volatiles from alamethicin-treated wild-type lines (ZH11) (Fig. 1) and untreated double-gene introgression lines, i.e. the homoterpene emissions from untreated plants requires overexpression of both PlTPS4 and CYP92C21 (Table 1), but not in the volatiles from the alamethicin-treated T0 homozygous knockout lines CYP92C21\(\triangle1\) and CYP92C21\(\triangle16\) (Fig. 3b).

Subcellular localization of OsCYP92C21 protein in endoplasmic reticulum.

To experimentally investigate the subcellular location of OsCYP92C21, the full length of \(OsCYP92C21\) gene was constructed into the N-terminus of YFP of the pXCSG-YFP vector (Feys et al., 2005), and the resultant fusion vector 35S-OsCYP92C21-YFP was transformed into rice protoplasts for transient expression. The analysis of transformed protoplasts by confocal laser-scanning microscopy showed that OsCYP92C21 was co-located with the endoplasmic reticulum marker 35S-Bip-RFP (Jin et al., 2001) (Fig. 4b), suggesting that the OsCYP92C21 protein is expressed in the endoplasmic reticulum of rice protoplasts.
Overexpression of *OsCYP92C21* gene in rice.

To overexpress *OsCYP92C21* in rice for functional characterization, its full-length coding region (CDS) was subcloned into a plant constitutive expression vector pTGH-3 under the control of the ubiquitin promoter (Cui et al., 2016), and then transformed into ZH11. Two T2 homozygous and single-copy lines OsCYP92C21-4 and OsCYP92C21-5 (Fig. S2) with high expression of *OsCYP92C21* (Figure. 4a) were selected for subsequent volatile collection and analysis. Although DMNT and TMTT were detected in the volatiles from the alamethicin-treated wild type plant CYP92C21WT (Fig. 1), they were not detected in the volatiles from untreated OsCYP92C21-overexpressing lines (OsCYP92C21-4 and OsCYP92C21-5) (Table 1).

**DMNT and TMTT emissions were enhanced by subcellular targeting terpene synthase expression.**

To boost the terpenoid precursor pool of the homoterpene biosynthesis in different subcellular compartments, the expression of the terpene synthase *PlTPS4* was targeted into the chloroplasts and mitochondria with the codon optimized chloroplast targeting signal peptide (ctp*) (Cui et al., 2016) and the mitochondria targeting signal peptide (mtp) (Luo et al., 2013). Then, *PlTPS4, ctp*- *PlTPS4* and *mtp*- *PlTPS4* were separately subcloned into pXCSG-YFP vector and transformed into rice protoplasts. The
microscopic observations showed that 35S-PlTPS4-YFP was co-located with the endoplasmic reticulum marker 35S-Bip-RFP, indicating that PlTPS4 protein is expressed in the endoplasmic reticulum. The 35S-ctp*-PlTPS4 was co-located with chloroplast auto-fluorescence, and the 35S-mtp-PlTPS4 was co-located with the mitochondrial marker F1-ATPase-RFP (Jin et al., 2003) (Fig. 5a), indicating that they are targeted into chloroplasts and mitochondria, respectively.

On this basis, PlTPS4, ctp*-PlTPS4 and mtp-PlTPS4 were respectively constructed into PTGH-3 vector and transformed into ZH11. The T₂ homozygous and single-copy lines, including PlTPS4-9, PlTPS4-22, ctp*-PlTPS4-10, ctp*-PlTPS4-20, mtp-PlTPS4-5 and mtp-PlTPS4-9, were selected for subsequent experiments (Fig. 5b; Fig. S3). Compared with the wild type plants, PlTPS4, ctp*-PlTPS4 and mtp-PlTPS4 transgenic lines showed higher emissions of linalool (Table 1). The expression of PlTPS4 in the endoplasmic reticulum resulted in the production of DMTT in rice plants, suggesting that DMTT emission can be initiated by boosting its terpenoid precursor pool in the endoplasmic reticulum where OsCYP92C21 is expressed (Fig. 4b). The DMNT and TMTT emissions from the ctp*-PlTPS4 and mtp-PlTPS4 transgenic lines were not affected compared with those from the PlTPS4 line in which PlTPS4 was expressed in the endoplasmic reticulum. However, the emission of nerolidol increased in the ctp*-PlTPS4 and mtp-PlTPS4 transgenic lines. The mtp-PlTPS-9 transgenic line produced
slightly higher DMNT (32 ± 9 ng plant$^{-1}$ day$^{-1}$) and nearly no TMTT. The ctp*-PtTPS4-20 transgenic line showed the highest linalool emission up to 184 ± 26 ng plant$^{-1}$ day$^{-1}$, which was about 14 folds that of the wild type ZH11 lines; only the ctp*-PtTPS4-10 transgenic line produced a trace amount of TMTT (1 ± 1 ng plant$^{-1}$ day$^{-1}$) (Table 1).

**Enhancement of DMNT and TMTT emissions by double-gene introgression.**

For introgression of the homoterpene synthesis gene and the terpene synthesis gene into the same rice plants, the OsCYP92C21-overexpressing transgenic line OsCYP92C21-4 was hybridized with the PtTPS4-overexpressing transgenic lines PtTPS4, ctp*-PtTPS4 and mtp-PtTPS4, resulting in PtTPS4/OsCYP92C21, ctp*-PtTPS4/OsCYP92C21 and mtp-PtTPS4/OsCYP92C21 introgression lines, respectively. In these cases, the DMNT and TMTT emissions from all double-gene introgression lines significantly increased compared with those from wild type (ZH11) and single-gene transgenic lines (Table 1). TMTT emission was detected only after the introgression. The DMNT emission increased to more than 100 ng plant$^{-1}$ day$^{-1}$ in the PtTPS4/OsCYP92C21 lines. The mtp-PtTPS4-9/OsCYP92C21-4 line had the highest emission of DMNT (943 ± 22 ng plant$^{-1}$ day$^{-1}$), which was 73–157 folds those of single-gene transgenic lines PtTPS4-9 and PtTPS4-22. The ctp*-PtTPS4-10/OsCYP92C21-4 line had the highest TMTT emission, which reached 179 ± 48 ng plant$^{-1}$ day$^{-1}$ and was
179 folds that of ctp*-PITPS4-10 line (Table 1).

**Female parasitic wasps C. chilonis were attracted by the GM rice plants.**

The olfactory responses of the wasp C. chilonis to the transgenic rice plants OsCYP92C21-4, PITPS4-9, mtp-PITPS4-9, ctp*-PITPS4-20, and the introgression plants PITPS4-9/OsCYP92C21-4, mtp-PITPS4-9/OsCYP92C21-4 and ctp*-PITPS4-20/OsCYP92C21-4 were measured using a Y-tube olfactometer (Fig. 6). There was no significant difference in the number of wasps attracted by OsCYP92C21-4 plant and the wild type ZH11 plant ($\chi^2 = 0.22$, df = 1, $P = 0.64$). The transgenic plants PITPS4-9 ($\chi^2 = 5.12$, df = 1, $P < 0.05$), mtp-PITPS4-9 ($\chi^2 = 3.92$, df = 1, $P < 0.05$) and ctp*-PITPS4-20 ($\chi^2 = 6.23$, df = 1, $P < 0.05$) attracted significantly more C. chilonis female wasps than the wild type ZH11 plants. The double-gene introgression lines PITPS4-9/OsCYP92C21-4 ($\chi^2 = 17.31$, df = 1, $P < 0.01$), mtp-PITPS4-9/OsCYP92C21-4 ($\chi^2 = 12.52$, df = 1, $P < 0.01$), and ctp*-PITPS4-20/OsCYP92C21-4 ($\chi^2 = 18.29$, df = 1, $P < 0.01$) were extremely more attractive to C. chilonis female wasps than the wild type ZH11 plants (Fig. 6).

**Discussion**

OsCYP92C21 is responsible for the biosynthesis of DMNT and TMTT in rice.
It was found that rice plants can emit volatile organic compounds including DMNT and TMTT after induction by alamethicin, a potent elicitor of volatile biosynthesis (Engelberth et al., 2001), jasmonic acid (JA) and brown planthopper treatment in this study (Fig. 1), which is similar to previous research results with JA treatment (Lou et al., 2005; Lou et al., 2006), indicating the existence of a homoterpene biosynthesis pathway in rice. The constitutive expression of AtCYP82G1 (At3g25180) could complement the inability of the T-DNA insertion GK377A01 mutant to produce TMTT from geranyllinalool in A. thaliana leaves, and the recombinant AtCYP82G1 protein expressed in yeast could convert (3S)-(E)-nerolidol and (3RS)-(E,E)-geranyllinalool to DMNT and TMTT, respectively (Lee et al., 2010). It has been suggested that the monocot CYP92 gene family has similar functions to the CYP82 gene family in dicots (Richter et al., 2016). Here, we identified OsCYP92C21 (LOC_Os03g44740) as a gene responsible for the biosynthesis of both DMNT and TMTT in rice. The CRISPR/Cas9 system was then used to knock out OsCYP92C21 gene in ZH11. As a result, these lines no longer are able to produce the homoterpenes upon induction by alamethicin, further confirming that OsCYP92C21 may be the only homoterpne synthase in rice. These are supported by subcellular targeting expression of a terpene synthase, genetic transformation and introgression of transgenic rice plants expressing the terpene synthase and OsCYP92C21, and by the result that the recombinant OsCYP92C21
protein expressed in yeast could convert \((E)\)-nerolidol and \((E,E)\)-geranyllinalool to DMNT and TMTT (Fig. 2).

**Subcellular localization of terpene synthase affects the volatile profile of rice plants.**

Plants biosynthesize terpenoid volatiles by various terpene synthases from the precursors, such as from geranyl diphosphate (GPP) to monoterpenoids, farnesyl pyrophosphate (FPP) to sesquiterpenoids, and geranylgeranyl diphosphate (GGPP) to diterpenoids through either the MVA pathway or the MEP pathway (Nagegowda, 2010). The homoterpene homoterpene DMNT and TMTT are synthesized from terpenoid precursors such as \((E)\)-nerolidol and \((E,E)\)-geranyllinalool by homoterpene synthases (Lee et al., 2010; Liu et al., 2017; Richter et al., 2016; Sohrabi et al., 2015). However, DMNT and TMTT emissions could not be detected from un-induced wild type (Fig. 1) and OsCYP92C21-overexpressing rice plants (Table 1) as in *A. thaliana* (Herde et al., 2008; Van Poecke, Posthumus, & Dicke, 2001). We speculated that the concentrations of the precursors \((E)\)-nerolidol and \((E,E)\)-geranyllinalool for the biosynthesis of DMNT and TMTT in rice plants might be too low or even absent under non-induced conditions. FPP and GGPP were found in the mitochondria and chloroplasts (Cunillera, Boronat, & Ferrer, 1997; Nagegowda, 2010; Okada, Saito, Nakagawa, Kawamukai, & Kamiya, 2000;
Sanmiya, Ueno, Matsuoka, & Yamamoto, 1999). PITPS4 is a multifunctional terpene synthase derived from Lima bean. Its recombinant protein is able to covert GPP, FPP and GGPP to linalool, (E)-nerolidol and (E,E)-geranyllinalool, respectively (Li et al., 2018). It is possible that targeting PITPS4 into rice subcellular compartments boosts the terpenoid precursor pool from FPP and GGPP and enhances the homoterpene biosynthesis. This speculation is supported by the increase in the emission of nerolidol, and the initiation of DMNT and TMTT emissions without any induction/treatment in the ctp*-PITPS4-10 and mtp-PITPS4 transformed rice lines (Table 1). These results are consistent with the results of previous studies, which showed that the expression of a dual linalool/nerolidol synthase (FaNES1) in A. thaliana mitochondria resulted in the generation of nerolidol and DMNT, while FaNES1 expression in chloroplasts led to the emission of linalool and its derivatives (Aharoni et al., 2003; Kappers et al., 2005). It is worthy to note that by targeting PITPS4 into mitochondria which have a low abundance of GPP (Nagegowda, 2010), mtp-PITPS4 transformed rice lines emitted more linalool than the wild type ZH11 and OsCYP92C21-overexpression lines (Table 1).

Further significant increases in DMNT and TMTT emissions as well as in linalool emission were achieved by the hybridization of OsCYP92C21-overexpression lines and PITPS4-overexpressing lines (Table 1). The emissions of DMNT, TMTT and linalool
are significantly higher than those obtained in single gene overexpression lines and previously reported PlTPS3 and PlTPS4 transgenic lines (Li et al., 2018). These results indicate that the ability of rice to produce DMNT and TMTT is dependent on the precursor pool, and imply that the up-regulation and/or induction of the OsCYP92C21 gene expression is necessary for DMNT and TMTT emissions in rice.

**Metabolic pathways of rice homoterpenes.**

The rice terpene synthases, namely OsTPS18 (Kiryu et al., 2018) and OsTPS13 (Cheng et al., 2007), were shown to be responsible for the production of nerolidol as minor volatiles. Only OsTPS3 (LOC_Os02g02930) was shown to catalyze the formation of (S)-linalool from GPP and (E)-nerolidol from FPP as major volatiles in rice (Yuan et al., 2008). So far, there is no report of rice terpene synthase that utilizes GGPP to generate (E,E)-geranyllinalool for TMTT biosynthesis. However, TMTT emission in rice was detected from the rice plants under alamethicin, BPH and JA treatment (Lou et al., 2005; Lou et al., 2006). Hence, there may be another un-determined terpene synthase responsible for the conversion of GGPP to (E,E)-geranyllinalool and then to TMTT by OsCYP92C21 in rice. This allows us to tentatively propose a metabolic pathway for the DMNT and TMTT biosynthesis in rice. Thus, OsCYP92C21 utilizes both (E)-nerolidol and (E,E)-geranyllinalool (Fig. 2), and converts these terpenoid precursors to DMNT.
and TMTT under the induction of herbivores, JA and alamethicin (Fig. 7). The formation of (S)-linalool from GPP is supported by the study of Yuan et al. (Yuan et al., 2008) and also by our previous study (Li et al., 2018) in rice, which can also be assumed in this study and in A. thaliana (Aharoni et al., 2003).

Application prospects of DMNT and TMTT in agriculture.

Genetic modification for nutrient enhancement in rice has been achieved by generating Golden Rice to biosynthesize beta-carotene, a precursor of vitamin A, in the edible parts of rice (Beyer et al., 2002; Paine et al., 2005). However, this does not solve the immense problems of rice pests such as brown planthopper N. lugens and striped rice stemborer C. suppressalis. Pest control in agriculture is mainly reliant on chemical pesticides, but the excessive use of pesticides has caused a series of negative impacts, such as pesticide residues and pest resistance to pesticides, leading to un-sustainability in agriculture (Ecobichon, 2001; Heong, Wong, & Reyes, 2015; Hong-xing et al., 2017). Companion planting strategies utilizing homoterpene-based plant indirect defense for sustainable pest control have been developed (Khan et al., 2014; Pickett, Woodcock, Midega, & Khan, 2014). In recent years, these strategies have been widely implemented as a push-pull strategy among smallholder farmers in sub-Saharan Africa with obvious effects against pests including the fall armyworm Spodoptera frugiparda (John A Pickett, 2019;
Midega, Murage, Pittchar, & Khan, 2016). Our study demonstrates that the genetically modified rice plants, by either subcellular targeting expression of terpene synthases or double-gene introgression of these terpene synthases with the homoterpene biosynthase OsCYP92C21, exhibit significant behavioral attraction to the female adult wasps *C. chilonis*, a natural enemy of the rice pest *C. suppressalis*, implying the application potential in rice fields. We found some differences in the volatile content of some other terpenes between WT and transgenic lines. However, since we have demonstrated that DMNT and TMTT were demonstrated to attract the parasitic wasps in our previous work (Li et al., 2018), we believe that DMNT and TMTT are the main volatiles affecting the behavior of the parasitic wasps by the transgenic rice plants. Of course, we could not determine whether there are some other terpenes in rice that affect the behavior of the parasitic wasps. Nevertheless, this study dissects the molecular mechanism of the homoterpene biosynthesis pathway in rice, and lays the foundation for utilizing plant indirect defense in the “push-pull” strategy in integrated pest management by genetic modification.

**Data availability**

All data that support these findings are available from Y.J.L. and F.Z. upon request. The raw sequencing data of *OsCYP92C21 (LOC_Os03g44740)* is available in the NCBI
GenBank under the accession number ABF97912.1.

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Author contributions

Y.J.L., F.Z., J.J.Z., J.A.P. and W.L. designed the research; W.L. and L.N.W. performed the experiments and collected the data; W.L., C.Y.L., W.H.M. and H.C. analyzed the data; Y.J.L., F.Z., G.R.W., J.A.P. and J.J.Z. contributed materials and analysis tools; Y.J.L., F.Z., J.J.Z., J.A.P. and W.L. wrote the manuscript; J.J.Z. and J.A.P. made critical revisions of the manuscript.

All authors reviewed the manuscript and contributed to the interpretation and manuscript revisions.
Competing interests

The authors declare no competing interests.

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Okada, K., Saito, T., Nakagawa, T., Kawamukai, M., & Kamiya, Y. (2000). Five Geranylgeranyl...


**Supporting Information**

**Fig. S1** Molecular characterization of rice homoterpene biosynthesis candidate genes.

**Fig. S2** Selection of single copy transgenic rice lines overexpressing OsCYP92C21 by Southern blot of pTGH-3-OsCYP92C21 lines.

**Fig. S3** Selection of PitPS4-overexpression lines with single copy number by Southern blot.

**Table S1** Primers used in this study.

**Figure Legends**

**Fig. 1 Induction of volatile emissions by ALA, JA and BPH in wild type rice plants**.

(a) the GC trace of the headspace volatiles from the alamethicin-treated ZH11 plants; 
(b) the GC trace of the headspace volatiles from the jasmonic acid-treated ZH11 plants; 
(c) the GC trace of the headspace volatiles from the brown planthopper-treated ZH11 plants; (d) the GC trace of the headspace volatiles from un-treated ZH11 plants. The headspace volatiles from 20-day old rice plants were collected by SPME after 24 h alamethicin treatment. The headspace volatiles from 20-day old rice plants were collected by Solid phase microextraction (SPME) after 24 h alamethicin-treatment, and
separated with the low-polarity SH-Rsi-5sil-MS column. Compounds giving peaks are:

1, Limonene; 2, Linalool; 3, DMNT; 4, Unknown; 5, Unknown; 6, Unknown; 7, Unknown; 8, α-Copaene; 9, Zingiberene; 10, Unknown; 11, Unknown; 12, (E)-Caryophyllene; 13, (E)-α-Bergamotene; 14, β-Sesquiphellandrene; 15, (E)-β-Farnesene; 16, α-Curcumene; 17, Cedrene; 18, Unknown; 19, β-Bisabolene; 20, (E)-Calamenene; 21, Unknown; 22, Nerolidol; 23, TMTT.

**Fig. 2 Conversion by recombinant OsCYP92C21 protein *in vitro* of (E)-nerolidol and (E,E)-geranyllinalool into DMNT and TMTT.** (a), GC trace from the yeast extract with the empty vector pYES2 provided with (E)-nerolidol; (b), GC trace from the yeast extract with pYES2-OsCYP92C21 provided with (E)-nerolidol; (c), GC trace from the yeast extract with the empty vector pYES2 provided with (E,E)-geranyllinalool; (d), GC trace from the yeast extract with pYES2-OsCYP92C21 provided with (E,E)-geranyllinalool. The OsCYP92C21 gene was overexpressed in the yeast *Saccharomyces cerevisiae* strain WAT11 with an *A. thaliana* P450 reductase. The peaks are labeled as: 1, DMNT; 2, nerolidol; 3, TMTT; 4, geranyllinalool. The DMNT and TMTT products were identified by comparison of the retention time and mass spectra with authentic standards. The headspace volatiles from the reactions were collected by SPME as Fig. 1.

**Fig. 3 The loss of DMNT and TMTT emissions in CYP92C21 deletion transgenic rice plants.** (a), the alignment (1st panel) and sequence of CYP92C21WT (2nd panel), CYP92C21△1 (3rd panel) and CYP92C21△16 (4th panel); (b), the gas chromatography traces (m/z = 69) of the volatiles collected from the un-treated wild type line ZH11, the ZH11 treated with alamethicin, OsCYP92C21-4 overexpressing line treated by alamethicin, CYP92C21△1 deletion line treated with alamethicin and CYP92C21△16 deletion line treated with alamethicin. The peaks are labeled as 1, linalool; 2, DMNT; 3, nerolidol; 4, TMTT. The headspace volatiles from the 20-day old
rice plants were collected by SPME after 24 h alamethicin treatment as Fig. 1.

**Fig. 4 Expression of OsCYP92C21 in transgenic rice lines and subcellular location of OsCYP92C21 in rice protoplasts.** (a), Confirmation of OsCYP92C21 gene in the transgenic rice lines by RT-qPCR. Relative expression levels of OsCYP92C21 in the wild type line ZH11, the transgenic lines OsCYP92C21-4, OsCYP92C21-5 are calculated as fold changes relative to the expression in the wild type line ZH11 and represented as the means ± s.d.m. The rice GAPDH gene was used as the reference gene for normalizing gene expression. Asterisks indicate significant differences between treatments (***P<0.01). (b), Subcellular localization of OsCYP92C21-YFP fusion protein in rice endoplasmic reticulum. YFP: yellow fluorescent protein, RFP: red fluorescent protein. Scale Bars = 5 μm.

**Fig. 5 Subcellular targeting of PITPS4 expression in rice protoplasts and expression in rice plants.** (a), Subcellular localization of PITPS4-YFP, ctp*-PITPS4-YFP and mtp-PITPS4-YFP in rice protoplasts. The genes were cloned into pXCSG-YFP and transformed in rice protoplasts. 35S-Bip-RFP: endoplasmic reticulum marker; F1-ATPase-RFP: mitochondrial marker. YFP: yellow fluorescent protein, RFP: red fluorescent protein. Scale Bars = 5 μm. (b), Relative expression levels of PITPS4 in wild type rice line ZH11 and the transgenic rice lines, PITPS4-9, PITPS4-22, ctp*-PITPS4-10, ctp*-PITPS4-20, mtp-PITPS4-5 and mtp-PITPS4-9. The genes were cloned into the plant over expression vector pTGH-3. The expression levels of T2 homozygous and single-copy transgenic lines were calculated relative to the expression level of PITPS4-9. Data are represented as the means±SD. Asterisks indicate significant differences between treatments (***P<0.01).

**Fig. 6 Enhanced attractiveness of the female *C. chilonis* adults to the transgenic rice lines OsCYP92C21-4, PITPS4-9, mtp-PITPS4-9 and ctp*-PITPS4-20, and the introgression rice lines PITPS4-9/OsCYP92C21-4, mtp-PITPS4-9/OsCYP92C21-4**
and ctp*-PlTPS4-20/OsCYP92C21-4. The numbers on each bar represent the numbers of wasps choosing the plant line. Sixty wasps were tested in each transgenic line. Chi-square analysis was performed to evaluate the significance of the test (**P < 0.01,*P < 0.05).

**Fig. 7 Overview of DMNT and TMTT biosynthesis in rice.** The biosynthesis of rice homoterpenes DMNT and TMTT is catalyzed through oxidative degradation by OSCYP92C21 (LOC_Os03g44740) from (E)-nerolidol and (E,E)-geranyllinalool (Figure 2), which are converted by OsTPS3 and another undetermined terpene synthase from farnesyl pyrophosphate (FPP) and geranylgeranyl diphosphate (GGPP). OsTPS3 also converts geranyl diphosphate (GPP) to (S)-linalool (Yuan et al., 2008)(Aharoni et al., 2003). The zig-zag arrow: Emissions of DMNT, TMTT, (S)-linalool, (E)-nerolidol and (E,E)-geranyllinalool are induced in rice leaves by the treatments with alamethicin, jasmonic acid and herbivores. * : the enantiomeric forms of linalool in our experiment were not determined but (S)-linalool is supported by Yuan’s study (Yuan et al., 2008) and our previous study (Li et al., 2018) in rice and in A. thaliana (Aharoni et al., 2003).

**Tables**

**Table 1. Emissions of TMTT, nerolidol, DMNT, and linalool from transgenic rice plants and wild-type plant Zhonghua11 (ng plant\(^{-1}\) day\(^{-1}\))**

<table>
<thead>
<tr>
<th></th>
<th>TMTT</th>
<th>Nerolidol</th>
<th>DMNT</th>
<th>Linalool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhonghua11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13 ± 1e</td>
</tr>
<tr>
<td>OsCYP92C21-4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>27 ± 9 e</td>
</tr>
<tr>
<td>OsCYP92C21-5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>35 ± 13 de</td>
</tr>
<tr>
<td>PlTPS4-9</td>
<td>-</td>
<td>-</td>
<td>6 ± 3 e</td>
<td>56 ± 9 de</td>
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<tr>
<td>PlTPS4-22</td>
<td>-</td>
<td>-</td>
<td>13 ± 4 e</td>
<td>60 ±20 de</td>
</tr>
<tr>
<td>mtp-PlTPS4-5</td>
<td>5 ± 3 b</td>
<td>-</td>
<td>22 ± 5 e</td>
<td>54 ± 3 de</td>
</tr>
<tr>
<td>mtp-PlTPS4-9</td>
<td>-</td>
<td>50 ± 22 a</td>
<td>32 ± 9 e</td>
<td>83 ± 19 de</td>
</tr>
<tr>
<td>ctp*-PlTPS4-10</td>
<td>1 ± 1 d</td>
<td>15 ± 3 b</td>
<td>6 ± 2 e</td>
<td>117 ± 16 d</td>
</tr>
<tr>
<td>ctp*-PlTPS4-20</td>
<td>-</td>
<td>7 ±1 b</td>
<td>3 ± 2 e</td>
<td>184 ± 26 cd</td>
</tr>
<tr>
<td>PlTPS4-9/OsCYP92C21-4</td>
<td>24 ± 5 d</td>
<td>-</td>
<td>130 ± 10 d</td>
<td>209 ± 57 cd</td>
</tr>
</tbody>
</table>
Volatile compounds were collected from the headspace of transgenic rice and wild type rice plants (Zhonghua 11) at the tillering stage as previously reported (Gao et al., 2015) with Tenax. The values in the table are the mean ± standard deviation (N = 3). The letters following each value in the same column show significant differences in certain volatiles between different rice materials (P<0.05, Duncan's multiple range test). "-" indicates that the emission of the volatile is below the detection level.

<table>
<thead>
<tr>
<th>Transgenic Line</th>
<th>Volatile Emission</th>
<th>Wild Type Emission</th>
<th>Duncan's Test</th>
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</thead>
<tbody>
<tr>
<td>PITPS4-22/OsCYP92C21-4</td>
<td>11 ± 6 d</td>
<td>100 ± 37 d</td>
<td>219 ± 10 c</td>
</tr>
<tr>
<td>mtp-PITPS4-5/OsCYP92C21-4</td>
<td>61 ± 13 c</td>
<td>210 ± 55 c</td>
<td>180 ± 52 cd</td>
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<tr>
<td>mtp-PITPS4-9/OsCYP92C21-4</td>
<td>119 ± 6 b</td>
<td>943 ± 22 a</td>
<td>596 ± 60 b</td>
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<tr>
<td>ctp*-PITPS4-10/OsCYP92C21-4</td>
<td>179 ± 48 a</td>
<td>231 ± 54 c</td>
<td>589 ± 157 b</td>
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<tr>
<td>ctp*-PITPS4-20/OsCYP92C21-4</td>
<td>87 ± 16 bc</td>
<td>616 ± 45 b</td>
<td>914 ± 72 a</td>
</tr>
</tbody>
</table>
Relative abundance (TIC × 10^6)

Retention time (min)

(a) OsCYP92C21 + (E,E)-geranylinalool

(b) pYES2 + (E)-nerolidol

(c) OsCYP92C21 + (E)-geranylinalool

(d) pYES2 + (E)-nerolidol