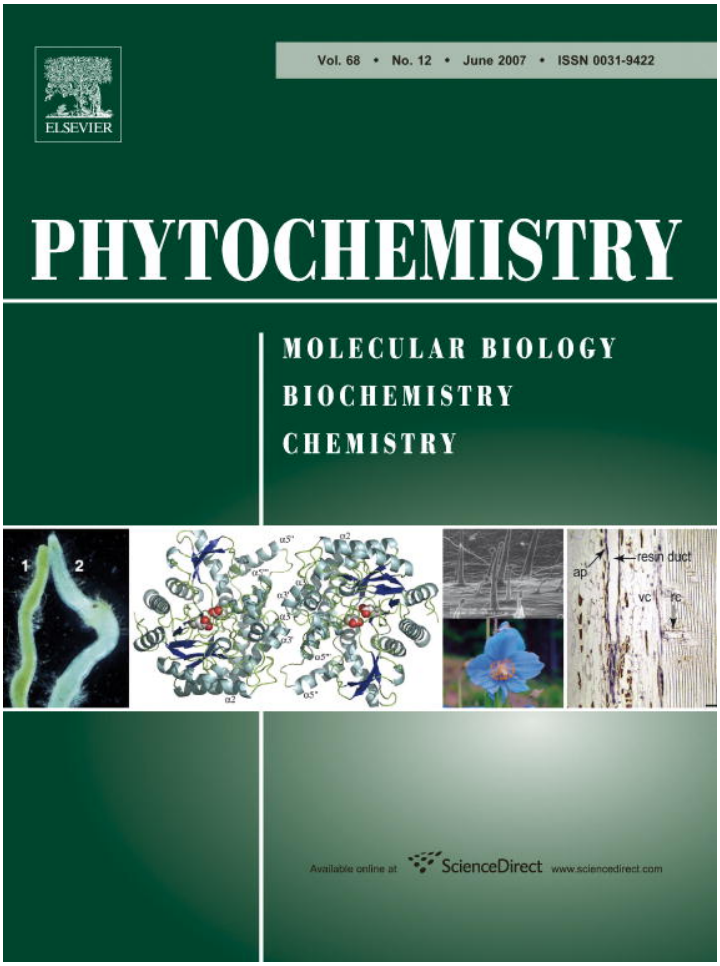

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The rice (*E*)-β-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes

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Abstract

Terpenoids serve as both constitutive and inducible defense chemicals in many plant species, and volatile terpenes participate in plant a indirect defense by attracting natural enemies of the herbivores. The rice (*Oryza sativa* L.) genome contains about 50 genes encoding putative terpene synthases (TPSs). Here we report that two of the rice sesquiterpene synthase genes, *OsTPS3* and *OsTPS13*, encode (*E*)-β-caryophyllene synthase and (*E*,*E*)-farnesol synthase, respectively. In vitro, the recombinant protein of OsTPS3 catalyzed formation of (*E*)-β-caryophyllene and several other sesquiterpenes, including β-elemene and α-humulene, all being components of inducible volatiles of rice plants. The transcript levels of *OsTPS3* exhibit a circadian rhythm of fluctuation, and its expression was also greatly induced by methyl jasmonate (MeJA). In addition, expression of *OsTPS3* in transgenic plants of *Arabidopsis thaliana* resulted in emitting high quantities of *OsTPS3* products. We also overexpressed *OsTPS3* in rice plants which then produced more (*E*)-β-caryophyllene after MeJA treatment. Finally, we found that the MeJA-treated transgenic rice plants attracted more parasitoid wasps of *Anagrus nilaparvatae* than the wild-type. These results demonstrate that OsTPS3, an enzyme catalyzing the formation of volatile sesquiterpenes, plays a role in indirect defense of rice plants.

Keywords: *Oryza sativa*; Gramineae; (*E*)-β-Caryophyllene synthase; (*E*, *E*)-Farnesol synthase; Sesquiterpene; Volatiles; Indirect defense

1. Introduction

Among plant secondary metabolites, terpenoids are structurally diverse compounds derived from the basic building blocks of isopentenyl diphosphate (IPP). According to the number of IPP units they contain, most terpenoids fall into three major groups: monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20). Sesquiterpenes form the largest and most diversified group of terpenoids and their biological and ecological significance lies in their involvement in plant–insect, plant–pathogen, and plant–plant interactions (Kant et al., 2004; Mercke et al., 2004; Kappers et al., 2005; Cheng et al., 2007). Monoterpenes and sesquiterpenes are usually the majority of plant volatile compounds released after herbivore damage, and they may play a role in attracting arthropods that prey upon or parasitize herbivores, thus minimizing further damage to plant tissues (Dudareva et al., 2003). In recent years, many attentions have been paid to this indirect defense of plants

Abbreviations: cv, cultivar; DMAPP, dimethylallyl diphosphate; FDP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GC, gas chromatography; GDP, geranyl diphosphate; GGDP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; MeJA, methyl jasmonate; MS, mass spectrum; OsTPS, *Oryza sativa* terpene synthase; SPME, solid-phase microextraction; TPS, terpene synthase; ZmTPS, *Zea mays* terpene synthase.

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against herbivores (Arimura et al., 2004; Degen et al., 2004; Schnee et al., 2006). A recent investigation revealed that caryophyllene (**1**, see Fig. 1) emitted from maize roots is an herbivore-induced belowground signal that strongly attracts entomopathogenic nematodes (Rasmann et al., 2005).

All terpenoids are derived from either the cytosolic mevalonate pathway or the plastidial 2-*C*-methyl-D-erythritol-4-phosphate pathway (Lichtenthaler, 1999). Terpene synthases (TPs) utilize geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP) to produce monoterpenes, sesquiterpenes and diterpenes, respectively. Investigation of TPSs has been an active field of plant volatile research and many genes have been isolated from various gymnosperms and dicots (Bohlmann et al., 1997; Cai et al., 2002; Lu et al., 2002; Chen et al., 2003; Mercke et al., 2004; Martin et al., 2004; Tholl et al., 2005). In addition, analysis of spatial and temporal patterns of gene expression has provided new information to the regulation of plant volatile emission (Lu et al., 2002; Dudareva et al., 2003; Aharoni et al., 2003; Arimura et al., 2004). In contrast to gymnosperms and dicots, the investigation of TPSs of monocots is rather limited. Only recently several sesquiterpene synthases from maize were cloned and characterized (Degenhardt and Gershenzon, 2000; Köllner et al., 2004; Schnee et al., 2002, 2006). Rice (*Oryza sativa*), one of the most important cereal crops in the world, is used as a model of monocots due to the availability of its genome sequence and the readiness for genetic transformation. Research of terpenoid biosynthesis in rice has been focused on diterpenes, including the phytohormone gibberellins (GA), and the diterpenoid phytoalexins and allelochemicals (Xu et al., 2007). Although the rice genome sequence implies quite a number of monoterpene and sesquiterpene synthases, till now there is no report on either gene expression or functional characterization of these enzymes.

Manipulation of plant terpene metabolism has long been a focus of interest in plant biotechnology (Degenhardt et al., 2003; Wu et al., 2006). Several monoterpene synthases have been modulated by overexpression in transgenic plants, resulting in the production of new monoterpenes that were emitted from vegetative and floral tissues (Lücker et al., 2004; Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003). In recent years, *Arabidopsis thaliana* has served as an excellent model plant for pioneering the metabolic engineering of terpenoids (D'Auria and Gershenzon, 2005). The maize *TPS10* (*ZmTPS10*) encodes an enzyme that catalyzes the formation β-farnesene (**2**), α-bergamotene (**3**) and other herbivory-induced sesquiterpenes; expression of *ZmTPS10* in *Arabidopsis* resulted in plants emitting high quantities of corresponding sesquiterpene products which were absent in the wild-type *Arabidopsis* leaves. When these transgenic plants were used as odor sources, females of the parasitoid *Cotesia marginiventris* learned to exploit the ZmTPS10 sesquiterpenes to locate their lepidopteran hosts (Schnee et al., 2006). Similarly, transgenic *Arabidopsis* plants expressing a strawberry nerolidol synthase gene attracted more carnivorous predatory mites of *Phytoseiulus persimilis* (Kappers et al., 2005).

Here, it is reported that rice plants release a blend of volatile sesquiterpenes after methyl jasmonate (MeJA) induction. Two rice sesquiterpene synthase, OsTPS3 and OsTPS13, were also functionally characterized as the (*E*)-

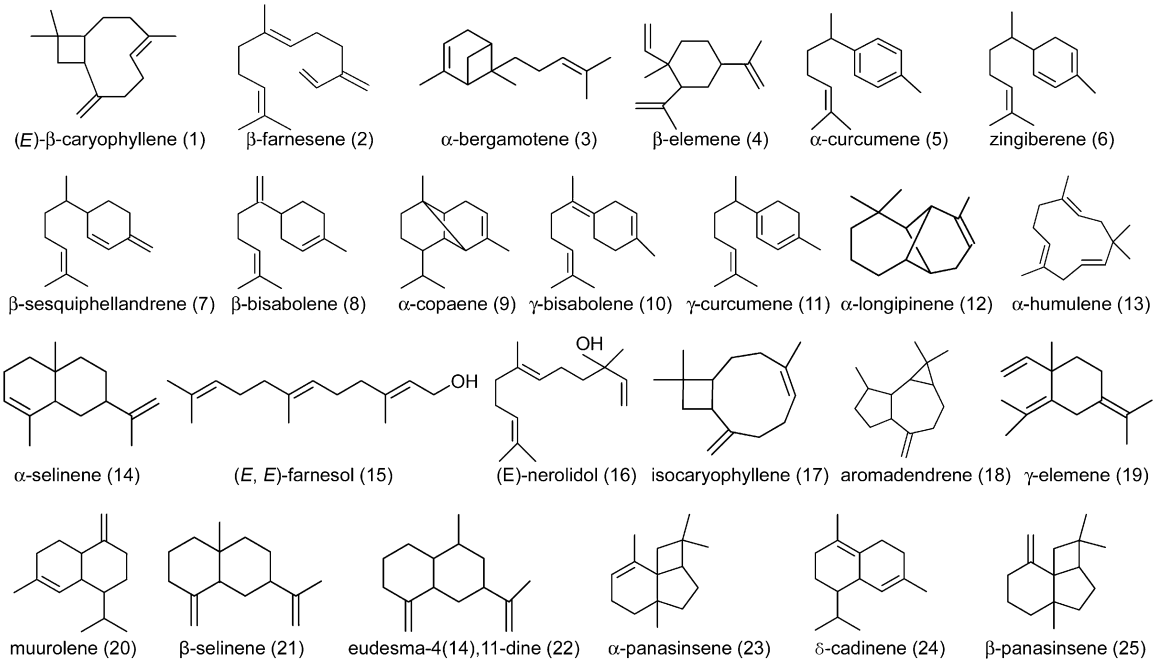

Fig. 1. Structures of sesquiterpenes released from rice seedlings and produced in in vitro assay of the two sesquiterpene synthases of rice, OsTPS3 and OsTPS13.

β-caryophyllene synthase and (*E*,*E*)-farnesol synthase, respectively. It is demonstrated that OsTPS3 catalyzes the formation of the major volatile sesquiterpenes of rice plants. Expression of *OsTPS3* gene is inducible by methyl jasmonate (MeJA) and is regulated in a circadian pattern. Furthermore, transgenic plants of both rice and *Arabidopsis* with higher levels of OsTPS3 product emission attracted more wasps of *Anagrus nilaparvatae* Pang et Wang, a main egg parasitoid of rice planthoppers (*Nilaparvata lugens*).

2. Results and discussion

2.1. Rice seedlings release a mixture of volatile sesquiterpenes after induction

To examine terpenes released from rice plants, volatile chemicals produced from 3-week-old seedlings were collected by a solid-phase microextraction (SPME) method and analyzed by gas chromatography-mass spectroscopy (GC-MS). Under normal conditions, only trace amounts of volatile sesquiterpenes of rice were detected. The seedlings were then treated with MeJA and the volatiles were collected 28 h after the treatment. It was found that rice seedlings produced a blend of sesquiterpenes after MeJA treatments. On the basis of their abundance, these compounds were (E)-β-caryophyllene (**1**), β-elemene (**4**), α-curcumene (**5**), zingiberene (**6**), β-farnesene (**2**), β-sesquiphellandrene (**7**), β-bisabolene (**8**), α-copaene (**9**), γ-bisabolene (**10**), γ-curcumene (**11**), α-Longipinene (**12**), α-humulene (**13**) and α-bergamotene (**3**) (Figs. 1 and 2, and Table 1).

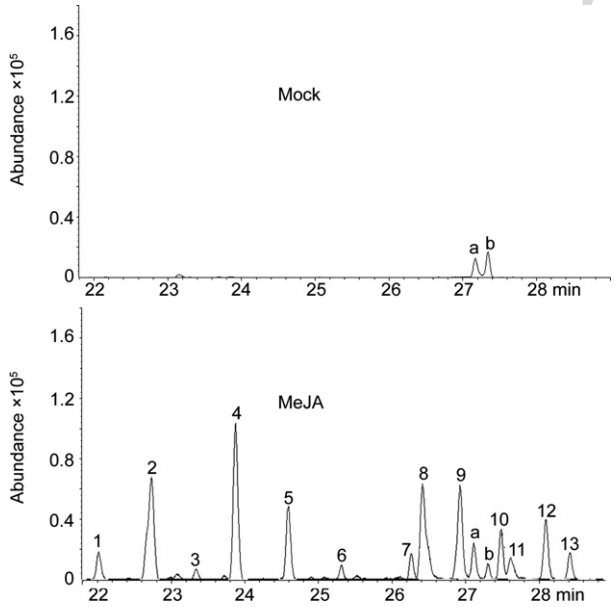

Fig. 2. Sesquiterpenes produced by rice seedlings after MeJA treatment. The 3-week-old seedlings were treated with buffer (Mock) or MeJA (MeJA) with volatiles were collected at 28 h after treatment by SPME trapping and analyzed by GC-MS. Peaks are: 1, α-copaene; 2, β-elemene; 3, α-bergamotene; 4, (*E*)-β-caryophyllene; 5, β-farnesene; 6, α-humulene; 7, α-Longipinene D; 8, α-curcumene; 9, zingiberene; 10, β-bisabolene; 11, γ-curcumene; 12, β-sesquiphellandrene; 13, γ-bisabolene; a, eicosane; b, silanamine.

Volatile sesquiterpenes were also collected from the crushed seedlings. Analysis by using SPME indicated that both intact and crushed plants produced similar chromatograms of sesquiterpenes (Supplementary Fig. 1). When the crushed tissues were used, much less material (about 1/7) and shorter collection time (1/3) were required to capture a similar amount of volatiles, Then crushed rice plants were employed for their analysis.

Herbivorous damage has been shown to induce accumulation of endogenous jasmonate in plants and this is accompanied by increased volatile emission. Thus, MeJA treatment can be used as a noninvasive mimic of insect attack (Martin et al., 2003). In the present investigation, MeJA treatment of rice plants stimulated production of (*E*)-β-caryophyllene (**1**) and other volatile sesquiterpenes (**1**–**13**). These results suggest that, as in many other plant species investigated, jasmonate is a signaling molecule in mediating the biosynthesis of volatile sesquiterpenes.

2.2. Isolation of rice sesquiterpene synthase cDNAs

Sequencing and annotation of the rice genome (http://CCR-081.mit.edu/GENSCAN.html) indicated that rice contains about 50 putative TPS genes. By using RT-PCR, full-length cDNAs of nine putative sesquiterpene synthases were isolated from the cultivar Zhonghua-11 (*O. sativa* ssp. *Japonica* L. cv. Zhonghua-11) (Supplementary Table 1), and the expression patterns were analyzed. It was found that, of the nine genes analyzed, *OsTPS3* had the highest steady-state level of transcripts in aerial organs (data not shown).

2.3. Identification of rice (*E*)-β-caryophyllene synthase and (*E*,*E*)-farnesol synthase

The nine putative sesquiterpene synthases were expressed in *Escherichia coli* as recombinant proteins for

Table 1
Volatile sesquiterpenes of the 3-week-old rice seedlings (28h after MeJA treatment)

	Compounds	ng/g FW[a]	%
1	α-Copaene	16.2 ± 3.0	3.2
2	β-Elemene	84.3 ± 12.8	16.7
3	α-Bergamotene	5.1 ± 0.9	1.0
4	(*E*)-β-Caryophyllene	97.9 ± 16.5	19.4
5	β-Farnesene	43.4 ± 7.3	8.6
6	α-Humulene	7.1 ± 1.1	1.4
7	α-Longipinene	13.6 ± 2.7	2.7
8	α-Curcumene	79.2 ± 14.9	15.7
9	Zingiberene	64.1 ± 14.1	12.7
10	β-Bisabolene	28.3 ± 3.7	5.6
11	γ-Curcumene	14.6 ± 2.8	2.9
12	β-Sesquiphellandrene	35.8 ± 6.1	7.1
13	γ-Bisabolene	15.1 ± 2.7	3.0

[a] Mean ± standard error from three independent experiments.

characterization of enzymatic activities. After incubation with either GDP or FDP, OsTPS3 and OsTPS13 converted FDP to sesquiterpene products (Fig. 3), and neither accepted GDP as a substrate. For an unknown reason, other proteins did not exhibit any TPS activities in our assay conditions.

In vitro, recombinant OsTPS3 protein catalyzed formation of at least 14 sesquiterpenes (Fig. 3a), of which the major products were (*E*)-β-caryophyllene (**1**) (45.9%), α-selinene (**14**) (15.5%) and β-elemene (**4**) (11.0%). The OsTPS13 converted FDP into (*E*,*E*)-farnesol (**15**) (84.2%), (*E*)-nerolidol (**16**) (9.7%) and an unknown compound (6.1%) (Fig. 3b). Thus, both *OsTPS3* and *OsTPS13* genes encode active sesquiterpene synthases that catalyze the formation of multiple products. The products of OsTPS3 are cyclic sesquiterpenes, whereas those of OsTPS13 are mainly acyclic alcohols.

A comparison of the results shown in Figs. 2 and 3a established that several OsTPS3 products, including (*E*)-β-caryophyllene (**1**), β-elemene (**4**) and α-humulene (**13**), were present in the sesquiterpene profiles of rice plants. Although certain in vitro products of OsTPS3 were not found in volatile sesquiterpenes of rice plants, possibly due to the different catalytic mechanisms of the sesquiterpene synthase in planta and in vitro, our data strongly suggest that OsTPS3 is at least one of the major sesquiterpene synthases responsible for the induced production of volatile sesquiterpenes of rice plants.

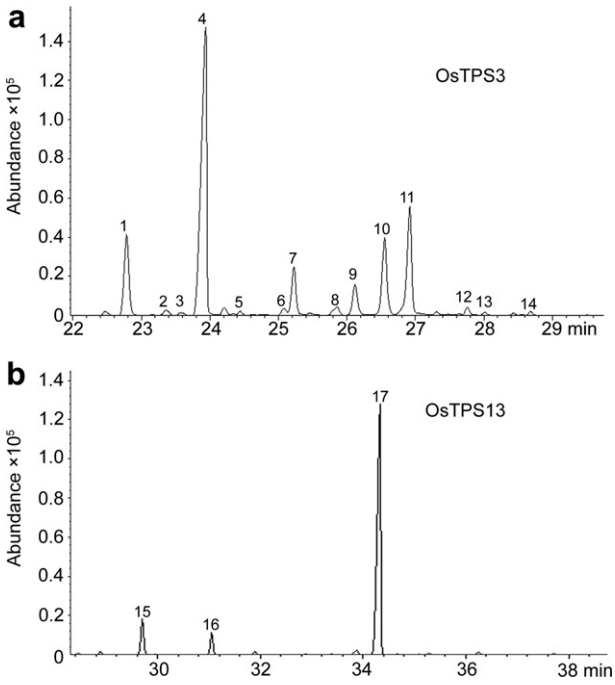

Fig. 3. Sesquiterpene products of OsTPS3 and OsTPS13. The recombinant protein, expressed in *E. coli*, was extracted and incubated with the substrate FDP. (a) Products of OsTPS3: 1, β-elemene (11%); 2, isocaryophyllene (0.8%); 3, aromadendrene (0.4%); 4, (E)-β-caryophyllene (45.9%); 5, γ-elemene (0.5%); 6, humulene-(vl) (0.9%); 7, α-humulene (6%); 8, muurolene (1.5%); 9, β-selinene (4.5%); 10, eudesma-4(14),11-dine (11%); 11, α-selinene (15.5%); 12, α-panasinsene (1%); 13, δ-cadinene (0.4%); 14, β-panasinsene (0.4%). (b) Products of OsTPS13: 15, nerolidol (9.7%); 16, unknown compound (6.1%); 17, (*E*,*E*)-farnesol (84.2%).

The *OsTPS3* and *OsTPS13* cDNAs encode proteins of 577 and 541 amino acid residues, respectively, and they share 31% identity with each other at the amino acid sequence level. The deduced amino acid sequences of both proteins contain highly conserved domains of terpene synthases, such as the DDxxD motif (Bohlmann et al., 1998). No signal peptide is apparent in either protein.

(*E*)-β-caryophyllene (**1**) is a common component of floral scent (Knudsen and Tollsten, 1993) and a constituent of the induced volatile blend of many plant species (Rasmann et al., 2005). In addition to rice, genes encoding (*E*)-β-caryophyllene synthase have been isolated from *Artemisia annua* (Cai et al., 2002), *A. thaliana* (Chen et al., 2003) and *Cucumis sativus* (Mercke et al., 2004). The OsTPS3 protein has a relatively low sequence identity (31-35%) to the previously identified (*E*)-β-caryophyllene synthase from dicots; however, it exhibits a much higher sequence identity (42–44%) to maize sesquiterpene synthases, including ZmTPS6, ZmTPS10 and ZmSTC1 (Schnee et al., 2002, 2006; Shen et al., 2000). OsTPS13 is most similar to ZmTPS7 and ZmTPS8 (Schnee et al., 2002) with 59% and 58% sequence identities, respectively. These data imply that sequence identity alone does not provide a clear clue to the catalytic properties of plant TPSs; rather, it is often a reflection of phylogenetic relationship between plants.

2.4. OsTPS3 and OsTPS13 are expressed in different organs

To further characterize the spatial expressions of *OsTPS3* and *OsTPS13*, transcripts of both genes were examined by Northern blots. In 3-month-old plants, *OsTPS3* gene had the highest steady-state level of transcripts in mature leaves, and a lower level in sheaths and spikelets. The transcripts were not detectable in roots, nor in leaves of the 3-week-old etiolated seedlings. For *OsTPS13*, expression was apparent only in leaf tissues of the etiolated seedlings (Fig. 4a).

2.5. Circadian rhythm of OsTPS3 expression

A number of plant monoterpene synthases show circadian patterns of gene expression, resulting in a fluctuated emission of their products (Lu et al., 2002; Dudareva et al., 2003). It was interesting to determine if the biosynthesis of volatile sesquiterpenes in rice plants was also modulated in a similar manner. The temporal expression pattern of *OsTPS3* in rice seedling leaves was investigated by quantitative real-time PCR at 4-h intervals during a 48-h period, which showed that the mRNA level of *OsTPS3* oscillated with the light/dark shifting (Fig. 4b). Levels of *OsTPS3* transcripts reached its lowest levels at ~7 PM, which was the switching point of light/dark periods. Transcript levels then increased during the dark period and peaked at the early stage of light period at ~11 AM, four hours in the light, followed by decreasing to the minimum

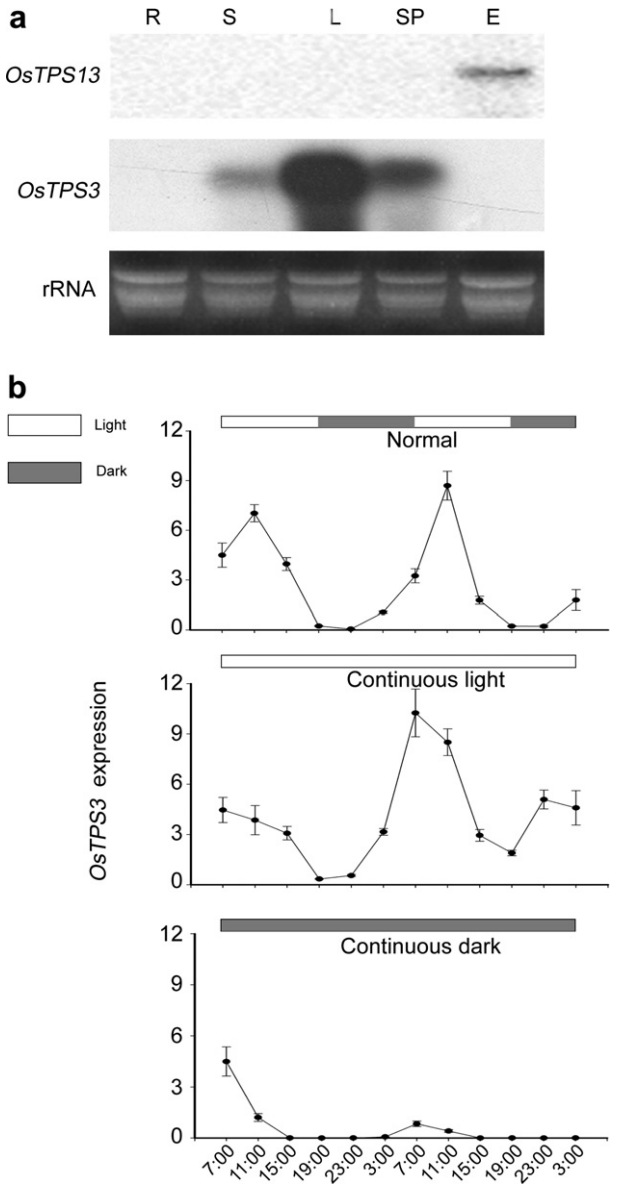

Fig. 4. Expression patterns of *OsTPS3* and *OsTPS13*. (a) Northern blot of *OsTPS3* and *OsTPS13*. Total RNA (12 μg per lane) was isolated from root (R), sheath (S), leaf (L), and spikelet (SP) of the 3-month-old plants, or from the 3-week-old etiolated seedlings (E). (b) Quantitative real-time PCR analysis of rhythmic changes of the steady-state level of *OsTPS3* transcripts in rice seedlings, which were grown under normal light/dark cycle (12 h/12 h), continuous light, or continuous dark conditions. Total RNA was isolated at 12 time points during a 48-h period.

at 7 PM. Under continuous light, the expression pattern was similar to that of the normal light/dark conditions, except that the expression peaked earlier from the second day. Under continuous dark conditions, expression was attenuated to an extremely low level (Fig. 4b).

2.6. MeJA stimulates OsTPS3 expression

As shown in Fig. 2, rice seedlings produced a complex mixture of volatile sesquiterpenes after MeJA stimulation. Because the sesquiterpenes released from the seedlings and the OsTPS3 products showed similar spectra to a certain extent, both with (*E*)-β-caryophyllene (**1**) as a predominant component, it is possible that the induced formation of volatile sesquiterpenes was, at least partly, a result of the enhanced *OsTPS3* gene expression. To ascertain the correlation of *OsTPS3* expression and (E)-β-caryophyllene (**1**) emission, *OsTPS3* transcript accumulation levels were measured as well as (*E*)-β-caryophyllene (**1**)/β-elemene (**4**) production of rice seedlings after MeJA treatment. Quantitative real-time PCR showed that MeJA stimulated expression of *OsTPS3* and the highest transcript level appeared approximately 24 h post-treatment (2. 5a). In accordance with *OsTPS3* expression, the production of (*E*)-β-caryophyllene (**1**) and β-elemene (**4**) reached the maximum level at 28 h post-treatment (Fig. 5b). It is interesting to note that, after MeJA treatment, both the *OsTPS3* expression and the (*E*)-β-caryophyllene (**1**)/β-elemene (**4**) emission still followed a circadian rhythm of fluctuation, although overall levels were substantially elevated. These results suggest that, in rice, both jasmonate signaling pathway and the circadian clock regulate *OsTPS3* gene expression and thereby the emission of its products.

The release of volatile monoterpenes and sesquiterpenes in plants displays a rhythmic pattern with maximum emis-

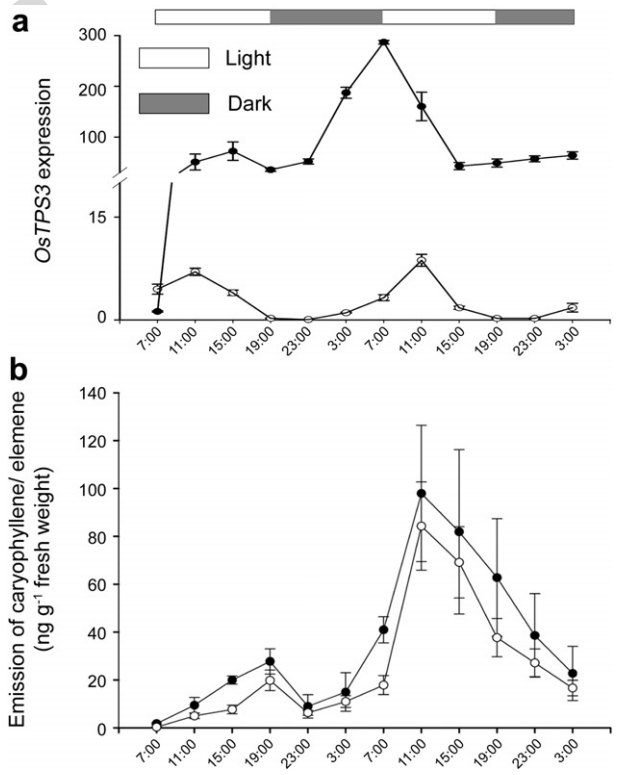

Fig. 5. MeJA-induction of *OsTPS3* expression and sesquiterpene emission. (a) Quantitative real-time PCR analysis of *OsTPS3* mRNA levels in rice seedlings after MeJA treatment during a normal light/dark cycle (solid circle). The *OsTPS3* mRNA levels in rice seedlings without treatment was also analyzed (open circle). (b) Changes of (*E*)-β-caryophyllene (solid circle) and β-elemene (open circle) emission after MeJA treatment during a normal light/dark cycle. Volatiles were collected from crushed leaves by SPME.

sion during the day, which generally coincides with the foraging activities of potential pollinators (Dudareva et al., 2003). It has been reported that *Arabidopsis* flower headspace has a clear diurnal pattern of (*E*)-β-caryophyllene (**1**) emission, with the peak level at ~9 PM, and the lowest level at ~8 AM (Aharoni et al., 2003). In *A. annua* the transcript abundance of a β-pinene synthase gene (*QH6*) fluctuates in a diurnal pattern with the highest mRNA level occurring in the day at ~3 PM, followed by a similar dynamics of β-pinene emission (Lu et al., 2002). This is similar to the pattern of rice *OsTPS3* expression and (*E*)-β-caryophyllene (**1**)/β-elemene (**4**) emission; the only difference lies in the exact timing of the rhythmic fluctuation.

2.7. Overexpression of OsTPS3 enhances (*E*)-β-caryophyllene emission

To further confirm the biochemical function of OsTPS3 in planta, this rice sesquiterpene synthase gene was expressed in *Arabidopsis* under the control of the CaMV 35*S* promoter. Rosette leaves of the 4-week-old plants from each individual line were screened by RT-PCR for *OsTPS3* expression (Fig. 6a), and by SPME for terpenoid emission. The results showed that, while no sesquiterpenes were detectable from the wild-type non-flowering *A. thaliana* plants (see also Aharoni et al., 2003), those of transgenic lines emitted a substantial level of the OsTPS3 blend of products, including (*E*)-β-caryophyllene (**1**), β-elemene (**4**) and α-humulene (**13**) (Fig. 6b).

The *OsTPS3* gene was also overexpressed in rice plants under control of the maize *UBIQUITIN1* promoter. The transgenic plants (lines O1 to O15) looked morphologically normal. PCR analysis indicated that the rice plants regenerated were transgenic (Fig. 7a). Leaves of the 3-week-old seedling were then examined for *OsTPS3*

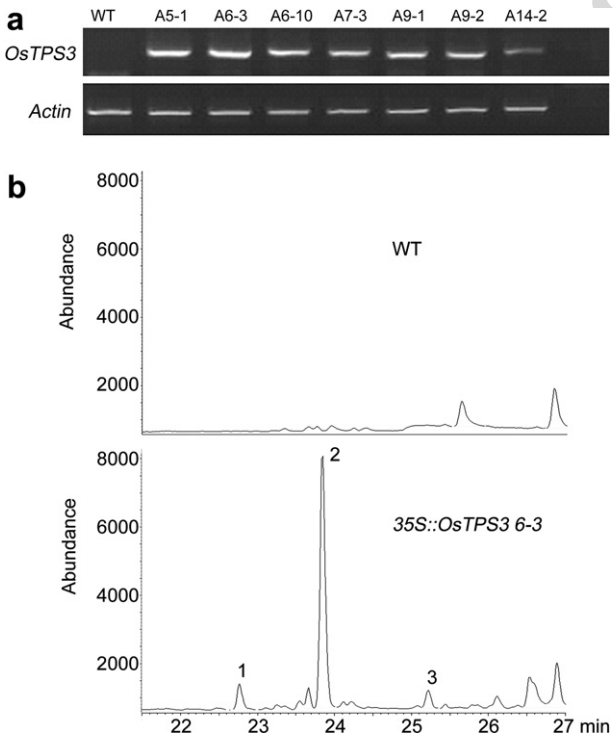

Fig. 6. Analysis of plants of *A. thaliana* transformed with *OsTPS3*. (a) RT-PCR analysis of *OsTPS3* transcripts in transgenic lines. PCR was performed by 24 cycles of amplification for *Actin* and 28 cycles for *OsTPS3*. (b) Volatiles from the wild-type and the transgenic Arabidopsis plants; 1, β-elemene; 2, (*E*)-β-caryophyllene; 3, α-humulene.

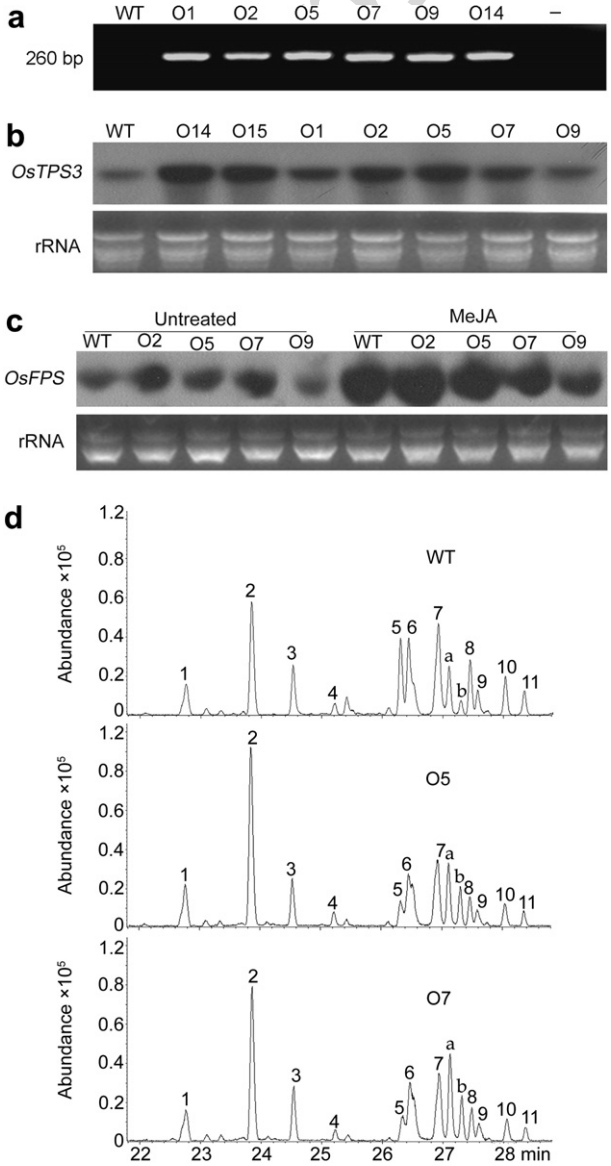

Fig. 7. Analysis of transgenic rice plants overexpressing *OsTPS3*. (a) PCR analysis of the transgenic rice plants using the *NOS* terminator primers. (b) Comparison of *OsTPS3* transcript levels in the 3-week-old transgenic (O) and wild-type (WT) rice plants by RNA gel blot. (c) RNA gel blot analysis of *OsFPS* transcript levels in the 3-week-old seedlings after MeJA treatment for 24 h, the expression was strongly induced. (d) Sesquiterpene volatiles of the transgenic (O5 and O7) and the wild-type (WT) rice plants after MeJA treatment. The volatiles were collected 24 h after treatment. Peaks are: 1, β-elemene; 2, (*E*)-β-caryophyllene; 3, β-farnesene; 4, α-humulene; 5, α-Longipinene D; 6, α-curcumene; 7, zingiberene; 8, β-bisabolene; 9, γ-curcumene; 10, β-sesquiphellandrene; 11, γ-bisabolene; a, eicosane; b, silanamine.

transcript accumulation and sesquiterpene production. Northern analysis showed that, to a different extent, transgenic plants of individual lines accumulated more *OsTPS3* transcripts than wild-type (Fig. 7b). Under normal conditions, however, elevated expression of *OsTPS3* in transgenic rice plants did not lead to emission of large quantities of (*E*)-β-caryophyllene (**1**), probably due to a limited availability of the substrate FDP. To overcome this bottleneck, the 3-week-old seedlings were treated with MeJA, which also strongly induced expression of a rice FDP synthase gene (*OsFPS*, D85317) in both transgenic and wild-type plants (Fig. 7c). Subsequent SPME analysis indicated that the transgenic seedlings emitted more (*E*)-β-caryophyllene (**1**) than the wild-type after MeJA elicitation (Fig. 7d). In the volatile samples collected from the wild-type seedlings, (*E*)-β-caryophyllene (**1**) accounted for ~18% of the total sesquiterpenes emitted, whereas in the volatiles from the transgenic lines of O5 and O7, this portion was increased to ~31% and ~27%, respectively (Table 2). For unknown reasons, the production of α-humulene (**13**), a minor product of OsTPS3, was lower in the transgenic plants than in the wild-type.

2.8. Elevated expression of OsTPS3 attracts more parasitoid wasps

Previous studies have shown that (*E*)-β-caryophyllene (**1**) is an important signaling molecule that mediates plant–insect interactions (Rasmann et al., 2005). To determine if the sesquiterpene products of OsTPS3 are involved in the indirect defense of rice plants, an olfactometer-mediated wasp-attractiveness assay was performed using wild-type and transgenic plants harboring the transgene of *35S::OsTPS3* as odor sources. *A. nilaparvatae* is an egg parasitoid of rice planthoppers, including the rice brown planthopper *N. lugens*, one of the most important insect pests of rice plants. *N. lugens* feeds on phloem sap, causing major physiological stress to the plant (Lou et al., 2005). When *Arabidopsis* plants were used, 62.5% of the female wasps tested walked into the arms of the Y-tube carrying the odor of the transgenic plants expressing *OsTPS3* (line A6-3), whereas the remaining wasps walked into the arms of the Y-tube carrying the odor of the wild-type plants (Fig. 8). Because under normal conditions neither wild-type nor transgenic rice seedlings emitted a considerable amount of sesquiterpenes, seedlings were treated with MeJA before the assay. We found that the MeJA-treated rice plants attracted more *A. nilaparvatae* than the untreated plants. Next, both transgenic and wild-type plants were treated by MeJA and the results compared. This showed that 72% of the female wasps walked into the arms carrying the odor of the transgenic line of O5, and 60% were attracted by the transgenic line of O7 (Fig. 8). These data point to a general tendency that female wasps of *A. nilaparvatae* prefer plants with higher levels of OsTPS3 products.

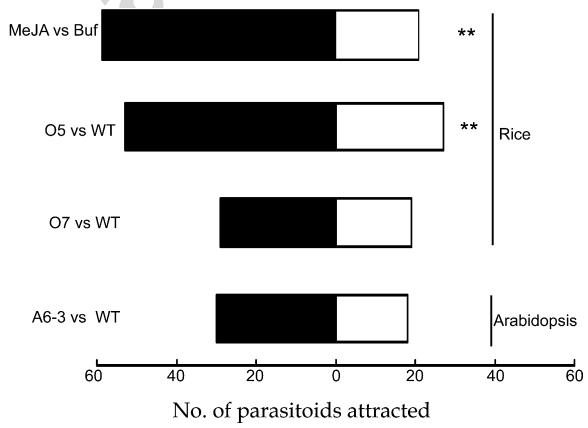

Fig. 8. Number (%) of *A. nilaparvatae* female adults attracted by volatiles released from plants of the following pairs of plant lines. MeJA-treated vs. untreated (Buf) rice plants; MeJA-treated transgenic line O5, or O7, vs. the MeJA-treated wild-type (WT) rice plants, respectively; transgenic *Arabidopsis* line A6-3 vs. the wild-type (WT) *Arabidopsis* plants. Asterisks indicate very significant differences between members of a pair ($P < 0.01$, χ^2 test).

Table 2
Volatile sesquiterpenes of the transgenic (O5 and O7) and the wild-type (WT) rice seedlings (24 h after MeJA treatment)

Compounds	WT		O5		O7	
	ng/g FW[a]	%	ng/g FW[a]	%	ng/g FW[a]	%
β-Elemene	9.8 ± 1.7	6.1	15.1 ± 3.2	8.9	12.1 ± 2.6	7.2
(E)-β-Caryophyllene	28.7 ± 5.2	17.9	52.9 ± 9.6	31.1	45.7 ± 9.4	27.2
β-Farnesene	12.0 ± 2.9	7.5	12.2 ± 2.1	7.2	14.8 ± 2.7	8.8
α-Humulene	5.0 ± 1.3	3.1	3.9 ± 0.6	2.3	3.2 ± 0.6	1.9
α-Longipinene	17.4 ± 3.6	10.9	8.3 ± 1.4	4.9	8.4 ± 1.4	5
α-Curcumene	27.4 ± 6.1	17.1	28.5 ± 4.9	16.8	32.3 ± 6.1	19.2
Zingiberene	28.8 ± 5.4	18	26.7 ± 5.1	15.7	27.4 ± 4.9	16.3
β-Bisabolene	11.5 ± 2.5	7.2	7.5 ± 1.2	4.4	8.4 ± 1.8	5
γ-Curcumene	5.3 ± 1.2	3.3	4.6 ± 0.8	2.7	5.4 ± 1.2	3.2
β-Sesquiphellandrene	8.6 ± 1.9	5.4	6.6 ± 1.4	3.9	6.5 ± 1.6	3.9
γ-Bisabolene	5.6 ± 1.0	3.5	3.7 ± 0.6	2.2	3.7 ± 0.7	2.2

[a] Mean ± standard error from three independent experiments.

3. Conclusions

In summary, two rice sesquiterpene synthases, OsTPS3 and OsTPS13, have been characterized. In vitro OsTPS3 catalyzed the formation of multiple cyclic sesquiterpenes, and OsTPS13 converted FDP into sesquiterpene alcohols. Of the OsTPS3 products (*E*)-β-caryophyllene (**1**) and β-elemene (**4**) are major components and are also present in large quantities in MeJA-inducible volatile sesquiterpenes of rice seedlings. *OsTPS3* is expressed in aerial organs of rice plants, including leaf, sheath and spikelets. The steady-state level of *OsTPS3* transcript in rice seedlings fluctuates in a circadian rhythm and is induced to increase by MeJA. When introduced into *Arabidopsis* plants, transgenic plants emitted a substantial level of the OsTPS3 products.

Furthermore, transgenic plants of both rice and *Arabidopsis* with higher levels of OsTPS3 product emission were more attractive to wasps of *A. nilaparvatae*, a main egg parasitoid of rice planthoppers. The results demonstrate that OsTPS3 produces volatile chemicals that play a role in protection of rice plants against herbivorous insects. The sesquiterpene synthase OsTPS3 is therefore an important component of the indirect defense response of rice plants.

4. Experimental

4.1. Plant material

Plants of *O. sativa* L. ssp. *Japonica* cv. Zhonghua-11 were grown in a greenhouse at 28 °C under a 12/12 h (light/dark) photoperiod at 150 μmol m^{-2} s^{-1}. For methyl jasmonate (MeJA) treatment, the 3-week-old seedlings were irrigated with MeJA (Sigma–Aldrich, St. Louis, MO) at 250 μM in distilled H_2O, and then harvested during a 48-h period at 4-h intervals, followed by volatile collection or RNA isolation. Plants of *A. thaliana* (ecotype Col-0) were grown in a greenhouse at 22 °C and under a 16/8 h photoperiod.

4.2. Isolation of cDNA and protein expression in E. coli

Based on the genome sequence of rice, cDNAs of putative TPSs were obtained by RT-PCR, and the primers used are listed in Supplementary Table 1. The resultant fragments were cloned into the vector pMD18-T (TaKaRa, Dalian, China). After confirmation by DNA sequencing, the fragments were subcloned into the vector pET32a (Novagen, Madison, WI) for protein production in *E. coli* strain BL21 Codon Plus (Stratagene, La Jolla, CA). Purification of His-tagged recombinant proteins on a Ni-NTA spin column (Qiagen, Valencia, CA) was performed as described by the manufacturer. Protein eluates were immediately desalted with a Bio-Rad Econo column, and the resulting eluates (in assay buffer containing 10 mM 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid, pH 7.0, 10% [v/v] glycerol, and 1 mM DTT) were used for activity assay. The volume of each assay was 1 mL, containing 10 μg protein, 20 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM $NaWO_4$, 0.1 mM NaF, and 40 μM geranyl diphosphate (GDP) or farnesyl diphosphate (FDP) (Sigma–Aldrich, St. Louis, MO), respectively. The assay was performed in a 4-mL vial with a solid-top polypropylene cap. After incubation at 30 °C for 3 h, a 65-μm solid-phase microextraction (SPME) PDMS-DVB fiber (Supelco, Bellefonte, PA) was inserted into the tube to collect volatiles for 30 min. The SPME fiber was injected into a gas chromatography–mass spectrometry (GC-MS) system (HP 6890/5973GC-MSD) for analysis. Alternatively, the assay was overlaid with pentane (0.3 mL) to trap volatile products and incubated for 3 h at 37 °C. Then the enzyme products were extracted three times with pentane (1 mL × 3), and the total organic extract was concentrated to ~100 μL for GC-MS analysis. The program included a ramp from 60 °C to 180 °C at 3 °C/min, and was then maintained for 10 min. Peak identities were confirmed by using authentic standards and NIST (National Institute of Standards and Technology) and Wiley libraries.

Assays with crude protein extracts of induced *E. coli* carrying the empty expression vector were performed as a negative control. The protein concentration was determined according to Bradford (1976) with BSA as a standard.

4.3. RNA isolation and analysis

Trizol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNAs from leaves or other tissues. For RT-PCR analysis, total RNA of 1 μg was used to synthesize cDNA with Oligo (dT) primers, using an Advantage RT-for-PCR kit (Toyobo, Osaka, Japan). For Northern analyses, total RNAs (12 μg) were separated on 1.0% formaldehyde denature agarose gels, and transferred onto a Hybond-N+ membranes (Amersham, Piscataway, NJ). Hybridization and washing were performed following standard procedures. The probes were isolated by PCR amplification of the corresponding cDNA fragments with specific primers (Supplementary Table 1), and randomly labeled with [^{32}P]dCTP.

Quantitative real-time RT-PCR (qRT-PCR) was performed using the TaqMan primers, and the assays were performed as previously described (Wang et al., 2004). The reaction parameters were 2-min of 50 °C, 30-min of 60 °C, and 5-min of 95 °C, followed by 35 cycles of 20-s of 94 °C melt and 1-min of 60 °C anneal/extension. Measurements were performed in triplicate, and data were analyzed with Rotor-Gene 6.0 software (Corbett Research, Australia) for relative quantification.

4.4. Plant transformation

The ORF of *OsTPS3* was amplified from the pET32a construct and inserted as a BamH I–EcoICR I fragment

into the binary vector pCAMBIA 1301 (CAMBIA, Australia). The resultant construct was introduced into *Agrobacterium tumefaciens* GV3101 cells, which were used to transform *A. thaliana* plants by a floral dip method (Clough and Bent, 1998). Rice plants were transformed with *A. tumefaciens* EHA105, as previously described (Xu et al., 2002). Transgenic plants were screened with PCR using the *NOS* terminator specific primers (Supplementary Table 1) and further analyzed by Northern.

4.5. Plant volatile analysis

Analysis of the volatiles of rice leaf was performed by using 600 mg of tissues ground in liquid N_2. The endogenous enzyme activities were terminated by addition of 5 M $CaCl_2$ (1.2 mL) solution, then (+)-2-carene (218 ng) was added as an internal standard. Subsequently, a 65-μm PDMS-DVB SPME fiber was used for the headspace. Alternatively, the intact rice seedlings was enclosed in a 15-mL glass tube and the 65-μm PDMS-DVB SPME fiber was used to capture the volatiles released from the plant tissue. Detached leaves of *Arabidopsis* were enclosed in a 15-mL glass tube and the 65-μm PDMS-DVB SPME fiber was used to capture the volatiles released from the plant tissue. Volatile sampling was carried out for 30 min at room temperature and the sample was analyzed by GC-MS as described for identification of enzymatic products.

4.6. Bioassays

Responses of *A. nilaparvatae* females to rice volatiles were measured in a Y-tube olfactometer (Lou et al., 2005). An air stream was generated and was divided into two, and each secondary air stream was led through a flowmeter, a tube with active charcoal, a humidifier bottle and one of the odor containers. Subsequently, the two airstreams were led through the two arms of the Y-tube olfactometer at 150 ml/min.

A. nilaparvatae females were raised with the eggs of *N. lugens*. *A. nilaparvatae* females had the choice between odors from: MeJA-treated rice plants vs. untreated WT plants; MeJA-treated rice plants of line O5 or line O7 vs. wild-type plants; and *Arabidopsis* plants of line A6-3 vs. wild-type plants, respectively. Ten plants for each rice line or 20 plants for each *Arabidopsis* line were cut off at soil level, the cut stem was wrapped with wet cotton and the entire plants were placed into one of the odor source containers. Mated female parasitoids were introduced individually into the base tube of the Y-shaped olfactometer and given 10 min to walk towards the end of either arm of the olfactometer. A choice for an odor source was defined as a female crossing a line 7 cm after the division of the base tube and staying there for at least 1 min. If a parasitoid did not make a choice within 10 min this was recorded as "no response". After testing 2 females, the olfactometer tube was washed with 98% alcohol and then heated at 80 °C. To eliminate the effects of asymmetrical bias, connections of the two arms of the olfactometer to the odor source containers were exchanged after testing 2 females, and the odor source containers were exchanged and supplied with a new set of plants after testing 16 females. For each odor source combination at least 48 females were tested for one experiment, which was repeated for at least three times. Differences in behavioral responses were determined by χ^2 tests (Lou et al., 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.04.008.

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