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SHORT COMMUNICATION

(*E*)-β-Farnesene synthase genes affect aphid (*Myzus persicae*) infestation in tobacco (*Nicotiana tabacum*)

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Abstract Aphids are major agricultural pests which cause significant yield losses of the crop plants each year. (*E*)-β-farnesene (EβF) is the alarm pheromone involved in the chemical communication between aphids and particularly in the avoidance of predation. In the present study, two EβF synthase genes were isolated from sweet wormwood and designated as *AaβFS1* and *AaβFS2*, respectively. Over-expression of *AaβFS1* or *AaβFS2* in tobacco plants resulted in the emission of EβF ranging from 1.55 to 4.65 ng/day/g fresh tissues. Tritrophic interactions involving the peach aphids (*Myzus persicae*), predatory lacewings (*Chrysopa septempunctata*) demonstrated that the transgenic tobacco expressing *AaβFS1* and *AaβFS2* could repel peach aphids, but not as strongly as expected. However, *AaβFS1* and *AaβFS2* lines exhibited strong and statistically significant attraction to lacewings. Further experiments combining aphids and lacewing larvae in an octagon arrangement showed transgenic tobacco plants could repel aphids and attract lacewing larvae, thus minimizing aphid infestation. Therefore, we demonstrated a potentially valuable strategy of using EβF synthase genes from sweet wormwood for aphid control in tobacco or other economic important crops in an environmentally benign way.

Keywords Sweet wormwood · (*E*)-β-Farnesene synthase · Transgenic tobacco · Aphid · Lacewing

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Introduction

Aphids (Aphididae) are major agricultural pests due to the serious physical and economic damage they cause to cultivated plants by sucking nutrients from the phloem and/or by transmitting plant viruses (Goggin 2007). For many crops, insecticides provide a simple strategy for aphid control. However, the application of such chemicals is not desirable partly because of the development of insecticide resistance and also to aim for more sustainable agricultural practices with less chemical inputs. Transgenic crops engineered for enhanced resistance to aphids could be an efficient alternative strategy. Some plant lectins, including *Galanthus nivalis* agglutinin (GNA), have been shown to be toxic to aphids in transgenic plants (Hilder et al. 1995). However, GNA could cause adverse effects on predatory ladybirds via aphids in the food chain (Birch et al. 1999), resulting in public concerns related to the biosafety in application of these genes in genetic engineering of agricultural important crops. Therefore, other safe

and more effective genes/genetic strategies for aphid control need to be exploited.

Orthologous genes of the (*E*)-β-farnesene (EβF) synthase which converts farnesyl diphosphate (FPP) to the acyclic sesquiterpene EβF has been isolated and characterized from Douglas fir (*Pseudotsuga menziesii*; Huber et al. 2005), Yuzu (*Citrus junos*; Maruyama et al. 2001), sweet wormwood (*Artemisia annua*; Picaud et al. 2005), and black peppermint (*Mentha* × *piperita*; Crock et al. 1997). Compared with the maize *terpene synthase 1*, which could produce three sesquiterpenes: (*E*)-β-farnesene (26%), (*E*)-β-nerolidol (29%), and (*E*, *E*)-farnesol (45%) (Schnee et al. 2002), the above four isolated EβF synthases produced only one major sesquiterpene, EβF, indicating these genes were good candidates for manipulating crop plants for aphid control. Indeed, overexpression of a peppermint EβF synthase gene in *Arabidopsis thaliana* not only repelled aphids but also attracted aphid parasitoids (Beale et al. 2006) and the EβF-emitting transgenic plants may have practical applications in agriculture as a result of increased predation on habituated aphids (De Vos et al. 2010), demonstrating that genetically engineering of plants to consecutively emit EβF for aphid control was feasible. Although the EβF synthase gene had been isolated from sweet wormwood and in vitro analysis indeed proved that it could convert FPP to the only product of acyclic sesquiterpene EβF (Picaud et al. 2005), the function of this gene in other plants remains unclear. In order to exploit the application of this gene in genetic engineering of agronomic important crop plants for aphid resistance, we reported here the isolation and characterisation of two EβF synthase genes from sweet wormwood. Furthermore, transgenic tobacco plants constitutively expressing these genes were characterized and the emission of EβF and its effects on aphid infestation and predation by lacewings were investigated.

Materials and methods

Materials

A. annua L. strain 025 were kindly provided by Prof. ShiHua Shen, Institute of Botany, Chinese Academy of Sciences. Tobacco (*Nicotiana tabacum* L., cv W38) seedlings grown on Murashige and Skoog MS medium were used for genetic transformation. Leaves were excised, frozen in liquid nitrogen and stored at −80°C for RNA extraction. Standard EβF was purchased from Tokyo Kasei Chemicals, Tokyo, Japan.

Cloning of the EβF synthase genes by reverse transcription polymerase chain reaction (RT-PCR) and sequence analysis

Plant materials stored at −80°C were used to extract total RNA, and the cDNA was synthesized using the M-MLV Reverse Transcriptase (TaKaRa, Dalian, China). PCR amplifications were done using specific primers designed from the published EβF synthase cDNA (AY835398; Picaud et al. 2005). The primers used here were: forward primer 5′-ATGTCGACTCTTCCTATTTCTAGTGT-3′ and reverse primer 5′-TTAGACAACCATAGGGTGAACG-3′. PCR fragments were cloned into pEASY-Blunt vector (Transgen Biotech, Beijing, China) and transformed into competent *Escherichia coli* DH5α cells. The inserts were sequenced by ABI 3130 XL DNA sequencer (ABI, Perkin-Elmer). DNA sequence data were analyzed using BLAST (http://www.ncbi.nlm.nih.gov/blast/), Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html), and iPSORT prediction (http://hc.ims.u-tokyo.ac.jp/iPSORT/). The alignment of the deduced protein sequences was performed using DNAMAN and CLUSTAL_X version 1.83.

Tobacco transformation and identification of positive transgenic lines

The recombinant plasmids (*AaβFS1*–pBI121 and *AaβFS2*–pBI121, as shown in Fig. 1a) were transferred into *Agrobacterium tumefaciens* strain AGL1. Transformation of tobacco was performed following the method of Horsch et al. (1985). Five- to six-week plantlets were identified by PCR and RT-PCR (forward primer 5′-TGGTTCCGACTCC TACGACA-3′ and reverse primer 5′-ATCCTTGC CAGCCTTCTCC-3′), and positive lines were transferred to pots, grew and self-pollinated in a green house. The T_2 lines with strong RT-PCR levels were transferred to pots for further analysis alongside transgenic lines harboring pBI121 blank vector as control.

Southern blot hybridization

Around 30 μg of genomic DNA from T_2 transgenic tobacco leaves was digested with *Eco*RV, the digested fragments were separated by electrophoresis in a 0.8% agarose gel at 30 V for 16 h and transferred to Hybond N+ membranes according to the manufacturer′s instructions (Amersham, Little Chalfont, UK). Membranes were hybridized with ^{32}P-radioactive probes generated by PCR using primers (forward primer 5′-AGAGGCCATGTTGCTTCAAGC-3′ and reverse primer 5′-CCATAGGGTGAACGAAGAACG-3′). Hybridization and membrane washing was performed according to standard protocols and signals were visualized by autoradiography.

Quantitative real time-PCR analysis

The expression of *AaβFS1* and *AaβFS2* genes in T_2 transgenic lines was further analyzed by quantitative real time (qRT)-PCR, which was performed with a Quant qRT-

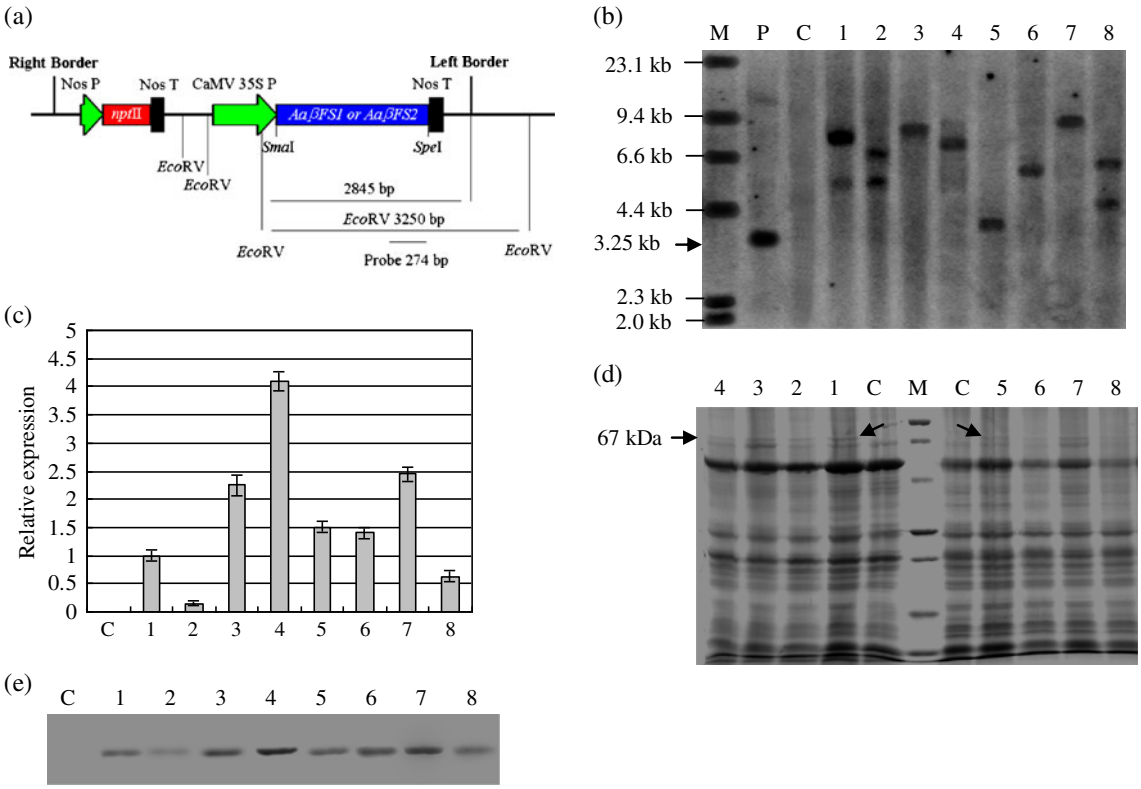

Fig. 1 Molecular analyses of tobacco plant lines transformed with *AaβFS1* and *AaβFS2*. **a** Schematic show of the *AaβFS1* and *AaβFS2* gene expression cassettes in the pBI121 plasmid, *Eco*RV restriction sites, and T-DNA borders. **b** Southern blotting of transgenic tobacco lines. *M* λDNA/HindIII, *P AaβFS1*–pBI121 plasmid, *C* blank vector control, *1–8* positive transgenic lines S1-1-1, S1-2-7, S1-3-4, S1-4-5, S2-1-2, S2-2-1, S2-3-6, and S2-4-4. The *arrowhead* indicated the expected band of 3,250 bp from plasmid. **c** qRT-PCR analysis of the expression of *AaβFS1* and *AaβFS2* in T_2 transgenic lines and vector control. The relative expression of the target gene was presented relative to the averaged level of S1-1-1 lines. The average and SE of three technical replicates were presented. *C* blank vector control, *1–8* positive transgenic lines S1-1-1, S1-2-7, S1-3-4, S1-4-5, S2-1-2, S2-2-1, S2-3-6, and S2-4-4. **d** SDS-PAGE analyses of total proteins extracted from T_2 transgenic lines and vector control. *Arrowhead* indicated the unique band of 67 kD in T_2 transgenic lines. *M* Protein marker, 94.0, 66.2, 45.0, 33.0, 26.0, 20.0, 14.4 kD from *top* to *down*, respectively. *C* blank vector control, *1–8* positive transgenic lines S1-1-1, S1-2-7, S1-3-4, S1-4-5, S2-1-2, S2-2-1, S2-3-6, and S2-4-4. **e** Western blots analyses of different *AaβFS1* or *AaβFS2* transgenic lines, *C* blank vector control, *1–8* positive transgenic lines S1-1-1, S1-2-7, S1-3-4, S1-4-5, S2-1-2, S2-2-1, S2-3-6, and S2-4-4

PCR Kit (Tiangen Biotech, Beijing, China) in ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Tobacco 18S rRNA gene was used as a standard control. Primers for tobacco 18S rRNA gene were forward primer 5′-GAAACGGCTACCACATCCA-3′ and reverse primer 5′-ATCCCAAAGTCCAACTACGAG-3′. Primers for *AaβFS1* and *AaβFS2* were forward primer 5′-TGGTTCCGACTCCTACGACA-3′ and reverse primer 5′-ATCCTTGCCAGCCTTCTCC-3′. qRT-PCR experiments were done in triplicate, and the target gene transcript level was normalized to the average amount of 18S rRNA transcript.

SDS-PAGE analysis and western blotting

After grinding T_2 transgenic tobacco leaves with a mortar and pestle, total proteins were extracted according to the manufacturer of Plant Total Protein Extraction Kit (Applygen, Beijing, China). Then proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). To identify the target protein, the total protein extract was separated on 10% SDS-PAGE and transferred to a polyvinylidine difluoride membrane (Nihon Millipore, Tokyo, Japan). After the membranes were blocked with Milk Blocking Buffer (CWbio, Beijing, China), they were incubated with the 1:1,000 diluted *AaβFS1* polyclonal antiserum for 2 h at room temperature. 1:10,000 diluted Peroxidase AffiniPure Goat Anti-Rabbit IgG (H + L; CWbio) was subsequently reacted for 1 h. Blots were visualized using the cECL Western Blot Kit as described by the manufacturer (CWbio).

Air entrainment and gas chromatography–mass spectra analysis

The volatile chemicals from the transgenic lines and control were collected and analyzed at Institute of Plant Protection, Chinese Academy of Agricultural Sciences, following the

method described by Bruce et al. (2008). After the 24-h collection period, the traps were rinsed with 400 μl methylene chloride containing 1,600 ng of *n*-octane as an internal standard. Of each sample, 1 μl was analyzed by HP6890/HP5973 gas chromatography–mass spectra (GC-MS; Hewlett-Packard). Hewlett-Packard Chemstation software was utilized for system control and data analysis. The quantification of all major components was based on comparisons with the internal standards.

Aphids and predator bioassays

The aphid and predator assays were performed at the Institute of Tobacco Research, Chinese Academy of Agricultural Sciences. T_2 transgenic lines with higher EβF emission were used for the assays with transgenic lines harboring pBI121 blank vector as control. For each assay, three independent experiments were performed and each was done in triplicate. All the bioassays were performed in an octagon arrangement (with a "diameter" of 1.5 m) with two *AaβFS1* plants, two *AaβFS2* plants, and four control plants inside as described by Kappers et al. (2005). *AaβFS1*, *AaβFS2*, and control lines were arranged in alternating angles of the octagon. This setup was totally enclosed by a white coarse-net cover in the green house. Responses of aphids to *AaβFS1* and *AaβFS2* lines were tested by introduction of 200 alate aphids into the chamber. The number of aphids on each plant was counted after 12 h. One hundred lacewing larvae starved for 6 h before the predators assay were released into the setup. The number of lacewings on each plant was counted after 15, 30, 45, and 60 min, respectively. To assess the preliminary effect of aphid control by predator foraging and repellence, 400 alate aphids and ten lacewing larvae were placed onto the midpoint of the setup for 12 h and then the number of aphids on the plants was counted.

Results

Isolation of EβF synthase genes from sweet wormwood

Sequencing of random-selected ten clones identified two distinct cDNAs. One sequence, *AaβFS1* (GenBank accession number GU294840), encoded a 574 amino acid protein with a theoretical pI of 5.18 and a molecular mass of 67.8 kDa containing four amino acids differing from AY835398 with 99.5% overall identity. Another cDNA sequence, *AaβFS2* (GenBank accession number GU294841), encoded a 575 amino acids protein with a theoretical pI of 5.25 and a molecular mass of 68 kDa, showed 99.5% identical to AY835398 protein. Neither of them possessed a signal peptide at the N-terminal according to an iPSORT prediction (http://hc.ims.u-tokyo.ac.jp/iPSORT/). Therefore, *AaβFS1* and *AaβFS2* were predicted to operate in the cytoplasm, the site where sesquiterpene was biosynthesized. Both cluster and phylogenetic analyses revealed that AaβFS1 and AaβFS2 were more closely related to the terpene synthase from sweet wormwood or relative plant species than that of their counterparts from distant species such as black pepper mint and maize terpene synthase 1 with only 35.5% and 23.3% identity at amino acid level, respectively (data not shown).

Generation and characterization of transgenic tobacco plants

The pBI121 plasmids containing cDNAs of *AaβFS1* or *AaβFS2* (Fig. 1a) were transferred into the tobacco cultivar W38 via *Agrobacterium*-mediated transformation. PCR and RT-PCR analysis was performed in T_0 transgenic lines. In total, 11 *AaβFS1* and nine *AaβFS2* transgenic plants were identified, respectively. These plants were advanced to T_2 lines by self-pollination of T_0 and T_1 lines.

The integration of the *AaβFS1* or *AaβFS2* gene was analyzed in eight T_2 lines (from different independent T_0 lines) by Southern blot analysis. Both plasmid and genomic DNA were digested by *Eco*RV and hybridized with a 274 bp cDNA fragment probe of *AaβFS1* gene. *Eco*RV cut six times in the *AaβFS1*–pBI121 plasmid and could release a fragment of 3,250 bp containing the *AaβFS1* sequence (Fig. 1a). Therefore, *Eco*RV digested *AaβFS1*–pBI121 plasmid showed the expected band of 3,250 bp in size when hybridized with the probe (Fig. 1b). Besides, *Eco*RV cut three times between the right and left border sequences of T-DNA and in transgenic plants containing only the T-DNA portion of the *AaβFS1*–pBI121 plasmid, the 274-bp probe would be expected to give hybridization bands of at least 2,845 bp due to the location of other *Eco*RV sites randomly situated in the flanking tobacco genomic DNA. The number of bands indicated approximately the number of transgene copies integrated. As indicated in Fig. 1b, two *AaβFS1* (S1-3-4 and S1-4-5) and three *AaβFS2* (S2-1-2, S2-2-1, and S2-3-6) T_2 lines showed a single hybridizing band; the other three lines (S1-1-1, S1-2-7, and S2-4-4) contained two bands, whereas control plants harboring the blank vector showed no hybridization at all. qRT-PCR analysis showed that the expression of *AaβFS1* and *AaβFS2* among the T_2 transgenic lines were different at transcription level, with that of S1-4-5 and S2-3-6 lines were much higher than S1-2-7 (Fig. 1c). SDS-PAGE showed that there was a unique band at about 67 kDa in transgenic lines compared with the control, the size was

consistent with the calculated molecular weight (67.8 kDa) and same as the recombinant AaβFS1 (data not shown), and this band was further confirmed by western blotting using AaβFS1 polyclonal antiserum (Fig. 1d, e).

Emission of EβF in *AaβFS1* and *AaβFS2* transgenic lines

Volatiles from the control and *AaβFS1* and *AaβFS2* transgenic lines at flowering stage were collected by air entrainment and analyzed by coupled GC-MS. The results showed that the control plants produced small amounts of tridecane and tetradecane but absence of EβF (Fig. 2a). In comparison, in addition to the similar amount of terpenes produced by the control, both *AaβFS1* and *AaβFS2* transgenic lines showed a unique peak, which was identified as EβF with its retention time and mass spectrum identical to that of the commercial pure EβF sample (Fig. 2b–d). EβF emission levels of the transgenic lines reached at 1.55–4.13 ng/day/g fresh tissues, respectively. Compared with the relative expression of *AaβFS1* and *AaβFS2* transgenic lines both at transcriptional and protein level in Fig. 1c, e, the EβF emission levels were not in a proportional relationship through the lines with higher expression level could emit more EβF.

Tritrophic interactions involving peach aphids, predatory lacewing and transgenic tobaccos expressing *AaβFS1* and *AaβFS2*

To test the efficacy of the transgenic lines in control of aphids, three independent evaluations were conducted as described in "Materials and methods" section (Supplementary Fig. S1a). Four lines with the higher EβF emission (S1-3-4, S1-4-5, S2-1-2, and S2-3-6), were selected for aphid and predator bioassays. In the first assay, the number of alate aphids present on each plant was recorded 12 h after release. The results indicated that transgenic plants harboring *AaβFS1* and *AaβFS2* repelled peach aphids (*Myzus persicae*), but not as strongly as expected. Compared with control, the number of aphids was reduced by approximately 10% on the highest EβF emission line S1-4-5 (4.65 ng/day/g fresh tissue; Supplementary Fig. S1b) while 22% aphids made no choice, mainly staying on the net cover. The repellence varied depending on different

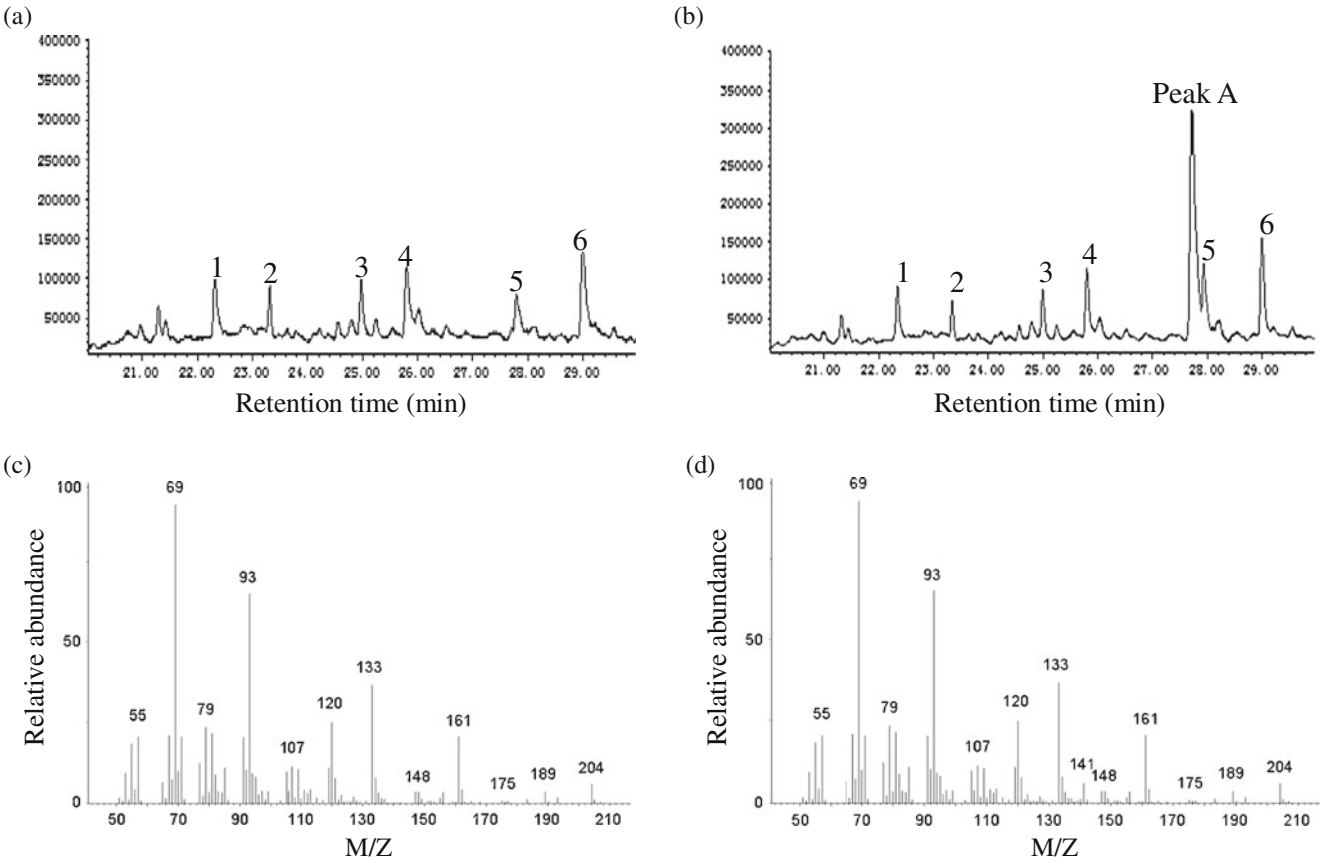

Fig. 2 GC-MS profile of volatiles emitted by transgenic tobacco plants. **a** The volatiles from nontransgenic control plants. **b** The volatiles from transgenic plants. The compounds were as follows: *1* tridecane, *2* and *6* siloxane contaminant, *3* and *5* unknown, *4* tetradecane, *peak A* EβF. **c** Mass spectrum of peak A. **d** Mass spectrum of authentic EβF

EβF emission level of the independent transgenic lines. Furthermore, the effect of transgenic plants on the foraging behavior of predators was also tested. Lacewings are effective predators of aphids because of their predacious habit and abundance in many agro-ecosystems in North China. Hence, lacewing larvae were selected for predator bioassay. Excitingly, *AaβFS1* and *AaβFS2* lines exhibited strong attraction to lacewings (Supplementary Fig. S1c). The number of lacewings that stayed on transgenic tobacco plants increased at all the four selected time points; the increase of lacewings that stayed on lines S1-3-4 and S1-4-5 reached a significant level at 30 min ($P<0.05$) and 45 min only for S1-4-5 line ($P<0.05$), demonstrating the potential function of *AaβFS1* and *AaβFS2* genes in indirect plant aphid defense. Then, 400 alate aphids and ten lacewings were simultaneously introduced into the setup and 12 h later, the number of aphids was reduced by approximately 16.4% in S1-3-4, 23.6% in S1-4-5 ($P<0.05$), 10.9% in S2-1-2, and 20.0% in S2-3-6, respectively, with 28.3% aphids displaying no choice and staying on the net cover (Supplementary Fig. S1d). In summary, *AaβFS1* and *AaβFS2* transgenic lines showed a pleiotropic effect on aphid behaviors, especially strong attraction to aphid predators, with one transgenic line S1-4-5 reached at significant level. EβF manipulation in tobacco plants here preliminarily presented an alternative strategy for aphid control.

Discussion

Metabolic engineering of terpenoids in plants could be an important alternative strategy for aphid control

To date, metabolic engineering of terpenoids in plants has met with some success, transgenic plants producing special mono-, sesqui-, and diterpenes could be served as an alternative tool for improving insect pest control. For example, *P450*-suppressed transgenic tobacco plants producing higher levels of diterpene cembratriene-ol could greatly diminish aphid colonization (Wang et al. 2001). Aphid infestation was minimized on transgenic *Arabidopsis* plants producing monoterpene linalool or sesquiterpene EβF (Aharoni et al. 2003; Beale et al. 2006). On the other hand, biological control methods are often considered to be alternatives to synthetic insecticides in control of pests; in this context, natural enemies of pests show great promise to limit crop damage in an environmentally safe manner (Degenhardt et al. 2003). For example, to enlist the natural enemies of pests in plant defense, Kappers et al. (2005) engineered *Arabidopsis* plants emitting (*E*)-DMNT, the homoterpene derivative of nerolidol. Transgenic plants significantly attracted carnivorous predatory mites (*Phytoseiulus persimilis*), the natural enemies of spider mites. Recent studies also showed that the transgenic *Arabidopsis* plants emitting EβF or TPS10 sesquiterpenes (containing EβF) attracted aphid parasitic wasp *Diaeretiella rapae* and *Cotesia marginiventris*, respectively (Beale et al. 2006; Schnee et al. 2006). Although lower EβF emission level from transgenic tobacco plants was observed in this study compared with that of transgenic *Arabidopsis* with black peppermint, EβF synthase gene described by Beale et al. (2006), the transgenic lines exhibited repellence to aphid and attraction to lacewing larvae which are more predacious on aphids than the adult ones (http://www.virginiafruit.ento.vt.edu/lacewings.html), especially after they were starved for 6 h, indicating that transgenic tobacco plants consecutively emitting fresh EβF could effectively recruit biological control agents (such as lacewing) to decrease density of aphids (Supplementary Fig. S 1c, d). However, Zhu et al. (1999) observed that lacewings did show a strong electroantennographic response to EβF but not successfully trapping lacewing adults in the field. The reason for this phenomenon might lie in the fact that EβF had high volatility and a short atmospheric life time (Bouvier-Brown et al. 2009), which might affect the lacewing trapping result.

The supply of FPP substrate might be a limiting factor in managing sesquiterpenes synthesis by genetic manipulation

In this study, we transferred *AaβFS1* and *AaβFS2* into tobacco under the control of a constitutive cauliflower mosaic virus 35S promoter and high level of EβF synthase was produced in transgenic lines as observed in SDS-PAGE and western blot analysis (Fig. 1d, e), but the level of EβF emission was relatively low and the nonlinear correlation between the EβF emission and *AaβFS1*/*AaβFS2* expression level indicated that the availability of the precursor (FPP) supply might be a major limiting factor in the biosynthesis of sesquiterpenes in tobacco plants.

In order to obtain enough of the expected sesquiterpenes, the following two strategies may be worth taken into account. Firstly, the availability of precursors plays an important role in plant sesquiterpene engineering, so this must be considered in the whole context of the pathway level, including the precursor synthesis and supply rather than single-gene engineering strategies. 3-Hydroxy-3-methylglutaryl CoA reductase (HMGR) catalyzes the irreversible conversion of HMG-CoA to mevalonate and FPP synthase catalyzes the synthesis of FPP from isopentenyl diphosphate and dimethylallyl diphosphate, these two enzymes have been proposed to be key regulatory step controlling isoprenoid metabolism (Cunillera et al. 1997). Coupled overexpression of a patchoulol synthase (PTS) with avian FPPs increased sesquiterpene patchoulol accumulation two- to fivefold

higher level over that of plants harboring only PTS, and this could be further augmented by another two- to sixfold by the concomitant overexpression of a truncated HMGR (Wu et al. 2006). Furthermore, sesquiterpene biosynthetic pathway could also be redirected from its natural cytosolic location to the plastids such as mitochondria and chloroplast. Wu et al. (2006) engineered high level of sesquiterpene production in tobacco plants by overexpressing both of an avian farnesyl diphosphate synthase and an appropriate sesquiterpene synthase in chloroplast, the transgenic plants could increase the synthesis of the sesquiterpenes patchoulol and amorpha-4, 11-diene more than 1,000-fold.

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