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Aphid-repellent pheromone E-β-farnesene is generated in transgenic *Arabidopsis thaliana* over-expressing *farnesyl diphosphate synthase2*

Varnika Bhatia[1], Jaya Maisnam[2], Ajay Jain[1], Krishan Kumar Sharma[2] and Ramcharan Bhattacharya[1,]*

[1]National Research Center on Plant Biotechnology, Indian Agricultural Research Institute Campus, New Delhi-110012, India and [2]All India Network Project on Pesticide Residues, Indian Agricultural Research Institute, New Delhi-110012, India
** For correspondence. E-mail rcb@nrcpb.org or rcbhattacharya1@gmail.com*

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• **Background and Aims** Plant-synthesized sesquiterpenes play a pivotal role in chemotactic interactions with insects. Biosynthesis of functionally diverse sesquiterpenes is dependent on the availability of a pool of the precursor farnesyldiphosphate (FDP). In *Arabidopsis thaliana*, *FPS2*, encoding cytosolic farnesyldiphosphate synthase, is implicated in the synthesis of cytosolic FDP, but it is not known whether enhanced levels of FDP have a commensurate effect on sesquiterpene-mediated defence responses. This study examined transgenic arabidopsis plants generated to over-express *FPS2* in order to determine if any effects could be observed in the response of aphids, *Myzus persicae*.
• **Methods** Transgenic arabidopsis plants were generated to over-express *FPS2* to produce FPS2 in either the cytosol or the chloroplasts. Morphochemical analyses of the transgenic plants were carried out to detremine growth responses of roots and shoots, and for GC-MS profiling of sesquiterpenes. Aphid response to hyrdo-distillate extracts and head-space volatiles from transgenic plants was assessed using a bioassay.
• **Key Results** Either over-expression of *FPS2* in the cytosol or targetting of its translated product to chlorplasts resulted in stimulatory growth responses of transgenic arabidopsis at early and late developmental stages. GC-MS analysis of hydro-distillate extracts from aerial parts of the plants revealed biosynthesis of several novel sesquiterpenes, including E-β-farnesene, an alarm pheromone of aphids. Both entrapped volatiles and hydro-distillate extracts of the transgenic leaves triggered agitation in aphids, which was related to both time and dose of exposure.
• **Conclusions** Over-expression of *FPS2* in the cytosol and targeting of its translated product to chloroplasts in arabidopsis led to synthesis of several novel sesquiterpenes, including E-β-farnesene, and induced alarm responses in *M. persicae*. The results suggest a potential for engineering aphid-resistant strains of arabidopsis.

Key words: *Arabidopsis thaliana*, *farnesyl diphosphate synthase2*, sesquiterpenes, E-β-farnesene, alarm pheromone, plant defence, plant–insect interactions, aphid resistance, transgenics.

INTRODUCTION

Insects identify their specific hosts largely by visual and/or olfactory cues that facilitate positive chemotactic responses and accurate landing on the host (Pickett *et al.*, 2006; Chittka and Döring, 2007). Olfactory cues comprise an array of volatile compounds including a prominent group of terpenes [isoprene (C5), monoterpenes (C10), sesquiterpenes (C15) and homoterpenes (C11, C16)] that are synthesized in glandular trichomes or epidermal cells of host plants (Wang *et al.*, 2008; Glas *et al.*, 2012). Many of the compounds are often released by plants to defend themselves from attack by pest-herbivores either by conferring direct antixenosis or by attracting predators and parasites that attack these herbivores (Kessler and Baldwin, 2001; Degenhardt *et al.*, 2009).

The available substrate pool shared by a diverse group of terpene synthases is pivotal for generating plant-specific terpenoids (Negre *et al.*, 2003; Boatright *et al.*, 2004; Pott *et al.*, 2004). Farnesyldiphosphate (FDP), a common substrate for the biosynthesis of an array of sesquiterpenes and precursors of di-, tri- and tetra-terpenes in lower and higher organisms, is generated by the cytosolic mevalonic acid (MVA) pathway (Lombard and Moreira, 2011). The distribution of cytosolic FDP to various downstream terpene biosynthetic pathways is governed by the relative substrate affinity of diverse terpene synthases. Interestingly, Nabeta *et al.* (1997) demonstrated an innate ability of liverwort cell cultures to transport cytosolic FDP into chloroplasts for the synthesis of carotenoids and chlorophylls. Whether a similar process occurs in higher plants remains a matter of conjecture and thus warrants detailed investigations. By contrast, the occurrence of the plastidic methyl-erythritol phosphate (MEP) pathway for generating geranyl-geranyl-pyrophosphate (GGPP), a common substrate for mono- and di-terpenes, is a well-documented phenomenon in lower and higher organisms (Schwender *et al.*, 1996, 1997; Rohmer, 1999; Hemmerlin *et al.*, 2012).

Terpene synthase engineering has been considered as a viable approach for developing plant-based terpene production platforms and for enhancing a plant's repellence as well as indirect defence to pest-insects (Beale *et al.*, 2006; Schnee *et al.*, 2006; Bleeker *et al.*, 2012; Nagel *et al.*, 2014). These strategies

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invariably resulted in accumulation of low levels of isoprenoid end products, thereby highlighting the lack of availability of carbon flux as substrates (Aharoni *et al*., 2003; Yu *et al*., 2013). Therefore, an increase in steady-state levels of FDP in cytosol and also its putative targeting to terpene-synthesizing organelles could potentially lead to either enhancement in terpene biosynthesis or the generation of novel sesquiterpenes in the volatile profile. In fact, an appreciable increase in the end product was observed upon supplementation of specific terpene synthases with biosynthetic enzymes, generating the substrate in both the cytosol and chloroplast (Wu *et al*., 2006). Therefore, it could be assumed that engineering genes in isoprenoid pathways could potentially result in enhanced production of C15-derived carbon flux.

In *Arabidopsis thaliana* (arabidopsis), *FPS1* (At5g47770.1) and *FPS2* (At4g17190), encoding cytosolic FDP synthase, showed differential expression patterns and have been implicated in the biosynthesis of FDP (Cunillera *et al*., 1996; Keim *et al*., 2012). However, neither of these two genes is expressed in the arabidopsis chloroplast. Over-expression of *FPS1*, which has a predominant role in almost all stages of the arabidopsis life cycle, triggered premature senescence, but this phenotype could be reverted by an application of mevalonic acid or 2-iP (Masferrer *et al*., 2002; Closa *et al*., 2010). However, it is not evident from these studies whether an enhanced level of FDP due to over-expression of *FPS1* altered the profile of downstream sesquiterpenes and/or exerted any effect on the responses of the transgenic plants to biotic stress. Neither there is any report on the effects of over-expressing cytosolic *FPS2* and/or its targeting to the chloroplast in transgenic arabidopsis with respect to responses toward insect pests and, in particular, aphids.

We report here on the generation of transgenic arabidopsis plants that either over-expressed *FPS2* in the cytosol or targeted the translated product to the chloroplast by a transit peptide. Compared with the wild-type, both the transgenic plants showed enhanced growth responses and their GC-MS profiles revealed the presence of several novel sesquiterpenes, including E-β-farnesene (E-β-f) which is an alarm pheromone for aphids. The consequent effects of an altered volatile profile of these transgenic plants on host–aphid interactions are discussed.

MATERIALS AND METHODS

Arabidopsis thaliana ecotype Columbia (Col-0) and a green peach aphid (*Myzus persicae*) colony reared on 3-week-old arabidopsis plants were grown under controlled growth conditions (16-h photoperiod, 22 °C day–night temperature and 75 % relative humidity).

Binary vectors and plant transformation

For generating constructs for cytoplasmic over-expression of *FPS2* under the constitutive promter (CaMV35S:*FPS2*), the *FPS2* sequence (At4g17190) was amplified from arabidopsis by PCR using gene-specific primers (FPS attB1_F and FPS attB2_R), on to which an 'att' sequence was added at their 5′ ends (Supplementary Data, Table S1). The purified amplicon was introduced into pDONR 221 entry vector of the Gateway system (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* DH5α electrocompetent cells. Among several recombinant clones identified through PCR and for which their fidelity had been validated by sequencing, one of them (hereafter the *FPS2* entry clone) was recombined into pEarleyGate100 destination vector (Earley *et al*., 2006) to generate CaMV35S:*FPS2*. For targeting *FPS2*-translated product into plastids, the construct (CaMV35S:Tp-FPS2) was generated by insertion of a transit peptide (Tp) sequence from *Brassica juncea* (GenBank accession number: FJ154097) upstream of the *FPS2* coding sequence with a *Kpn*I site inserted by PCR using primers TP_FP and TP_RP (Table S1). Subsequently, the entry clone was recombined into pEarleyGate100 destination vector to generate CaMV35S:Tp-*FPS2*. Several of these clones were sequenced to confirm the correct reading frame. The BioEdit program and SignalP 3.0 server were employed to validate the correct reading frame of the translation and the cleavage site of the transit peptide, respectively. The two constructs (CaMV35S:*FPS2* and CaMV35S:Tp-*FPS2*) were mobilized into *Agrobacterium tumeifaciens* (strain GV 3101) by electroporation. Floral dip transformation was carried out as described by Clough and Bent (1998). For selection of transgenic plants, the transformed seeds (T_0) of arabidopsis were sown on Petri dishes containing MS agar medium supplemented with glufosinate ammonium (6 μg mL^{-1}). Several transgenic plants were recovered with an average transformation efficiency of 5·8 %.

Analyses of morphological characters

Wild-type and transgenic plants, grown for 2–3 weeks under controlled conditions, were evaluated for their height, numbers of branches and siliques per plant. For determination of dry weight, 4-week-old plants were uprooted, and their roots were washed with water, blot-dried and dried to a constant weight at 80 °C in hot air oven. For revealing root system architecture (RSA), seedlings were grown on vertically orientated Petri dishes containing full-strength MS medium + 0·8 % (w/v) agar for 7 d. Roots were excised at the hypocotyl–root junction, gently spread to reveal RSA, and scanned at 600 d.p.i. (HP Scanjet G2410). Rosette leaves were dissected from shoots and scanned at 600 d.p.i.; ImageJ (Collins, 2007; http://rsb.info.nih.gov/ij) was used to measure total shoot area.

Estimation of chlorophyll content

Leaves (500 mg) from 2-week-old plants were ground in 1 ml of 80 % (v/v) acetone containing a pinch of calcium carbonate to minimize the action of chlorophyllase. The homogenate was centrifuged at 5000 r.p.m. (Thermo Scientific, Waltham, MA, USA) for 5 min. The supernatant was collected and the pellet was subjected to pigment extraction repeatedly in acetone until it appeared colourless. The extracts were pooled and the volume was recorded. Chlorophyll a [12·7 (A_{663}) − 2·69 (A_{645})], chlorophyll b [22·9 (A_{645}) − 4·68 (A_{663}) and total chlorophyll [20·2 (A_{645}) + 8·02 (A_{663})] were quantified as described by Mackinney (1941).

ICP-MS analysis

The whole plant was washed thoroughly with sterile deionized water, blot-dried and then air-dried for 2–3 d at 60 °C in a

hot air oven. Dried samples (approx. 25 mg) were transferred to Pyrex tubes and digested overnight with concentrated HNO_3 (3 mL) at 80 °C. The Pyrex tubes were capped with marbles during digestion. Digested sample was diluted to 15 mL with deionized water and the concentrations of macronutrients (N, P, K) and micronutrients (Zn, Fe, Mn, Cu) were analysed along with indium as an internal standard using inductively coupled plasma optical emission spectroscopy (iCAP 6500 Series ICP Emission Spectrometer, Thermo Fisher Scientific). National Institute of Standards and Technology traceable calibration standards were used for the calibration.

Molecular analyses

Genomic DNA was isolated from leaves of the transgenic lines using DNAzol (Molecular Research Center, Inc., Cincinnatti, OH, USA). PCR analysis of genomic DNA was performed using primers specific for the selectable marker gene BASTA (BASTA_1F and BASTA_1R). Genomic DNA from untransformed arabidopsis plants and plasmid DNA of the recombinant binary vector were used as negative and positive controls, respectively. Semi-quantitative reverse transcriptase PCR (RT-PCR) of the transgenic lines was performed with *FPS2* gene-specific primers (FPS_1F and FPS_1R). *Tubulin* (At5g62690) was used as an internal control to ensure an equal amount of cDNA for each reaction. The cycle number for each of the transcripts was optimized in PCR to ensure a linear response in amplification. Table S1 provides a list of primers.

Extraction of hydro-distillate

Fresh plant tissues (approx. 250 g) comprising leaves, flowers and stems were suspended in approx. 3 volumes of deionozed water, heated at 70 °C and refluxed for 5–6 h in a Clavenger apparatus. Condensed vapour formed an emulsion with water which was then extracted with n-hexane for extraction of hydro-distillate.

GC-MS analysis

Extracted hydro-distillate was identified in select ion mode GC-MS with a Quadrupole mass analyser in electron ionization, at 70 eV ionization energy (Shimadzu GC-2010 equipped with an AOC-20i autoinjector coupled with an MS-QP 2010 mass detector). This analysed the mass and scanned at up to 10 000 μs^{-1}. The capillary column used was a non-polar stationary phase DB-1 of 30 m, 0·25 mm i.d., 0·25 μm film thickness (J&W Scientific, Agilent, Santa Clara, CA, USA). The column oven temperature was programmed at 70 °C (4-min hold) followed by ramp of 3 °C min^{-1} to 200 °C, forming a chromatographic run of 52·33 min. The injection port temperature was kept at 250 °C. The ion source and MS interphase temperature were 250 and 280 °C, respectively. The total helium gas (mobile phase) flow rate was 40·2 ml min^{-1} and the column flow was maintained at 0·91 ml min^{-1}. An aliquot of 1 μL was injected in splitless mode for 2 min followed by split mode with a split ratio of 1 : 40. The detector voltage was 1 kV in absolute mode in the autotuning report. The analytes were identified on the basis of their retention times and by comparison of their mass spectra with respect to the National Institute of Standards and Technology library (Shimadzu, Kyoto, Japan). Analytical-grade E-β-f (Sigma-Aldrich, St Louis, MO, USA) was used for authentication and quantification of E-β-f in the samples. For quantification, a standard curve was developed by plotting the peak areas generated by different concentrations of E-β-f standard. A linear relationship ($y = 8 \times 10^6 x$) with a correlation coefficient of $r^2 = 0{\cdot}999$ was obtained. The amount of E-β-f in hydro-distillates was determined by extrapolating the peak area on the standard curve.

Aphid behavioural assay with hydro-distillate extract

A custom-designed glass tube apparatus was employed for an aphid behavioural assay with hydro-distillate extract (Supplementary Data Fig. S1). Hydro-distillate extract (100 μL) in n-hexane from control and different transgenic lines was pipetted on a cotton ball and 50 *M. persicae* nymphs were exposed to air flow emanating from it. For full details see Fig. S1. The number of aphids showing agitation upon exposure to volatiles was recorded at an interval of 2 min until the time when no perceptible agitation was observed. The control assays were also performed by exposing nymphs either only to the air stream or the one saturated with n-hexane vapours.

Aphid behavioural assay with head-space volatiles

Head-space volatiles from 3-week-old plants (three plants per 4-inch pot) were entrapped in a customized flat-bottomed bell-jar type glass apparatus (6·5 × 18 inch) sealed with petroleum jelly and maintained under controlled conditions (22 °C, 75 % relative humidity and 16-h photoperiod) for 40 h (Supplementary Data Fig. S1). Initially, 100 nymphs (3–5 d old) were allowed to acclimatize for 30 min in tube A under a charcoal-filtered air stream passed at a rate of 0·5 mmHg through the apparatus. Subsequently, head-space volatiles was administered on the aphid nymphs by connecting the outlet tap (T4) and the inlet tap (T5) of the bell-jar apparatus to tube B and tube X, respectively. The number of aphids that showed perceptible agitations were recorded at intervals of 5 min for up to 40 min. The entire set up of the glass tube apparatus was flushed with charcoal-filtered air before analyses of each sample. The actual percentage agitation was computed by subtracting the percentage agitation of the transgenic plant from that of the control plant and is represented as per gram dry weight of transgenic tissue.

Statistics

For each experiment, data are presented from two independent biological experiments with 2–3 technical replicates each. The statistical significance of differences between mean values was determined using Student's *t* test. Different letters on bar charts were used for indicating means that were statistically different at $P < 0{\cdot}05$.

RESULTS

Generation of cytosolic and plastidic FPS2 *transgenic plants and their morphometric analysis*

Transgenic arabidopsis plants over-expressing *FPS2* were generated through *Agrobacterium*-mediated incorporation of the two chimeric constructs CaMV35S:*FPS2* for cytosolic localization of FPS2 and CaMV35S:Tp-FPS for chloroplast-targeting of the FPS2 protein (hereafter cytosolic and plastidic transgenics, respectively). Based on segregation analysis of BASTA resistance traits in conjuction with PCR and RT-PCR analyses, several T_2 homozygous lines for each of the cytosolic and plastidic constructs were recovered. Segregation data indicated probable single copy transgene integration in cytosolic (Cy3T, Cy11T and Cy12T) and plastidic (Pl3T, Pl8T and Pl9T) transgenic lines. For determination of variations, if any, in various developmentally regulated morphometric traits in both the transgenics compared with their respective controls, they were grown for 2–3 weeks under controlled conditions and also up to maturity under greenhouse conditions (Fig. 1). There were significant ($P < 0{\cdot}05$) increases in the total number of leaves in the shoots of different lines of both the transgenics compared with their respective controls (Fig. 1A, B), which was also reflected in a commensurate increment in their shoot areas (Fig. 1C). As anticipated, there were also noticeable variations across different lines of both cytosolic and plastidic transgenic lines. Interestingly, there were significant ($P < 0{\cdot}05$) increases in total chlorophyll contents of these transgenic lines compared with their respective controls (data not shown). To determine whether the elevated chlorophyll content in these transgenics exerted any commensurate effect on their carbon fixation ability, we further assayed the transcript levels of genes encoding phosphoglycerate kinase (*PGK1*; *At3g12780*), glyceraldehyde-3-phosphate dehydrogenase A (*GAPA*; *At3g26650.1*) and glyceraldehyde-3-phosphate dehydrogenase B (*GAPB*; *At1g42970.1*). Levels of transcripts for these genes were comparable across different transgenic lines and their respective controls. Further studies are warranted to determine the probable causes for increased shoot area and chlorophyll content in these transgenic lines. As increased shoot area of these transgenic plants suggested a possible shift in the source–sink relationship, an effect on their root growth was assumed. As anticipated, there were apparant accentuated growth responses of the root system in different lines of both transgenics (Fig. 1D, E). In addition, transgenics and their respective controls were also grown to maturity for up to 4 weeks under greenhouse conditions to compare differences, if any, in different morphometric traits (Fig. 1F–I). Compared with their respective controls, different lines of both the transgenic plants exhibited significant ($P < 0{\cdot}05$) increases in the values of these traits. The results thus provided conclusive evidence towards stimulatory effects of over-expressing *FPS2* on growth responses of the transgenics. However, there were no consistent differences in growth responses of the transgenics over-expressing *FPS2* in the cytosol or its product being targeted to plastids.

Expression analyses of transgenic lines

Levels of *FPS2* transcripts were determined in control and transgenic plants by semi-quantitative RT-PCR and estimation of integrated density values (IDVs) of their amplicons (Fig. 2). Higher levels of *FPS2* transcript accumulation in different transgenic (cytosolic and plastidic) lines compared with their respective controls were evident both by semi-quantitative RT-PCR (Fig. 2A) and by their respective IDVs (Fig. 2B). Variations in transcript levels were also apparent across the independent lines of both the transgenics (Fig. 2). A similar trend in the variability of transgene expression was also observed by semi-quantitative RT-PCR using BASTA-specific primers (data not shown). Therefore, the observed variations in the expression of *FPS2* across different transgenic lines could be attributed to a positional effect of transgene integration.

Characterization of volatile terpene profile of transgenic plants

The hydro-distillate extracts from the aerial parts of 3-week-old transgenic (cytosolic and plastidic) plants and their respective controls were analysed by GC-MS for the detection of, if any, novel sesquiterpenes (Fig. 3). The analysis revealed several novel sesquiterpenes ($C_{15}H_{24}$) in both the transgenics (Table 1, Fig. 3A–C). Among these, E-β-f, β-sesquiphellandrene and α-zingiberene were detected in all the transgenic lines. Although epi-bicyclosesquiphellandrene, β-bisabolene and ϒ-cadinene were detected in all the plastidic transgenic lines, they were not detected uniformly across different cytoplasmic transgenic lines. Furthermore, Z-β-farnesene was detected only in plastidic lines. The study clearly suggested differential effects of manipulating expression of *FPS2* on the biosynthesis of sesquiterpenes. The lack of detection of a few sesquiterpenes across all the cytopasmic lines remains enigmatic and warrants further investigation. Interestingly, among the sesquiterpenes detected in transgenics, E-β-f has been well documented as an alarm pheromone and induces a flee response in aphids (Beale *et al.*, 2006; Yu *et al.*, 2013). Therefore, we subsequently validated the fidelity of the detected E-β-f in the transgenics by comparing its mass spectrum with that of the reference standard (Fig. 3D, E), and quantified it in hydro-distillate extracts from all the transgenic lines (Fig. 3F). As anticipated, there were significant variations in the level of E-β-f across the hydro-distillate extracts from different transgenic lines. The results indicate significant effects of intrinsic genetic variability on the responses of the manipulated gene across the transgenics.

Aphid behavioural response on exposure to E-β-f standard

Aphid nymphs were exposed temporally (2, 4, 6, 8, 10 and 12 min) to different concentrations of E-β-f ($0{\cdot}05 \times 10^{-2}$ to 10 p.p.m.) to determine the temporal and dose-dependent responses of aphids (Table 2). At $0{\cdot}05 \times 10^{-2}$ p.p.m., with an increase in exposure time up to 8 min there was a consistent and commensurate increment in percentage agitations of aphids that peaked at 6·66 %. With an increase in the concentration of E-β-f, there was a gradual reduction in the time required to achieve optimal agitation by aphids. The maximum agitation (33·3 %) by aphids was recorded during exposure at 10 p.p.m. for 12 min. The results thus clearly suggested that trigerring of the agitation response by aphid nymphs is dependent on both

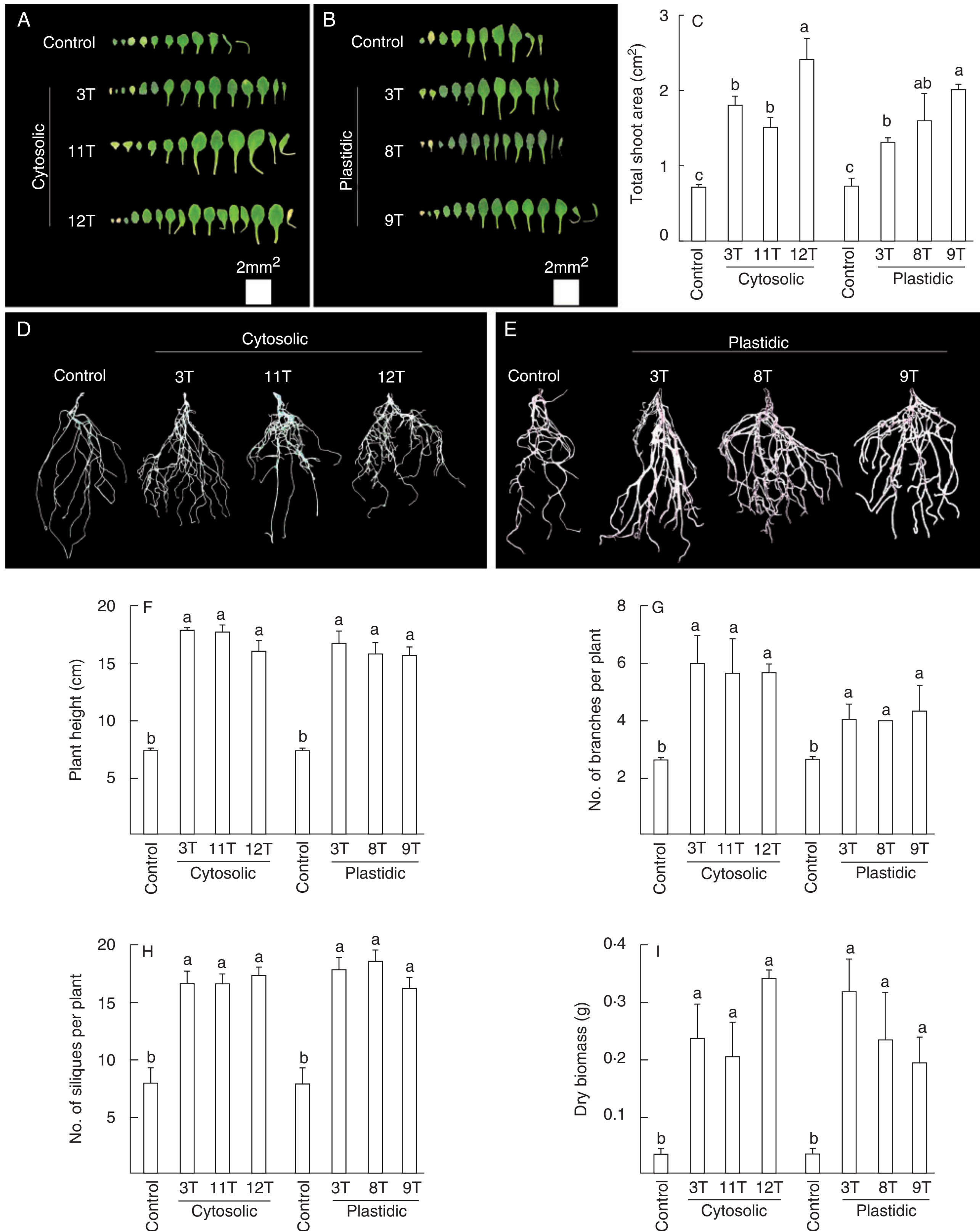

FIG. 1. Morphometric analyses of transgenics. Cytosolic and plastidic transgenics were grown under controlled conditions on (A–E) vertically orientated agar Petri dishes for 2–3 weeks, and (F–I) up to maturity. Seedlings grown on agar Petri dishes were dissected at the root–shoot junction for displaying all the dissected leaves from shoots of control and transgenics (A, B), their total shoot area (C) and root system (D, E). Mature plants were scored for plant height (F), number of branches per plant (G), number of siliques per plant (H) and dry biomass (I). Data presented are mean ± s.e. ($n = 3$), and different lower-case letters on bars indicate that values are statistically significant ($P < 0{\cdot}05$).

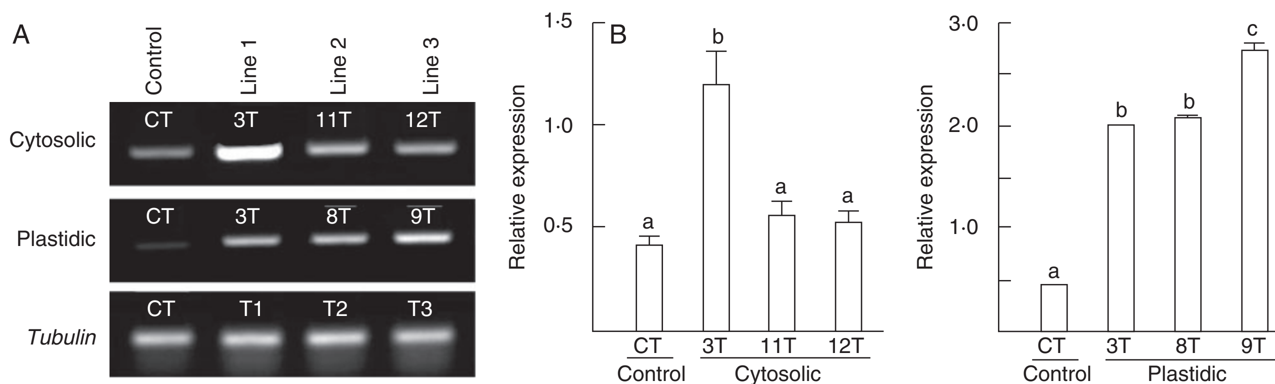

FIG. 2. Expression analysis of *FPS2* in the transgenics. (A) Total RNA (5 µg) from leaves of cytosolic and plastidic transgenic lines, grown to maturity under controlled conditions, was used for synthesis of cDNA and PCR amplified with *FPS2*-specific primers. *Tubulin* was used as an internal control, showing similar RNA concentrations in different samples. (B) Relative expression of *FPS2* in control and transgenic samples was computed from IDVs of the PCR products.

the concentration of E-β-f and the duration for which it is exposed.

Aphid behavioural study in response to hydro-distillate of the transgenics

Hydro-distillate extracts from the aerial parts of 3-week-old transgenics and respective control plants were used to determine whether the hydro-distillate triggers any agitation in the aphids when exposed to its vapour for different time intervals (Fig. 4). Interestingly, noticeable agitation (5·3–9·3 %) was observed when the aphids were exposed to either cytoplasmic or plastidic hydro-distillate extracts for 6 min. There were significant ($P < 0·05$) increases in percentage agitation by the aphids upon exposure to these transgenic extracts for longer durations (10 and 14 min). As anticipated, there was no perceptible agitation by the aphids when they were exposed to the hydro-distillate extract from the control plants for 6, 10 and 14 min. Overall the hydro-distillate extracts from all six transgenics showed the ability, albeit with some variations, to trigger agitation by the aphids in a temporal manner.

Effect of entrapped volatile terpenes from the transgenics on aphid nymphs

One hundred *M. persicae* nymphs were exposed to the entrapped volatiles from the headspaces of 3-week-old control, cytoplasmic and plastidic transgenic plants for 20 and 35 min to determine if they triggered any agitation (Fig. 5). The entrapped volatiles from the transgenic lines induced a detectable and significant agitation in feeding nymphs in contrast to their respective control plants. The number of aphids showing agitation increased with increasing duration of exposure in all the transgenic lines and were significantly higher compared with their control plants at each time point. However, the responses induced by different transgenic plants were variable and paralleled the differential level of E-β-f detected in these transgenic lines. The maximum agitation of 30 % by the aphids was observed when they were exposed for 20 min to the entrapped volatile terpenes from cytoplasmic transgenic line 11T. The relative percentage agitation by the aphids was significantly lower when exposed for same duration to the entrapped volatile terpenes from cytoplasmic transgenic line 3T. By contrast, the entrapped volatile terpenes from cytoplasmic transgenic line 12T induced only 5·59 % agitation by the aphids. Likewise, a variable response in agitation (12·18–19·9 %) by the aphids was evident when exposed to the entrapped volatile terpenes from different plastidic transgenic lines. Although there were significant increases in percentage agitation by the aphids upon longer exposure for 35 min to entrapped volatiles from different transgenic lines, the variable trend across them remained the same.

DISCUSSION

In the MVA pathway, FDP supplies carbon flux to an array of sesquiterpenes ($C_{15}H_{24}$) exhibiting significant variations in their structure and stereochemistry that consequently governs their biological functions (Lange and Ahkami, 2013; Yeo *et al*., 2013). In arabidopsis, *FPS1* and *FPS2* encode isoforms of FDP synthase that are involved in the generation of FDP in cytosolic and mitochondrial branches of isoprenoid biosynthesis (Cunillera *et al*., 1997; Masferrer *et al*., 2002), and are required for growth and development, defence responses, and signal transduction (Lange and Ghassemian, 2003; Bouvier *et al*., 2005; Crowell and Huizinga, 2013). Therefore, manipulating expression levels of *FPS1* and/or *FPS2* could be an attractive paradigm for increasing the FDP pool and its consequent effects on specific isoprenoid end products. In this context, transgenic arabidopsis plants over-expressing *FPS1* were generated to determine its contribution to the FDP pool in the MVA pathway (Masferrer *et al*., 2002). Contrary to expectation, the study instead revealed compromisation of the upstream cytokinin biosynthetic pathway that triggered a premature cell death/senescence-like phonotype. By contrast, loss-of-function mutants of *FPS1* and *FPS2* revealed that either of the two is sufficient to support isoprenoid biosynthesis for normal growth and development (Closa *et al*., 2010). However, it is not apparent from studies on transgenics over-expressing *FPS1* or loss-of-function mutants of *FPS1* and *FPS*2 whether they have any alterations in the profile of sesquiterpenes and/or responses to

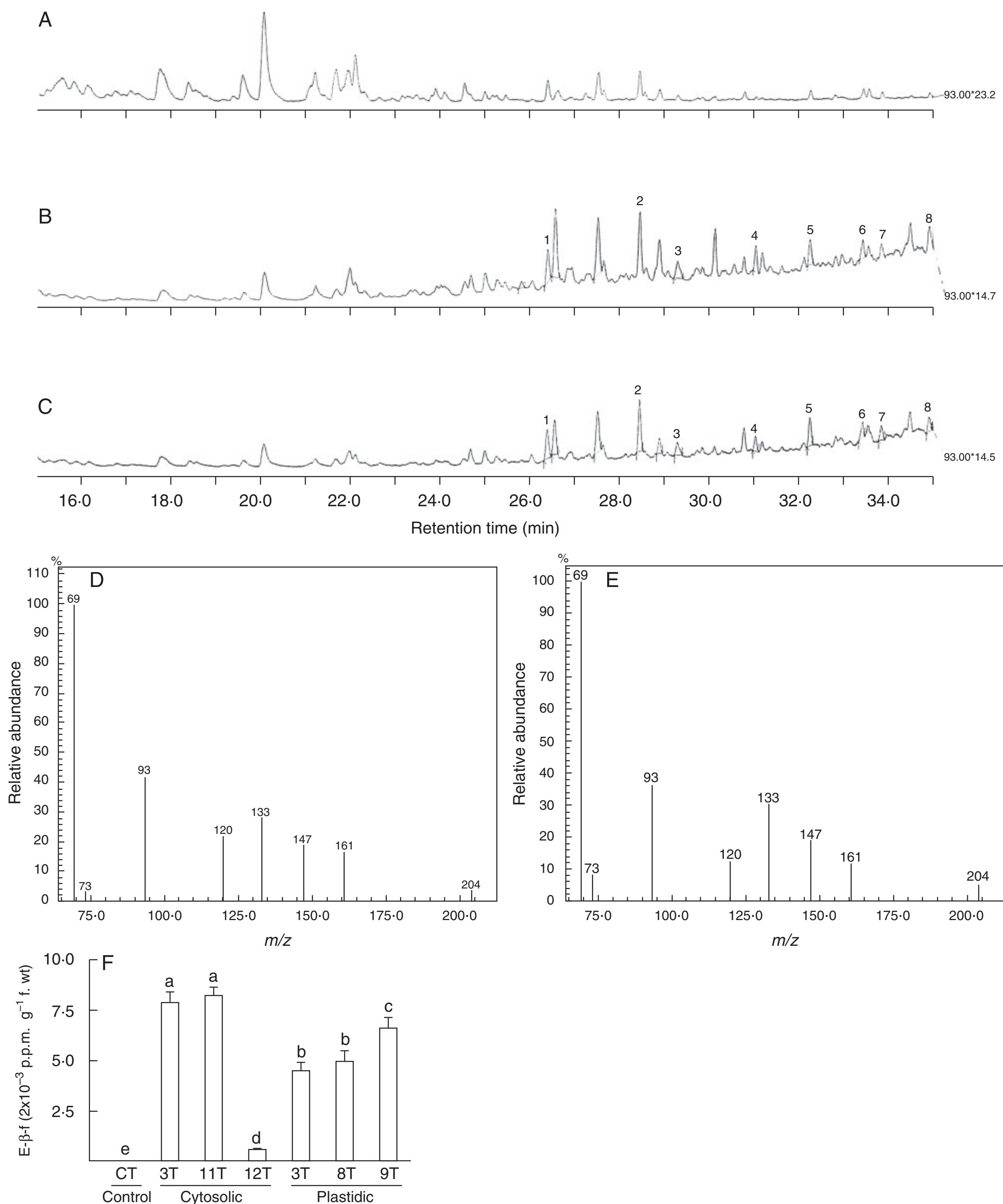

FIG. 3. GC-MS chromatograms of sesquiterpenes. Representative GC-MS chromatograms of hydro-distillate extract of 3-week-old control (A), cytosolic [Cy11T] (B) and plastidic [Pl9T] (C) transgenic lines. Several novel sesquiterpenes ($C_{15}H_{24}$) were specifically detected in both the transgenics, seven of which could be identified by comparison of their retention times and mass spectra in accordance with the National Institute of Standards and Technology library, and are indicated by numbers on the representative peaks: 1, E-β-farnesene (E-β-f); 2, 5, β-sesquiphellandrene; 3, epi-bicyclosesquiphellandrene; 4, β-bisabolene; 6, α-zingiberene; 7, ϒ-cadinene; 8, Z-β-farnesene. Mass spectrum of E-β-f in cytosolic [Cy11T] (D) and plastidic [Pl9T] (E) transgenic lines. (F) Quantification of E-β-f in control and transgenic plants. Values represent mean ± s.e. ($n = 3$). Different lower-case letters on bars indicate significantly ($P < 0{\cdot}05$) different values.

TABLE 1. *Novel sesquiterpenes identified in GC-MS chromatograms of the transgenic lines*

Sesquiterpene	Peak no.	Retention time (min)	Peak area (%)					
			Cytosolic transgenics			Plastidic transgenics		
			3T	11T	12T	3T	8T	9T
E-β-farnesene	1	26·4	11·4	12·5	10·4	11·2	13·4	11·4
β-Sesquiphellandrene	2	28·4	19·3	9·3	18·1	20·1	22·8	26·8
	5	32·2						
α-Zingiberene	6	33·4	5·0	6·5	3·6	7·3	4·5	5·5
epi-Bicyclosesquiphellandrene	3	29·2	ND	9·3	8·3	6·2	7·6	6·0
β-Bisabolene	4	31·1	6·4	8·5	ND	4·5	8·1	4·9
ϒ-cadinene	7	33·8	ND	ND	5·8	6·2	4·4	5·2
Z-β-farnesene	8	34·9	ND	ND	ND	4·4	4·8	5·2

None of the listed sesquiterpenes was detected in the respective control plants. ND, not detected.

TABLE 2. *Temporal agitation response of aphid nymphs to different concentrations of E-β-farnesene*

Serial no.	E-β-f in farnesene standard	No. of aphids agitated from a total of 100 (mean ± s.e.)					
		2 min	4 min	6 min	8 min	10 min	12 min
1.	$0{\cdot}05 \times 10^{-2}$ p.p.m.	1·11 ± 1·11	2·22 ± 1·11	5·55 ± 1·11	6·66 ± 0	6·66 ± 0	6·66 ± 0
2.	$0{\cdot}5 \times 10^{-2}$ p.p.m.	5·55 ± 1.11	7·77 ± 1·11	10 ± 0	10 ± 0	10 ± 0	11·11 ± 1·11
3.	5×10^{-2} p.p.m.	7·77 ± 1·11	11·11 ± 1·11	13·33 ± 0	14·44 ± 1·11	17·77 ± 1·11	17·77 ± 1·11
4.	10×10^{-2} p.p.m.	8·88 ± 1·11	12·22 ± 1·11	14·44 ± 1·11	17·77 ± 1·11	18·88 ± 1·11	21·11 ± 1·11
5.	50×10^{-2} p.p.m.	8·88 ± 1·11	12·22 ± 1·11	15·55 ± 1·11	18·88 ± 1·11	21·11 ± 1·11	22·22 ± 1·11
6.	1 p.p.m.	10 ± 0	12·22 ± 1·11	16·66 ± 1·92	18·88 ± 1·11	23·33 ± 1·92	24·44 ± 1·11
7.	5 p.p.m.	12·22 ± 1·11	15·55 ± 1·11	17·77 ± 1·11	21·11 ± 1·11	24·44 ± 1·11	25·55 ± 1·11
8.	10 p.p.m.	12·22 ± 1·11	15·55 ± 1·11	18·88 ± 2·22	28·88 ± 1·11	31·11 ± 1·11	33·33 ± 0

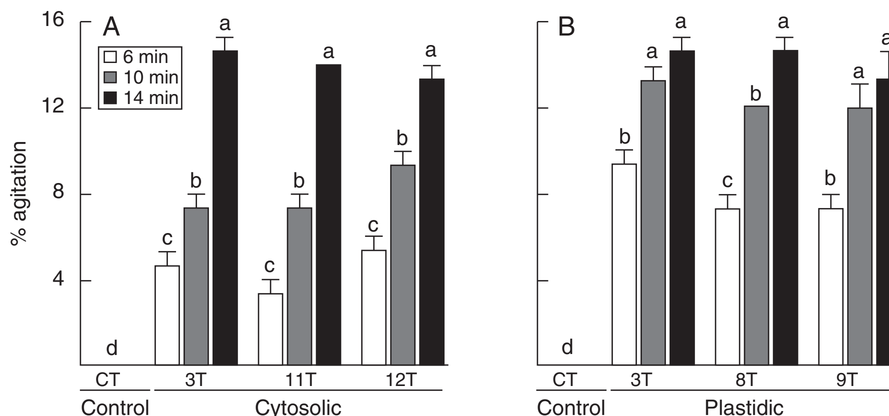

FIG. 4. Effect of hydro-distillate extract on agitational response of aphids. A temporal bioassay was performed in a custom-designed apparatus (Fig. S1) to determine the potency of hydro-distillate extract from 3-week old shoots of cytosolic (A) and plastidic (B) transgenic lines in inducing an agitation response in alate aphids upon exposure for 6, 10 and 14 min (as indicated in the key). The transgenic-induced responses were compared with their respective controls. Values ($n = 6$) are means ± s.e. and different lower-case letters on bars indicate that the values are statistically ($P < 0{\cdot}05$) significant.

any biotic stress. Many of the sesquiterpenes constitute plant defence compounds (Köllner *et al.*, 2008; Huang *et al.*, 2012) and their synthesis is presumably regulated in induced defence responses (Mumm *et al.*, 2008).

Although in lower organisms transmembrane exchange of cytosolic FDP to the plastid-bound MEP pathway is well documented (Suire *et al.*, 2000; Hemmerlin *et al.*, 2003; Green *et al.*, 2012), neither this process nor the likelihood of generating an additional pool of isoprene diphosphate from the *de novo* presence of FDP synthase in plastids is known. Although *FPS1* and *FPS2* exhibit more than 90 % identity in their amino acid sequences, the latter is more catalytic and thermostable

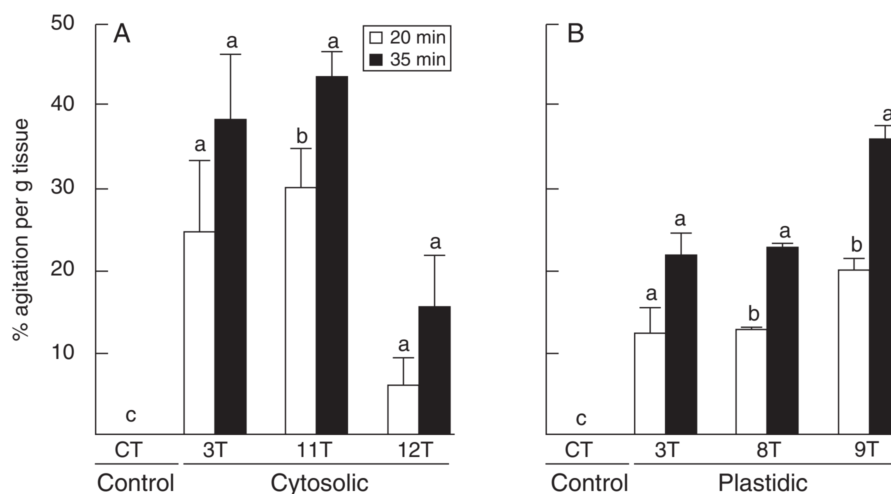

FIG. 5. Effect of head-space volatiles on agitational responses of aphids. A temporal bioassay was performed in a custom-designed apparatus (Fig. S1) to determine the potency of head-space volatiles from 3-week-old shoots of cytosolic (A), and plastidic (B) transgenic lines in inducing an agitation response in alate aphids upon exposure for 20 and 35 min (as indicated in the key). The transgenic-induced responses were compared with their respective controls. Values ($n = 6$) are means ± s.e. and different lower-case letters on bars indicate that the values are statistically ($P < 0·05$) significant.

(Keim *et al*., 2012). Therefore, it was intriguing to see whether over-expressing *FPS2* and/or targeting of its translated product to chloroplast would exert any influence on the sesquiterpene biosynthetic pathway and consequent altered responses of the transgenic plant to biotic stresses, particularly aphids. Interestingly, temporal expression of *FPS2* remained constitutive in uninfected and aphid-infested aerial parts of arabidopsis (data not shown). Thus, it was apparent that *FPS2* may not be directly involved in responses to aphid infestation in arabidopsis. This led us to ask whether over-expression of this gene in the cytosol or targeting of its translational product to the plastid would exert any influence on the profile of plant defence compounds, particularly sesquiterpenes. Therefore, we attempted to over-express *FPS2* under control of CaMV35S, which significantly increased, albeit with variations, its transcript level across the independent cytoplasmic and plastidic transgenic lines (Fig. 2). To determine the effects, if any, of the elevated expression of *FPS2* on developmental responses of the transgenic lines, they were grown up to seedling and maturity stages under sterile and greenhouse conditions, respectively (Fig. 1). Irrespective of the growth conditions and developmental stages, all the transgenic lines exhibited exaggerated stimulatory effects on ontogenetically distinct morphometric traits (Fig. 1F–I). This could be attributed to the probably involvement of FDP, which is a broad-spectrum substrate to an array of compounds including sterols and brassinosteroids that are known to play a pivotal role in different aspects of growth and development (Bouvier *et al*., 2005). In an earlier study, over-expression of *FPS1* in arabidopsis triggered premature senescence in leaves (Masferrer *et al*., 2002). However, over-expression of *FPS2* in either cytoplasm or plastid did not elicit any visible symptoms of early senescence. Moreover, no apparent detrimental effect was observed in growth and developmental responses of different transgenic lines of *FPS2*. This clearly suggested that *FPS1* and *FPS2* influence distinct pathways governing growth and development. This could be further corroborated by comparable expression of *FPS1* in *FPS2* transgenics and their respective controls. Although it is not evident from the study of Masferrer *et al*. (2002) whether over-expression of *FPS1* exerted any influence on the expression of *FPS2*, based on our study it could be assumed to be an unlikely effect, although further studies providing more empirical evidence are warranted.

Although attempts were made for transgenic expression of different terpene biosynthetic genes to engineer *de novo* synthesis of novel sesquiterpenes conferring protection against insect-pests, their trace levels impeded realizing the full potential of this approach (Bleeker *et al*., 2012; Yu *et al*., 2012, 2013). This could be due to tight control of the cytosolic FDP pool (Aharoni *et al*., 2003). However, there have been conflicting views about the impact of an increased FDP pool on isoprenoid end products. For instance, the sterol profile was found to remain unaltered in transgenic arabidopsis over-expressing *FPS1* (Masferrer *et al*., 2002). By contrast, sequestration of C-flux for generating additional FDP in cytosol or plastid led to increased synthesis of sesquiterpenes, sterols and carotenoids (Daudonnet *et al*., 1997; Wu *et al*., 2006). In this context, hypothetical prediction on the fate of downstream sesquiterpenes in transgenics over-expressing *FPS2* would be ambiguous. Therefore, to provide empirical evidence we investigated the effect of manipulating the expression of *FPS2* on the profile of sesquiterpenes and consequent responses, if any, of the transgenics towards an aphid-pest (*Myzus persicae*).

GC-MS analysis of the C15 sesquiterpene profile of cytosolic and plastidic transgenics revealed synthesis of several novel sesquiterpenes, a few of which were detected only in the latter (Table 1). This suggested that synthesis of some of the novel sesquiterpenes could be governed by subcellular localization of the FDP pool. Interestingly, some of the novel sesquiterpenes, including E-β-f, detetcted by over-expressing *FPS2* in the present study were found in the head-space collection of insect-infested *Sorghum bicolor*, and constituted predominant volatile cues in tritrophic interactions (Zhuang *et al*., 2012). E-β-f has been well documented as an alarm pheromone and induces a

'run-away' response in feeding aphids upon predator attack (Pickett and Griffiths, 1980; Avé *et al*., 1987; Francis *et al*., 2005). Presumably, elevated levels of E-β-f in *FPS2* transgenics could be due to increased substrate availability for E-β-f synthase. Enginerring repellence to *Myzus persicae* by increasing levels of E-β-f could also be achieved by generating transgenic arabidopsis expressing the E-β-f synthase gene of *Mentha* × *piperita* (Beale *et al*., 2006). Our study clearly suggested the inherent presence of a biosynthetic pathway for E-β-f and other novel sesquiterpenes in arabidopsis, dependent on the availability of a threshold level of the FDP pool. As E-β-f synthase has a large K_m value and a consequent reduced affinity towards its substrate FDP (BRENDA enzyme information system; BRaunschweig ENzyme Database; http://www.brenda-enzymes.org), it would have a limitation in synthesizing detectable levels of E-β-f under the steady-state level of FDP in arabidopsis. Our study thus corroborated earlier studies highlighting a decisive role of levels of available substrate for generating terpenoid end products (Negre *et al*., 2003; Boatright *et al*., 2004; Pott *et al*., 2004).

We also examined the behavioural responses of *M. persicae* upon exposing them to either entrapped volatiles emitted by live transgenics (cytosolic and plastidic) or hydro-distillate extracts from their leaves (Figs 4 and 5). Although both the assays revealed perceptible agitation in feeding aphid nymphs, the response was relatively higher with entrapped volatiles comprising E-β-f that was emitted continuously from live plants. A similar agitation response by aphids upon exposure to standard E-β-f further validated the potency of this sesquiterpene synthesized by *FPS2* transgenics.

It remains a matter of conjecture whether a blend of other novel sesquiterpenes produced by the transgenics could have any contributory influence towards the repellence response of aphids. Interestingly, the alarm potency of E-β-f, a predominant component of the essential oil of *Hemizygia petiolata*, was markedly attenuated due to inhibitory effects of minor components such as isomers of bicyclogermacrene and germacrene D present in the oil (Bruce *et al*., 2005). Whether this reduced potency is due to species-specific inhibition by other components or due to an adaptive response of aphids to a blend of E-β-f compared with its pure form and thus more of a generic nature is presently uncertain, and warrants detailed investigations across taxonomically diverse species. However, regarding the general adaptability of aphids to a blend of E-β-f to realize an engineered alarm response for plant protective purposes, production of pure E-β-f would be more desirable. Resistance towards aphids is generally not an innate characteristic of cultivated crop species and conventional breeding as well as transgenic approaches to achieve this have met with only limited success. Although several genes encoding lectins and protease inhibitors have been thought to have the potential to deter sap-sucking insects, their transgenic expression in agronomic crop species did not yield applicable resistance to aphids (Bhatia *et al*., 2011; Bala *et al*., 2013). Moreover, concerns regarding their possible undesired effects on higher animals have deterred much progress in this direction. Therefore, metabolic enginerring of the plant-derived E-β-f synthase gene has been considered as an environmentally friendly, potent strategy for developing aphid resistance in plants (Yu *et al*., 2012). Significant progess has already been taken in this direction with a global effort towards developing transgenic wheat conferring resistance to aphids (www.rothamsted.ac.uk). In addition, metabolic engineering and/or genetic manipulation of other sesquiterpenes for developing insect resistance has been widely researched (Bleeker *et al*., 2012; Lange and Ahkami, 2013; Nagel *et al*., 2014).

In summary, this study has empirically demonstrated the effects of over-expressing *FPS2* in cytosol and targeting its translational product to chloroplast not only on growth and development of arabidopsis but also on the synthesis of some novel sesquiterpenes, including E-β-f. Bioassays performed with head-space volatiles and extracts showed significant repellence of *Myzus persicae*. Overall the study provided evidence towards an alternative strategy of engineering aphid-resistant arabidopsis.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used in cloning, genomic PCR and RT-PCR experiments. Fig. S1: custom-designed glass-tube apparatus employed for aphid behavioural assays.

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