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ORIGINAL PAPER

Production of moth sex pheromone precursors in *Nicotiana* spp.: a worthwhile new approach to pest control

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Abstract

Pheromones are environmentally friendly alternatives to traditional pesticides for pest control. They are widely applied for insect monitoring, mating disruption and mass trapping. *Nicotiana benthamiana* and *N. tabacum* are potential green biomass production platforms of moth sex pheromones. Using these two *Nicotiana* species as plant factories, we expressed biosynthetic genes of plant and insect origin in leaf tissue. Moth sex pheromone precursors (*E*)-11-tetradecenoic acid, (*Z*)-11-tetradecenoic acid and (*Z*)-11-hexadecenoic acid were produced by introducing the acyl-ACP thioesterases *CpuFatB1* from *Cuphea pulcherrima* or *CpaFatB2* from *C. palustris* and the fatty acyl desaturases *AveΔ11* from *Argyrotaenia velutinana*, *CpaE11* from *Choristoneura parallela* or *AtrΔ11* from *Amyelois transitella*, under the control of CaMV-35S promoter. Among the *Nicotiana* spp. transformants, the best line produced (*Z*)-11-hexadecenoic acid at 17.6% of total fatty acids in leaves, during flowering stage, corresponding to 335 µg of (*Z*)-11-hexadecenoic acid per gram of fresh leaf. The (*Z*)-11-hexadecenoic acid production lines from *N. benthamiana* were selected for further propagation to obtain homozygous lines. In the *N. benthamiana* T2 generation, the production quantity of (*Z*)-11-hexadecenoic acid was stable. Our study demonstrates the feasibility of stable transformation of *N. benthamiana* for production of moth pheromone precursors in vegetative tissue.

Keywords Pest control · Insect pheromone precursors · *Nicotiana* spp. · Acyl-ACP thioesterase · Fatty acyl desaturase · Stable transformation

Key message

- Pheromones are environmentally friendly alternatives to traditional pesticides for pest control. We established a novel approach to produce moth pheromone precursors stably.
- This is the first report on production of insect pheromone precursors over generations in plants.
- We produced (*E*)-11-tetradecenoic acid, (*Z*)-11-tetradecenoic acid and (*Z*)-11-hexadecenoic acid, the fatty acid precursors of pheromones used by hundreds of moth pest species.
- Worthwhile amounts of (*Z*)-11-hexadecenoic acid may be produced by cultivating our most productive *N. benthamiana* line under field conditions.

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Introduction

The insect order Lepidoptera contains more than 160,000 described moth and butterfly species (Nieukerken et al. 2011). Species of moths are among the most damaging pests of food and fiber crops. Moths also have the capability of adapting fast and evolving resistance to insecticides (Simmons et al. 2010). Female moths emit species-specific sex pheromone component blends that attract males of the same species over long distances for mating (Wyatt 2003).

Due to the numerous problems associated with use of conventional pesticides (Brittain and Potts 2011; Bull 1982), synthetic pheromones have emerged as an alternative for insect control (Reddy and Guerrero 2000). Currently, tons of synthetic pheromones are produced for application in pest management (Weatherston and Stewart 2002). However, use of hazardous chemicals and generation of by-products during conventional chemical synthesis of pheromones (Mori 2007, 2010) may cause pollution problems. High costs for synthesis also limit the use of pheromones in many crops.

Since the techniques for genetically engineering of plants were developed in the early 1980s, numerous research projects have focused on utilizing transgenic plants to produce high-value recombinant proteins or compounds (Boehm 2007; Karg and Kallio 2009; Lienard et al. 2007; Ma et al. 2005; Mett et al. 2008). During the past 20 years, producing insect pheromones or their biosynthetic precursors in genetically modified plant factories has been attempted. A moth pheromone precursor was produced in *Nicotiana tabacum* by the introduction of a moth desaturase (Nešněrová et al. 2004), and an aphid alarm pheromone was produced from endogenous plant sesquiterpene by expression of a (*E*)-β-farnesene synthase in Arabidopsis (Beale et al. 2006). Moreover, Ding et al. (2014) proved that transient expression of genes coding for consecutive pheromone biosynthetic steps in *N. benthamiana* resulted in production of biologically active multi-component sex pheromones. The sex pheromone mixtures prepared from the acetylated fatty alcohol fractions produced by the genetically modified plants has the same activity for trapping of male small ermine moths *Yponomeuta evonymella* and *Y. padella* compared to conventionally produced synthetic pheromones (Ding et al. 2014). These studies have demonstrated that it is feasible to produce highly attractive and species-specific moth pheromones in genetically modified plants. However, the use of stably transformed *N. benthamiana* for semi-synthetic preparation of pheromones was so far never explored.

The majority of known moth pheromones are unsaturated C_{10}–C_{18} straight chain primary alcohols, acetates or aldehydes (Ando et al. 2004; Löfstedt et al. 2016). The biosynthesis of these compounds starts from de novo synthesis of palmitic acid or stearic acid, followed by desaturation, chain elongation or shortening to produce specific chain lengths, double bond positions and stereochemistry and finally the formation of the terminal functional groups (Löfstedt et al. 2016). In plant leaf tissue, the fatty acid biosynthesis takes place primarily in the chloroplasts utilizing C3 products of photosynthesis as carbon source (Bao et al. 2000). The palmitic and oleic acids so produced are then released into the cytosol as precursors for extra-plastid lipid synthesis and provide a pool of substrates that pheromone biosynthetic enzymes might utilize for moth pheromone production. Furthermore, the enzymes deployed for the biosynthesis of extra-plastid lipids and the insect desaturases introduced for the biosynthesis of moth pheromones in this study are located in the endoplasmic reticulum (Heinemann and Ozols 2003; Li et al. 2013), which also offers advantages for producing moth pheromones in plant leaves.

Nicotiana spp. are suitable to use for lipid metabolic engineering as they can be transformed by *Agrobacterium* with relative ease and high efficiency as well as having a fairly large green biomass (Aslan et al. 2014; Naim et al. 2012; Reynolds et al. 2015). In the present study, we used genes for *Nicotiana* spp. transformation that have been functionally characterized in heterologous expression systems in previous studies. These genes include: two plastid acyl-ACP thioesterases, *CpuFatB1* from *Cuphea pulcherrima* (Myrtales: Lythraceae) (Kim et al. 2015a) and *CpaFatB2* from *C. palustris* (Dehesh et al. 1996a) that can increase the amount of palmitic acid and myristic acid, respectively, by hydrolyzing the thioester bond of acyl-chain-ACP synthesized by the fatty acid synthase complex; three Δ11 fatty acyl desaturases, *i.e.*, *Ave*Δ*11* from *Argyrotaenia velutinana* (Lepidoptera: Tortricidae) and *CpaE11* from *Choristoneura parallela* (Lepidoptera: Tortricidae) producing (*Z*)-11-tetradecenoic acid (Z11-14:acid) (Liu et al. 2002) and (*E*)-11-tetradecenoic acid (E11-14:acid) (Liu et al. 2004), and *Atr*Δ*11* from *Amyelois transitella* (Lepidoptera: Pyralidae) producing (*Z*)-11-hexadecenoic acid (Z11-16:acid) (Ding et al. 2014). We used *Agrobacterium*-mediated leaf-disc stable transformation (Clemente 2006) on *N. tabacum* to allow pheromone precursors Z11-14:acid, E11-14:acid and Z11-16:acid production. The three pheromone precursors were produced in all regenerated transformants. Subsequently, the same set of genes were introduced into *N. benthamiana*. We then propagated and selected *N. benthamiana* lines for the production of Z11-16:acid, an immediate pheromone precursor for many moth species (Ando et al. 1979; Kehat and Dunkelbum 1990; Lynch et al. 1984; Rothschild et al. 1982). Our study proves the feasibility to produce a high quantity of moth pheromone precursors in stably transformed plants.

Materials and methods

Construction of plant expression vectors for tobacco stable transformation

CpaFatB2 (GeneBank accession number: AAC49180), *Atr*Δ*11* (JX964774), *Ave*Δ*11* (AF416738), *CpaE11* (AF518014) were amplified from entry clones (Ding et al. 2014). *CpuFatB1* (AGG79283) was a gift from E. Cahoon (University of Nebraska, Lincoln). *Cauliflower mosaic virus* 35S promoter (*35S*) and Octopine Synthase gene terminator (*OCS*) were used to regulate gene expression. For assembling multiple genes into one expression cassette, each gene,

including promoter and terminator, was first amplified by PCR, with primers (Table S1) spanning from the start codon to the stop codon of the opening reading frame (ORF), on a Veriti Thermo Cycler. The conditions used were: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 40 s, 72 °C for 1 min, followed by a final extension for 10 min at 72 °C. The reactions were performed in a total volume of 50 µL, containing 25 µL of Maxima polymerase master mix (Thermo Scientific™), 2.5 µL for each primer (10 µM) (Table S1), 1 µL of sample cDNA (15 ng/µL), 19 µL of sterilized H_2O. The PCR products were analyzed by electrophoresis on 1.5% w/v agarose gel in TAE buffer (40 mM Tris–acetate, 2 mM Na_2EDTA-H_2O), and gel purified with a GeneJET Gel Extraction Kit (Thermo Scientific™). Subsequently, fusion PCR (phusion®Taq, Thermo Scientific) was used to perform truncation and gene fusion for gene assembly (Atanassov et al. 2009). All genes with promoters and terminators were cloned into plant expression vector pXZP393 by Gateway recombination cloning technology (Invitrogen).

Assembly of moth pheromone biosynthetic pathways

Three plant expression vectors (Fig. 1a) were constructed to produce different pheromone precursors by assembling various transgene combinations. The expression of the exogenous genes was controlled by the *35S* promoter (Gatz et al. 1992) and *OCS* terminator (Schünmann et al. 2003). The engineered pathways for pheromone precursors production are shown in Fig. 1b. The thioesterases encoded by *CpaFatB2* and *CpuFatB1* take the myristate and palmitate plastid acyl carrier protein (ACP) from chain elongation, respectively, to form the corresponding myristic (14:0) (Dehesh et al. 1996) and palmitic (16:0) acid (Kim et al. 2015). The fatty acids are acylated to 14:CoA and 16:CoA when transported out of the plastid into the cytosol. The 14:CoA and 16:CoA are then subsequently converted into the corresponding pheromone precursors Z11-14:CoA, E11-14:CoA and Z11-16:CoA, by a desaturase encoded by *AveΔ11*, *CpaE11* or *AtrΔ11* (Ding et al. 2014). The expression vector with the functional transgene combination of *CpuFatB1-AtrΔ11* was transformed both into *N. tabacum* and *N. benthamiana*, cultivating the line for production of Z11-16:CoA. The other expression vectors that contained *CpaFatB2-AveΔ11* or *CpaFatB2-CapE11* were only transformed into *N. tabacum*, cultivating two different lines for production of Z11-14:CoA and E11-14:CoA, or E11-14:CoA alone, respectively.

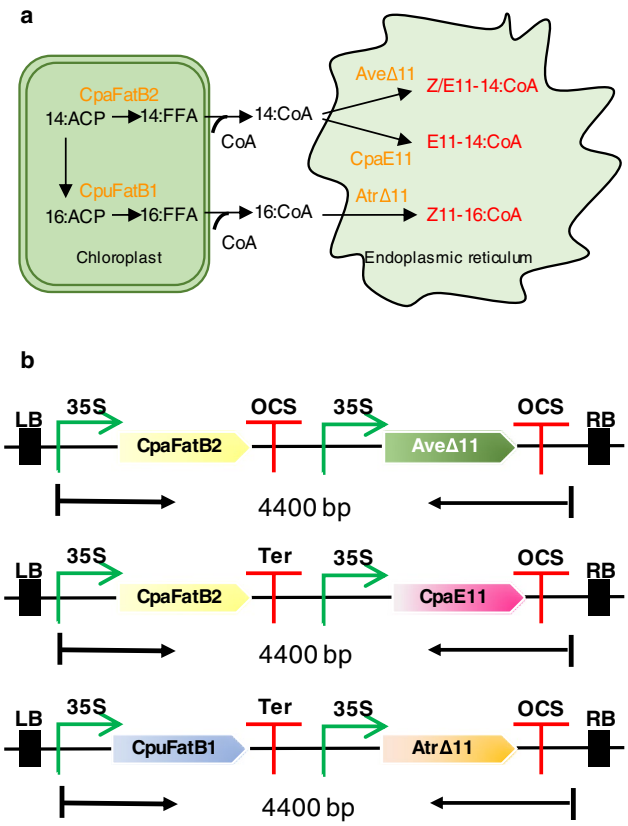

Fig. 1 Engineering strategy toward pheromone precursor production in *Nicotiana* spp. **a** Scheme of expression vectors for pheromone precursor production in *Nicotiana* spp. by stable transformation. pXZP393, plant expression vector pXZP393. 35S, *Cauliflower mosaic virus* 35S promoter. OCS, Octopine Synthase gene terminator. Pheromone biosynthetic genes are shown in bold. RB and LB are the T-DNA border sequences for *Agrobacterium* which are shown in black background. The sequence length between RB and LB is ca. 4400 bp. CpaFatB2, *Cuphea palustris* 14:ACP thioesterase; CpuFatB1, *C. pulcherrima* 16:ACP thioesterase; AveΔ11, *Argyrotaenia velutinana* Δ11 desaturase; CpaE11, *Choristoneura parallela* E11 desaturase; AtrΔ11, *Amyelois transitella* Δ11 desaturase. **b** Engineered metabolic pathways of pheromone precursor production in *Nicotiana* spp. leaves. ACP, acyl carrier protein; FFA, free fatty acids. The introduced enzymes are indicated in orange, and the target pheromone precursor is shown in red. Acyl intermediates in the pathway (also throughout the article) are given as short forms. E/Z11-14:CoA refers to the fatty acyl coenzyme A with a chain length of 14-carbon atoms and a double bond at Δ11 position in 'E' or 'Z' configuration; Z11-16:CoA refers to the fatty acyl coenzyme A with a chain length of 16-carbon atoms and a double bond at Δ11 positions in 'Z' configuration

Plant material and growth condition

Wild-type *N. benthamiana*, wild-type *N. tabacum* (Wisconsin 38) and transgenic plants were grown either in the greenhouse under 16 h/8 h or in a climate chamber under 14 h/10 h light/dark conditions. Growth temperature and relative humidity in greenhouse were set at 24 °C/18 °C in day/night and 40%, respectively. In the climate chamber, the temperature was set at 22 °C.

Transformation of *Agrobacterium*

All the constructed expression clones in pXZP393 were confirmed by sequencing and then electroporated into *A. tumefaciens* GV3101 (MP90RK) with settings of 1700Vmm^{-1}, 5 ms (Eppendorf 2510).

Agrobacterium-mediated leaf-disc transformation

For each construct, 30 ml of *Agrobacterium* solution was incubated at 30 °C in LB medium supplemented with 50 mg/L rifampicin, 50 mg/L gentamicin and 50 mg/L spectinomycin for 24 h (30 °C, 300 rpm (INNOVA®42, Incubator Shaker Series)). The bacteria were spun down at 3,300 g for 5 min at room temperature and resuspended in LB medium without any antibiotic. Optical density (A600nm) of each *Agrobacterium* culture was adjusted to 0.9–1 in a total volume of 30 ml with LB medium prior to tobacco leaf-disc transformation. Plant material was obtained from 4–5-week-old *Nicotiana* plants grown under sterile conditions on MS medium (Murashige and Skoog 1962) in a climate chamber.

Transgenic lines were obtained by *Agrobacterium*-mediated leaf-disc transformation. Leaf discs (20 mm × 20 mm) were cut out and incubated 5 min in an *A. tumefaciens* solution, dried with sterile napkin paper and transferred to Petri dishes with MS medium (Horsch el al. 1985). After 48 h incubation in darkness, leaf discs were transferred to selection medium (MS medium supplemented with 50 mg/L kanamycin, 250 mg/L cefotaxime, 1 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L naphthalene acetic acid (NAA)). After 2–3 weeks of incubation, the calli produced on the leaf edges were transferred to shoot-inducing medium (the selection medium supplemented with 100 mg/L kanamycin). After 2–3 weeks of incubation, the shoots were transferred to root-inducing medium (identical as shoot-inducing medium, but without hormones) with 100 mg/L kanamycin and 250 mg/L cefotaxime. The shoots were finally transferred into soil and grown in greenhouse until maturity.

Sampling for leaf lipids analysis

The T0 plants regenerated from calli were used for lipid analysis. From each T0 plant at the age of 1.5 months, three pieces of different mature leaves (size ca. 50 mm × 50 mm) were randomly taken. Samples from each T0 plant were analyzed in triplicate. Then, the T0 plants were kept growing in greenhouse until maturity of T1 seeds. Subsequently, T1 seeds collected from the best target compound-producing T0 plant were sown on MS medium supplemented with 100 mg/L kanamycin. For *N. tabacum* and *N. benthamiana*, eight and thirty T1 plants from each T0 plant were cultivated, respectively. Seedlings from each T1 plant surviving the selection were transferred to soil and grown to maturity for producing T2 seeds. Thirty *N. benthamiana* T2 seeds from each plant were collected and cultivated to T2 plants for analysis. For T1 and T2 plant analysis, at the same age to T0 plant, three pieces of mature leaves (size was same to T0 plant) were randomly taken from each plant and mixed as one sample.

Total fatty acids analysis

For fatty acid analysis, 100–300 mg fresh leaf tissue per sample was treated by 1 mL 2% sulfuric acid in methanol containing 3.12 µg methyl nonadecanoate (19:Me) as internal standard, incubated at 90ºC for 1 h. Subsequently, 1 mL water and 1 mL heptane were added and the mixture was vigorously vortexed and centrifuged at 2000 rpm (Heraeus™ Sepatech-3760) for 2 min. Finally, ca. 1 mL heptane phase containing the fatty acids in the form of corresponding methyl esters was transferred to a new glass vial for GC/MS analysis.

TLC separation of leaf neutral lipids

For neutral lipid analysis, total lipids from 1 g fresh leaf were extracted in 4 mL of methanol/chloroform (2:1, v/v) using a glass tissue grinder. The crude extract was transferred to a glass tube, and the grinder was washed with 1 mL of chloroform, which was then transferred to the extract. One milliliter of water was added to produce a biphasic mixture, which was then vortexed vigorously and centrifuged at 2000 rpm (Heraeus™ Sepatech-3760) for 2 min. Total leaf lipids extract was then present in ca. 2.3 mL chloroform phase. From this phase, 300 µL was taken and concentrated to ca. 40 µL under gentle nitrogen flow and then loaded at 20 mm from the bottom of a thin-layer chromatography (TLC) plate without fluorescent indicator (Silica gel 60, Merck, Germany), along with standard (TLC Mix 34, Larodan, Sweden). The plate was developed in a solvent system of heptane/diethyl ether/acetic acid (60:40:1, v/v/v). The obtained lipid bands were scraped off and extracted with 1.5 mL of methanol/chloroform (2:1, v/v) containing 3.12 µg 19:Me in a sonication bath until the gel bands were totally shattered. One mL of water was added to the tube to partition the lipids into the chloroform phase, which was then transferred to a new tube and evaporated to dryness, followed by acid methanolysis as described above.

Gas chromatography/mass spectrometry (GC/MS)

Plant leaf samples were analyzed by using an Agilent 5975 mass-selective detector, coupled to an Agilent 6890 series gas chromatograph either equipped with a polar column (HP-INNOWax, 30 m × 0.25 mm, 0.25 µm film thickness) or a nonpolar column (HP-5MS, 30 m × 0.25 mm, 0.25 µm film

thickness), and helium was used as carrier gas. For analysis of fatty acid methyl esters (FAMEs), the oven temperature was set at 80 °C for 1 min, then increased to 230 °C at a rate of 10 °C/min and held for 10 min.

To determine the position of double bonds in target compounds, DMDS derivatization was performed according to Dunkelblum et al. (1985). The DMDS adducts were analyzed by GC/MS on a nonpolar column (HP-5MS) using the following oven temperature program: 80 °C for 2 min, then increased at a rate of 15 °C/min to 140 °C, then increased at a rate of 5 °C /min to 260 °C and held for 3 min.

Statistical analysis

Data were subjected to analysis of variance, and means were compared by unpaired *t*-test with two-tailed *P* value by using the Prism software program (Prism 8). $P < 0.05$ indicates a significant difference.

Results

Fatty acid composition in *N. tabacum* and *N. benthamiana* leaves

The GC/MS analysis showed that wild-type *N. tabacum* leaves contained 54 ± 5% linolenic acid (18:3), 12 ± 3% palmitic acid (16:0), 10 ± 3% linoleic acid (18:2), 8 ± 2% (*Z,Z,Z*)-7,10,13-hexadecatrienoic acid (16:3) and 2 ± 1% stearic acid (18:0) (Table 1 and Fig. 2a). In addition, oleic acid (18:1), (*Z,Z*)-7,10-hexadecadienoic acid (16:2) and (*Z*)-13-hexadecenoic acid (16:1(13)) and arachidic acid (20:0) were found in the leaf (Table 1 and Fig. 2a). Wild-type *N. benthamiana* leaves showed a similar fatty acid composition as in *N. tabacum*, the majority of the total fatty acids were 18:3, 18:2, 16:0 and 16:3, followed by

Table 1 Fatty acid compositions (weight %) of total leaf lipids in the wild type and in T0 transgenic *Nicotiana tabacum* expressing acyl-ACP thioesterases and desaturases

Genotype	14:0	14:1(Z11)	14:1(E11)	16:0	16:1(Z11)	16:1(13)	16:2	16:3	18:0	18:1	18:2	18:3	20.0
Wild type	0.1	–	–	12.2	–	2.4	1.1	7.6	1.7	1.0	10.8	54.2	0.3
CpaFatB2-AveΔ11													
#001	0.2*	0	0	14.0*	–	1.6	0.9	10.5	1.5	1.0	7.6	54.0	0.2
#002	0.2*	0	0	14.0*	–	2.1	0.8	11.0	1.3	0.7	7.1	53.6	0.2
#003	0.2*	0	0	13.3*	–	2.4	1.0	10.0	1.0	0.9	10.4	53.5	0.2
#004	0.2*	0	0	13.8*	–	2.0	0.9	12.0	1.2	0.8	8.0	52.3	0.2
#005	0.5*	0	0	16.4**	–	1.7	0.8	10.9	1.2	0.7	7.7	49.9	0.1
#006	0.8**	0.1	0	28.7***	–	0.8	0.4	2.1	1.7	0.4	8.2	48.1	0.4
#007	1.7**	0	0	14.1*	–	1.7	0.5	14.8	1.6	0.3	7.0	60.2	0.2
#008	0.8**	0	0	12.3	–	2.6	0.9	10.7	0.8	0.7	9.4	56.8	0.2
#009	1.9**	0.1	0	32.7***	–	1.4	0.5	3.7	1.2	0.5	12.0	43.5	0.2
#010	3.1***	0.4	0.1	36.3***	–	0.5	0.5	1.9	3.5	0.8	15.3	34.8	0.8
#011	0.6**	0	0	12.9	–	2.3	0.8	10.0	1.1	0.5	7.8	54.9	0.2
#012	2.9***	0	0	15.7**	–	1.5	1.1	3.9	2.6	1.1	9.8	44.8	0.4
CpaFatB2-CpaE11													
#001	5.5***	–	0.2	38.3***	–	1.0	0.5	1.6	1.9	0.7	14.9	33.0	0.2
#002	3.6***	–	0.2	35.4***	–	0.8	0.6	2.7	1.7	0.6	11.2	39.5	0.2
#003	2.6***	–	0.1	36.0***	–	0.8	0.4	2.2	2.9	0.6	14.4	37.3	0.4
#004	2.0**	–	0.1	32.0***	–	0.8	0.6	4.0	1.8	0.5	12.8	35.4	0.8
CpuFatB1-AtrΔ11													
#001	0.3*	–	–	20.1**	0.7	2.0	0.8	11.5	1.1	0.6	6.9	47.4	0.3
#002	0.4*	–	–	18.4**	0.2	2.1	0.6	8.7	1.1	0.9	9.8	52.7	0.3
#003	0.3*	–	–	19.8**	0.9	1.7	1.0	11.7	0.8	0.5	7.1	49.6	0.5
#004	0.2*	–	–	15.0**	0.4	2.1	0.9	11.8	0.9	0.5	7.9	55.1	0.3
#005	0.3*	–	–	13.0**	0.1	2.2	1.0	11.6	0.8	0.7	8.7	57.3	0.5
#006	1.2**	–	–	14.8**	0	2.2	1.0	7.6	1.6	1.0	8.3	51.4	0
#007	1.6**	–	–	30.0***	0.5	3.6	1.3	6.4	1.4	1.2	12.4	44.4	0

Values are the means of at least three biological replicates. Significance analysis was only applied to compare 14:0 and 16:0 between transformants and wild type by using unpaired t-test. *, **,*** indicate $P < 0.05$, 0.01, 0.001, respectively

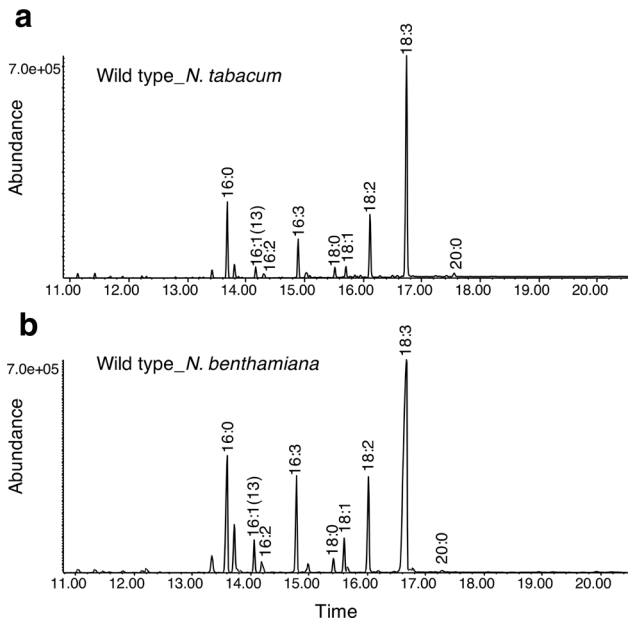

Fig. 2 Chromatograms of total fatty acids in wild-type **a** *Nicotiana tabacum* and **b** *N. benthamiana*, in the form of corresponding methyl esters by GC/MS analysis. 16:0, palmitic acid; 16:1(13), (*Z*)-13-hexadecenoic acid; 16:2, (*Z*,*Z*)-7,10-hexadecadienoic acid; 16:3, (*Z*,*Z*,*Z*)-7,10,13-hexadecatrienoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:0, arachidic acid

16:1(13), 18:0 and 18:1, and besides that 16:2 and 20:0 were also detected (Table 2 and Fig. 2b).

A large variation in the production of 14:0, 16:0 and unsaturated pheromone precursors was observed in both T0 and T1 *N. tabacum* lines (Table 1 and Fig. 3). For production of C_{14} pheromone precursor in *N. tabacum*, *CpaFatB2-AveΔ11* and *CpaFatB2-CpaE11* transformants contained 14:0 and 16:0 from 0.2 to 5.5% and 12.3 to 38.3%, respectively (Table 1), higher than the wild type which produced 0.1% of 14:0 and 12.2% of 16:0. *CpaFatB2-AveΔ11* transformants produced Z11-14:acid and a smaller amount of E11-14:acid (Fig. 4a), and *CpaFatB2-CpaE11* transformants produced E11-14:acid (Fig. 4b). For production of C_{16} pheromone precursors in *N. tabacum*, *CpuFatB1-AtrΔ11* transformants produced 0.2 to 1.6% of 14:0 and 13.0 to 30.0% of 16:0, higher than that from the wild-type plants (Table 1). Z11-16:acid was produced in the transformant lines up to 0.9% (Table 1 and Fig. 4c). Taking these transformant lines to a second generation did not improve the levels of pheromone precursor production (Fig. 3). Eight T1 plants from *CpuFatB1-AtrΔ11* #001 T0 parent produced 13.1 to 22.5% of 16:0 (Fig. 3a) and 0.1 to 1.0% of Z11-16:acid (Fig. 3b).

CpuFatB1-AtrΔ11 transformants of *N. benthamiana* also showed a large variation in T0 and T1 lines. T0 *N. benthamiana* transformants contained 15.0 to 31.6% of 16:0 (with the exception that #027 plant produced 10.0%), higher than the wild-type plants that produced an average of 14.8% (Table 2). These transformants produced Z11-16:acid (Fig. 4d), ranging from 0.1 to 4.8% (Table 2). Among the twenty-eight T0 transformants, six plants accumulated more than 2.0% of Z11-16:acid (Table 2). The percentage of Z11-16:acid varied significantly (0.3 to 4.2%) among different leaves from same plant at the same stage, indicating presence of chimerism in the plant (Fig. S1). More than 150 T1 plants from five T0 parent plants (at least 30 T1 from each selected T0) were grown to the next generation, and the production of Z11-16:acid ranged from 0.1 to 10.1% (Fig. 5), thus in many cases reaching higher percentages as compared to the T0 plants. Besides this, the T1 plant #025-16 produced even higher proportion of Z11-16:acid in the mature leaves during plant flowering (Fig. 6). This line produced 17.6% Z11-16:acid, corresponding to 335 µg per gram fresh leaf and equaling ca. 50 mg Z11-16:acid in the entire plant. The highest 16:0 production recorded in T1 plants was 38.1% (Fig. 5), higher than the best T0 plant (Table 2). Five T1 plants from each line are propagated to next generation. Two T2 lines (#00621, #00830) still showed big variation in Z11-16:acid production (Fig. 5a–b). The other three T2 lines (#00925, #02516, #02615) showed smaller variation (Fig. 5c–e), and in the #02615 T2 line, we consider the Z11-16:acid production stable (Fig. 5e).

Fitness of transgenic *N. tabacum* and *N. benthamiana*

Compared to the wild-type *N. tabacum*, the transgenic tobacco was growing much slower, and the leaves were less green when the *CpaFatB2* was introduced. There were also small tissue lesions on the surface of leaves (data not shown). In addition, such deleterious effect on the leaves as well as delayed growth rate and chlorosis were more often observed in the plants that produced higher amounts of Z/E11-14:acid, and more than one-fifth of T1 plants of *CpaFatB2-CpaE11* transformants did not survive during development (Fig. 3). However, no developmental problem was observed in *CpuFatB1-AtrΔ11* transformed *N. tabacum* and *N. benthamiana*.

Lipid fractions in transgenic *N. benthamiana* leaves

In order to understand the distribution of pheromone precursors in transgenic *N. benthamiana* leaves, total lipids were separated by TLC into different molecular classes analysis (Fig. S2). The results showed that the wild-type leaves contain about 80% polar lipids (PL), 10% sterol esters (SE), 4% monoacylglycerols (MAG), 3% triacylglycerols (TAG) and diacylglycerols (DAG) plus 1% free fatty acids (FFA) (Fig. 7a). Compared to the wild type, the amount of TAG and SE in the transgenic plants was significantly increased.

Table 2 Fatty acid compositions (weight %) of total leaf lipids in the wild type and in T0 transgenic *Nicotiana benthamiana* expressing acyl-ACP thioesterases and desaturases

Genotype	14:0	16:0	16:1(Z11)	16:1(13)	16:2	16:3	18:0	18:1	18:2	18:3	20.0
Wild type	0.3	14.8	–	2.3	1.2	7.6	1.6	1.3	6.2	55.6	0.2
CpuFatB1_AtrΔ11											
#001	0.8**	25.0***	1.0	1.7	1.0	2.1	3.3	1.1	4.9	36.1	0.4
#002	0.7**	18.0**	0	1.6	0.9	2.3	3.5	0.9	4.4	39.1	0.7
#003	0.3	30.5***	0.6	0.7	0.8	1.8	3.5	1.6	9.6	38.6	0.4
#004	0.9**	26.1***	0.8	0.7	0.7	1.6	3.7	2.8	12.8	34.5	0.7
#005	0.2	19.8**	0.1	0.2	1.0	0.5	10.1	13.9	13.0	27.2	1.7
#006	0.4*	25.1***	3.2	0.8	1.2	1.1	2.9	0.2	4.8	40.1	0.5
#007	0.6*	28.1***	1.8	2.0	0.7	1.7	2.5	0.4	3.8	39.6	0.3
#008	0.3	24.3***	2.3	0.6	1.0	0.9	4.0	0.3	5.6	38.4	0.9
#009	0.5*	28.1***	4.8	1.5	0.8	1.7	2.5	2.1	5.0	44.4	0.3
#010	0*	27.0***	1.1	0.7	1.3	1.0	7.4	1.1	8.5	42.7	0.8
#011	0.1*	20.6***	0.1	1.4	1.0	1.4	6.7	1.2	7.1	50.3	0.2
#012	0.4*	28.6***	2.2	0.9	0.7	2.0	1.9	0.3	3.5	55.9	0.3
#013	0.4*	20.1**	0.5	1.5	0.9	1.8	3.8	2.2	9.4	48.6	0.9
#014	0.3	17.9**	0.1	1.3	0.5	2.1	3.3	1.2	8.5	56.1	0.4
#015	0.5*	17.8**	0	1.4	0.6	2.4	2.6	0.8	5.7	55.0	0.5
#016	0.6*	31.6***	0	1.2	1.6	0.9	4.3	0.8	7.2	44.2	0.6
#017	0.6*	28.8***	1.8	1.6	0.9	2.7	1.5	0.7	5.7	45.3	0.3
#018	0.4*	23.0**	1.9	2.0	0.7	4.1	1.1	1.0	6.6	51.7	0.1
#019	0.5*	29.1***	1.8	2.0	1.3	3.0	2.5	2.1	8.2	40.5	0
#020	0.4*	27.5***	1.9	0.6	0.8	1.1	3.7	0.5	8.0	39.6	0.5
#021	0.4*	24.8***	1.7	1.5	0.6	2.6	2.1	0.3	3.1	49.1	0.4
#022	0.6*	29.0***	2.3	1.1	0.5	1.3	2.9	0.9	4.0	42.0	0.5
#023	0.4*	17.7*	0.2	1.5	0.5	1.4	4.8	0.2	6.9	43.0	0.6
#024	0.3	26.1***	1.9	1.4	0.7	4.8	1.7	0.2	6.7	46.7	0.5
#025	0.3	24.4***	1.7	1.8	0.8	4.7	1.6	1.1	6.7	45.9	0.5
#026	0.5*	28.0***	4.4	1.5	0.2	5.2	1.6	1.3	4.4	49.1	0.5
#027	0.2	10.0**	0	0.7	0.3	0.7	2.9	0.8	4.4	26.0	1.8
#028	0.5*	29.6***	1.8	1.5	0.9	3.9	2.4	2.3	10.6	39.3	1.1

Values are the means of at least three biological replicates. Significance analysis was only applied to compare 14:0 and 16:0 between transformants and wild type by using unpaired *t*-test. *, **,*** indicate $P < 0.05$, 0.01, 0.001, respectively. The unusually high percentage of fatty acids are indicated by underline

In wild-type leaves, nearly 80% of 16:0 was present in PL, and small amount of 16:0 was found in SE, TAG, MAG and DAG, whereas the transformed leaves showed a significant enrichment of 16:0 in the TAG and SE, at about 20–40%. A small fraction of 16:0 was present in the MAG, DAG and FFA, at a similar level as in the wild type. The pheromone precursor Z11-16:acid shows a similar distribution to 16:0 (Fig. 7b).

Discussion

The successful production of several insect sex pheromone precursors in *N. tabacum* and *N. benthamiana* by stable transformation was demonstrated in this study. All of the transformed plastid thioesterases and fatty acyl desaturases were functionally active in producing pheromone precursor in both *Nicotiana* species. This is the first report of Z/E11-14:acid production in a plant by stable transformation and levels of up to 17.6% Z11-16:acid of the total fatty acids was achieved. The Z11-16:acid production in *N. benthamiana* was better than in *N. tabacum* (Tables 1 and 2). The average value of Z11-16:acid production in T0 *N. tabacum* was 0.2% (Table 1), whereas in T0 *N. benthamiana*, it was 1.8% (Table 2). The best *N. benthamiana* transgenic line #025 produced as high as 13.6% of Z11-16:acid of the total fatty acids in T2 plants (Fig. 5), which is much higher than the production reported by Nešnerová et al. (2004), who claimed that 6% of Z11-16:acid of total fatty acids was produced in their transgenic *N. tabacum* NtD15B line. The quantity of Z11-16:acid in *N. benthamiana* was calculated as 335 µg per gram fresh leaf, compared to 32 µg per gram of *N. tabacum* fresh leaf in Nešnerová et al. 2004. The results suggest that *N. benthamiana* has potential to be more efficient than *N.*

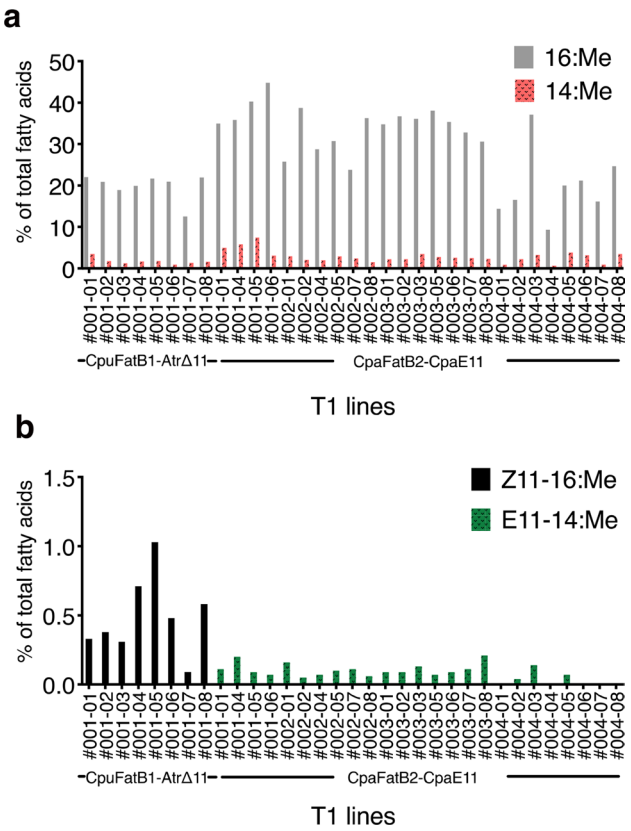

Fig. 3 Percentage of **a** palmitic acid (16:0) and myristic acid (14:0), and **b** pheromone precursors Z11-16:acid and E11-14:acid of total fatty acids in T1 leaves of *Nicotiana tabacum*. Fatty acids were analyzed in form of corresponding methyl esters. 16:Me, Methyl palmitate; 14:Me, Methyl myristate; Z11-16:Me, (*Z*)-11-hexadecenoic acid methyl ester; E11-14:Me, (*E*)-11-tetradecenoic acid methyl ester. Eight T1 plants for each line were numbered and cultivated from the beginning, but some of them died during the growing process, that is the reason for absence of some CpaFatB2-CpaE11 T1 plants

tabacum as a plant factory for Z11-16:acid production. In the study of Ding et al. (2014), 381 µg per gram fresh leaf of Z11-16:acid was produced in *N. benthamiana* by transient expression, which is a massive overexpression of exogenous genes over a few days that ignores the health of the plant. Here, the production of 335 µg per gram leaf of Z11-16:acid by stable transformation shows the ability of vegetative material to function with the expression of *CpuFatB1* and *AtrΔ11* and yield compounds over development, providing the potential for further commercial production.

Interestingly, although at low levels, we detected the elongation product (Z)-13-octadecenoic acid (Z13-18:acid) in both transformed *N. benthamiana* and *N. tabacum* lines when the Z11-16:acid was produced. This indicates that the novel fatty acids are accepted by an endogenous fatty acyl elongase in both species. Additionally, it is interesting that both wild-type *N. tabacum* and *N. benthamiana* produce more than 2% monounsaturated (Z)-13-hexadecenoic acid (Z13-16:acid) in their fatty acid profiles. Z13-16:acid is a pheromone precursor of the moth *Herpetogramma submarginale* (Yan et al. 2015b), which uses (Z)-13-hexadecenyl acetate (Z13-16:OAc) as its major sex pheromone component.

Moreover, it is observed that in transgenic *N. benthamiana* T1 plant #025–16, the amount of Z11-16:acid increased from 10.1% of total fatty acids in 6-week-old plants to 17.6% in 3-month-old flowering plants (Fig. 6). This may be due to some of the fatty acids being re-allocated from polar lipids to neutral lipids during the plant development. The fatty acids in the leaves reach a maximum at early flowering (Chu and Tso 1968; Leech et al. 1973), which provides an improvement of substrates to desaturase during leaf oil accumulation. The overexpression of acyl-ACP thioesterases has previously been reported to modify oil profile in transgenic plants (Mandal et al. 2000; Salas et al. 2002; Voelker et al. 1996). Overexpression of *Arabidopsis thaliana* thioesterases in transgenic *N. benthamiana* leaf was confirmed to increase the TAG as well (EI-Tahchy et al. 2017). Our results of lipid distribution in *N. benthamiana* leaves also indicate that the thioesterase gene *CpuFatB1* elevated the TAG amount, leading to an increase in pheromone precursor levels (Fig. 7). Medium-chain fatty acids (MCFA) produced in leaves result in unbalanced membrane lipid profiles and undesirable chlorosis and cell death (Reynolds et al. 2017), which we think might be the reason for low productivity of pheromone precursors and insignificant increase of 14:0. Also, the deleterious effect on the *CpaFatB2-CpaE11 N. tabacum* transformants is likely caused by the E11-14:acid product rather than 14:0, because no deleterious effect was observed in *CpuFatB1-AtrΔ11* transformed *Nicotiana* spp., which also produced higher 14:0 than the wild-type plants. Reynolds et al. (2017) demonstrated that overexpression of the KENNEDY pathway genes can push the MCFA to the TAG pool to increase the MCFA production in leaf oils without disturbing membrane homoeostasis and cell death. Therefore, to improve TAG accumulation could be a means to increase the production of pheromone precursor by sequestering products into TAG instead of ending up in membranes with a negative influence on plant function and health.

Typically TAG accounts for less than 1.5% of the total leaf fatty acids and takes up less than 3% of de novo synthesized fatty acids (Fan et al. 2013a; Yang and Ohlrogge

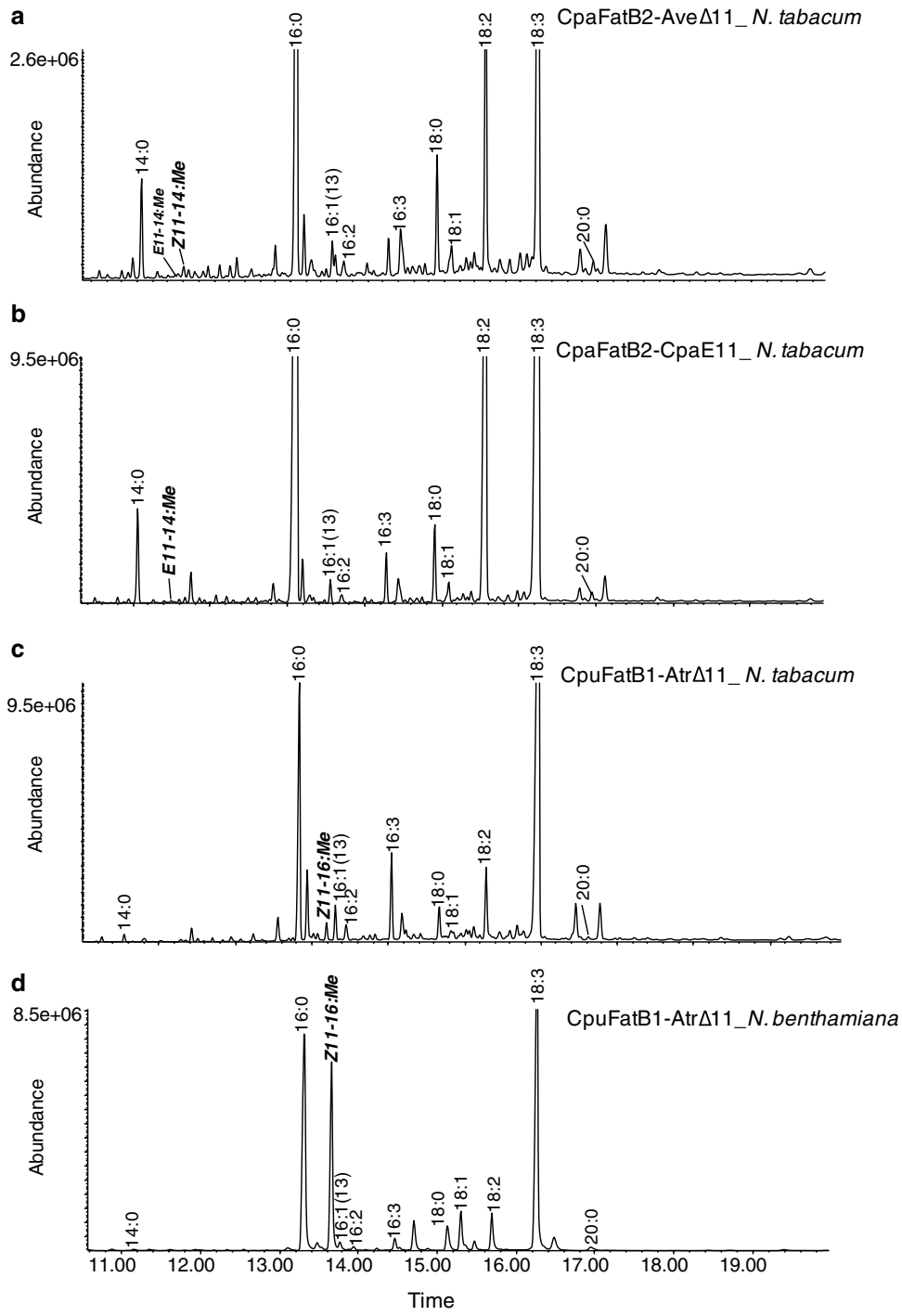

Fig. 4 Total lipids GC chromatograms of **a** CpaFatB2-Ave11, **b** CpaFatB2-CpaE11, **c** CpuFatB1-AtrΔ11 transformants from *Nicotiana tabacum* and **d** CpuFatB1-AtrΔ11 transformants from *N. benthamiana*. GC/MS analysis of fatty acids in the form of corresponding methyl esters. The target pheromone precursors specifically produced by transformants were indicated in italic and bold. Z11-14:Me, (Z)-11-tetradecenoic acid methyl ester; E11-14:Me, (E)-11-tetradecenoic acid methyl ester; Z11-16:Me, (Z)-11-hexadecenoic acid methyl ester; 14:0, myristic acid; 16:0, palmitic acid; 16:1(13), (Z)-13-hexadecenoic acid; 16:2, (Z,Z)-7,10-hexadecanoic acid; 16:3, (Z,Z,Z)-7,10,13-hexadecanoic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:0, arachidic acid

2009). However, benefitting from the development of biotechnology and utilizing bioengineering approaches the levels of TAG in the leaves may increase up to 30–33% TAG (Vanhercke et al. 2017; Cernac and Benning 2004; Eastmond 2006; Kelly et al. 2011; Santos-Mendoza et al. 2008; Hu et al. 2017; Nguyen et al. 2015; Pidkowich et al. 2007). This may be implemented as existing possibilities in order to optimize the plant factory in future studies. Furthermore, increasing the exogenous gene activities by modification of promoters and enhancer (Kay et al. 1987; Minetoki et al.

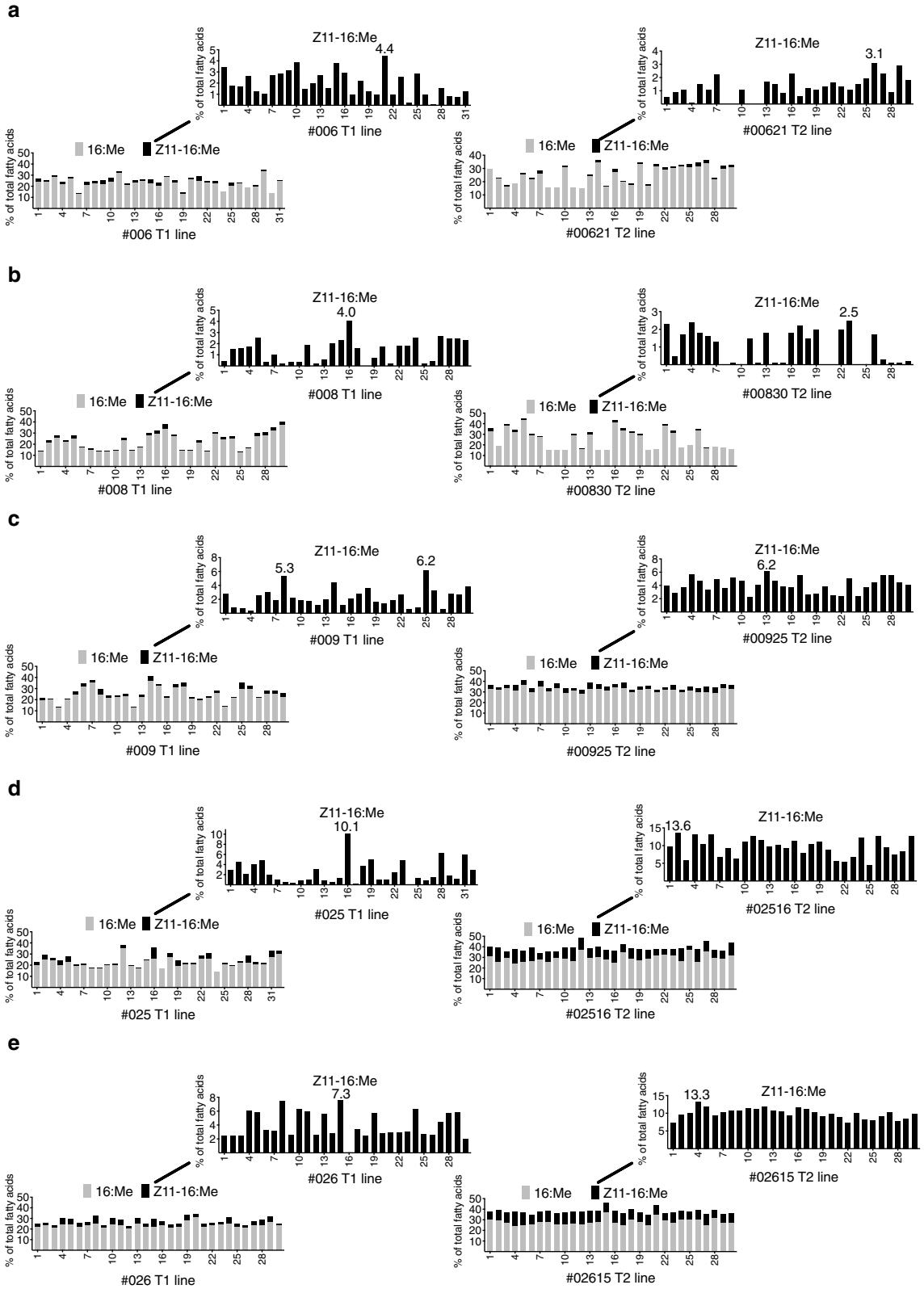

Fig. 5 Percentage of palmitic acid (16:0) and (Z)-11-hexadecenoic acid (Z11-16:acid) of total fatty acids in the form of corresponding methyl esters in T1 and T2 leaves of *Nicotiana benthamiana* from **a** #006 and #00621 line; **b** #008 and #00830 line; **c** #009 and #00925 line; **d** #025 and #02516 line; **e** #026 and #02615 line Fatty acids were analyzed in form of corresponding methyl esters. 16:Me, Methyl palmitate; Z11-16:Me, (Z)-11-hexadecenoic acid methyl ester. The histogram at the top right corner for each line is the zoomed in y-axis for Z11-16:acid. Fatty acids were analyzed in form of corresponding methyl esters

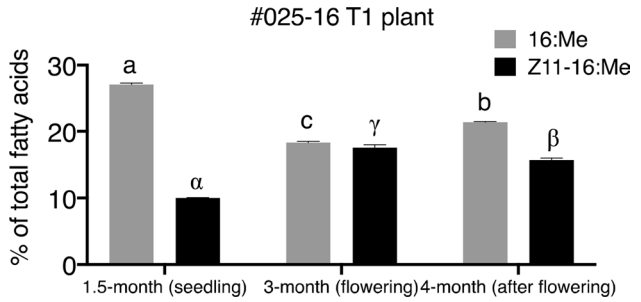

Fig. 6 Percentage of palmitic acid (16:0) and (Z)-11-hexadecenoic acid (Z11-16:acid) of total fatty acids in T1 plant #025-16 leaves during different development stages of plant. Fatty acids were analyzed in form of corresponding methyl esters. 16:Me, Methyl palmitate; Z11-16:Me, (Z)-11-hexadecenoic acid methyl ester. Values are the means ± standard error of the mean (SEM) from three replicates. The different letters above the standard error bar indicate a significant difference between the means. The P value less than 0.05 is statistically significant

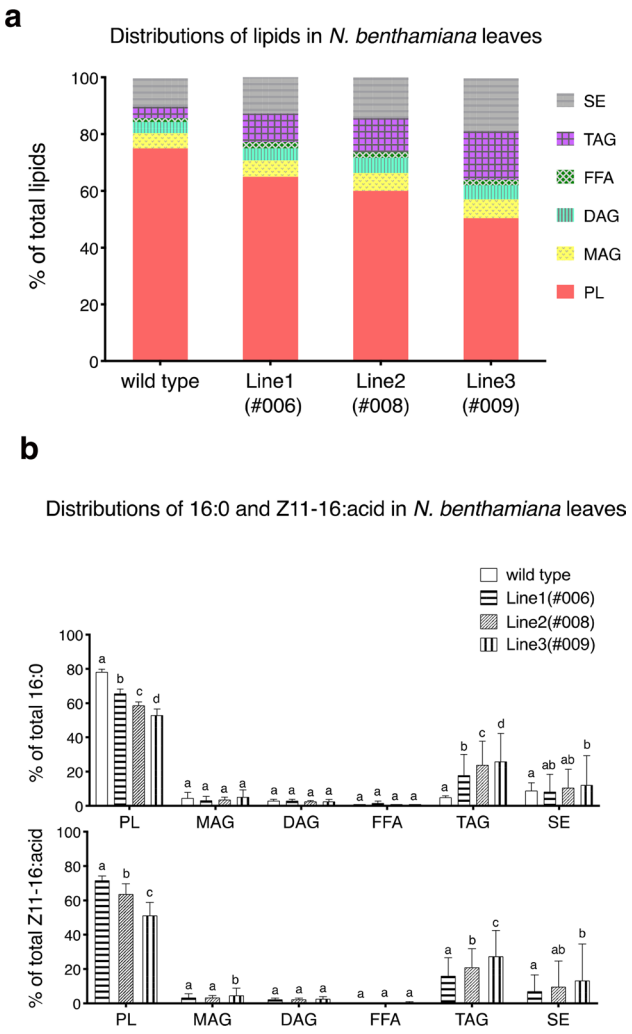

Fig. 7 Leaf fatty acid distribution in *Nicotiana benthamiana CpuFatB1-AtrΔ11* transformants and wild-type plant. **a** Comparison of total lipids distributions between transformed lines and wild type. **b** Comparison of 16:0 and Z11-16:acid distributions between transformed lines and wild type. Fatty acids were analyzed in the form of methyl esters. SE, sterol esters; FFA, free fatty acids; TAG, triacylglycerols; DAG, diacylglycerols; MAG, monoacylglycerols; PL, polar lipids. 16:0, palmitic acid; Z11-16:acid, (*Z*)-11-hexadecenoic acid. Values are the means ± standard error of the mean (SEM) from three replicates. The different letters above the standard error bar indicate a significant difference between the means. The *P* value less than 0.05 is statistically significant

1998), transformation of multiple transgene copies (Carrier et al. 1998; Mansur et al. 2005; Nguyen et al. 2015; Schultz et al. 1987; Fath et al. 2011) and transformation of virus silencing suppressor genes (Naim et al. 2016) might also contribute to higher quantity of pheromone precursor in plant factories.

In conclusion, in the present study we established a novel approach for stable production of moth sex pheromone precursors. *Nicotiana* spp. were genetically modified for production of pheromone precursors via integration of genes using *Agrobacterium*-mediated leaf-disc transformation. This is the first report on an extended production of insect pheromone precursors over generations in plants. Our best line of *N. benthamiana* produced 335 µg of the target compound per gram of fresh leaf, demonstrating it a good platform species for production of C_{16} pheromone precursors, which can be simply converted into pheromones by a semi-synthetic approach. Considering that the production of *N. benthamiana* green biomass is as high as 100–300 tons per hectare (Sheen, 1983; Werner et al. 2011), a worthwhile amount of pheromone precursors may be produced by cultivating our most productive *N. benthamiana* line under field conditions. Also, our study is a step forward toward our long-term vision of producing moth pheromones in stably transformed plants that can be used for direct emission of the pheromones for attraction or mating disruption.

Author contributions statement

YHX, BJD, HLW, CJS, PH and CL conceived and designed the study. YHX and BJD carried out vector design and sequencing; YHX performed leaf-disc transformation,

plant cultivation and all the sample analysis; YHX drafted the manuscript, and all authors reviewed and edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest for the current study.

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