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Engineering of insect juvenile hormone III biosynthesis in the plant *Nicotiana benthamiana*

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ABSTRACT

Juvenile hormones (JHs) are farnesoic acid-derived sesquiterpenoids that play a crucial role in regulating various developmental processes in insects. Based on these reported biological activities, JHs and their synthetic analogs have been utilized as insecticides with significant commercial success over the past years. Here we describe the engineering of the JH pathway of the yellow fever mosquito (*Aedes aegypti*) by transient gene expression in the plant *Nicotiana benthamiana*. This approach led to the successful production of JH III in *N. benthamiana* leaves at a concentration of *ca.* 10 μg/g fresh weight. The co-expression of a feedback-insensitive version of 3-hydroxy-3-methylglutaryl coenzyme A reductase from *Arabidopsis thaliana* further increased the titer eight-fold from 10 to 80 μg/g fresh weight. Our efforts also revealed that the rich endogenous metabolic background of *N. benthamiana* can generate farnesoic acid, a key precursor to JH III, and thus, only 3 genes need to be expressed to provide high titers of this compound. Our study demonstrates the production of high titers of JH III in *N. benthamina* via heterologous expression of insect JH biosynthetic genes.

1. Introduction

Juvenile hormones (JHs) are a group of structurally related acyclic sesquiterpenoids biosynthesized by all insects and a number of non-insect arthropods (Riddiford, 2020; De Kort and Granger, 1996; Chang, 1993). These molecules are produced in the corpora allata, a pair of endocrine glands located in the brain of these animals. The eight known natural JHs exhibit a variety of epoxidation and/or methylation derivatizations on the farnesoic acid backbone (Riddiford, 2020). Juvenile hormone III (JH III) (Fig. 1a) is the most prevalent of the JH derivatives and has been detected in the majority of insects studied to date (Cheong et al., 2015).

Juvenile hormones play key roles in the regulation of various physiological processes in insects, most notably in development and reproductive maturation (Denlinger et al., 2012; Roy et al., 2018). Therefore, JHs are currently being developed for use as insecticides for pest control. In fact, artificial application of JHs or synthetic analogs of JHs has been shown to cause a range of developmental and reproductive defects in insects. For example, fenoxycarb, a synthetic JH analog, causes prolonged instar duration, deformed pupae and larval-pupal intermediates, sterility, inhibited egg production, and abnormal caste differentiation in a number of different insects (Dhadialla et al., 1998; Wilson, 2004). Although JHs have a broad spectrum of insect toxicity, these molecules are less toxic to fish, birds and mammals, making this an attractive compound class for commercial use as insecticides (Dhadialla et al., 1998)

The JH biosynthetic pathway (Fig. 1a) branches from the mevalonate pathway, which provides the starting precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). IPP and DMAPP are converted by the enzyme (*E,E*)-farnesyl pyrophosphate (FPP) synthase to (*E,E*)-FPP, which in turn is converted to (*E,E*)-farnesol, then (*E,E*)-farnesal, and finally (*E,E*)-farnesoic acid by the enzymes farnesyl diphosphate pyrophosphatase, farnesol dehydrogenase, and farnesal dehydrogenase, respectively (Noriega, 2014; Bellés et al., 2005). The subsequent steps vary depending on the insect order. In Orthoptera, Blattodea and Hymenoptera, (*E,E*)-farnesoic acid is methylated by an *O*-methyltransferase to give (*E,E*)-methyl farnesoate, which is then oxidized by an epoxidase to form JH III (Fig. 1a). In Lepidoptera, however, epoxidation precedes methylation (Bellés et al., 2005; Tsang et al., 2020). Although JHs are found in all insects, the yellow fever mosquito (*Aedes aegypti*) is the only species in which the entire biosynthetic pathway has been fully elucidated. However, except for the

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epoxidase, which has been demonstrated to be involved in the formation of JH III by a CRISPR/Cas-mediated knockout line (Nouzova et al., 2021), the other enzymes have only been characterized *in vitro* (Mayoral et al., 2009a; Mayoral et al., 2009b; Rivera-Perez et al., 2013; River-a-Perez et al., 2015; Nyati et al., 2013; van Ekert et al., 2014).

Surprisingly, JH III is not only produced by insects but has also been identified in a number of plants, such as the sedges *Cyperus iria* and *C. aromaticus* (Cyperaceae) (Chan et al., 2010; Toong et al., 1988), *Cananga latifolia* (Annonaceae) (Yang et al., 2013), and the grass *Euclasta condylotricha* (Poaceae) (Bohounton et al., 2023). Grasshopper nymphs fed on *C. iria* leaves showed abnormal development and morphology (Toong et al., 1988), while chopped leaves resuspended in water had lethal effects on mosquito larvae (Schwartz et al., 1998). Similar larvicidal effects have also been observed in *E. condylotricha* against the mosquito *Anopheles gambiae* (Bohounton et al., 2023). These results suggest that JH III biosynthesis has evolved to play a role in the defense systems of these plants. However, how plants produce JH III still remains unclear.

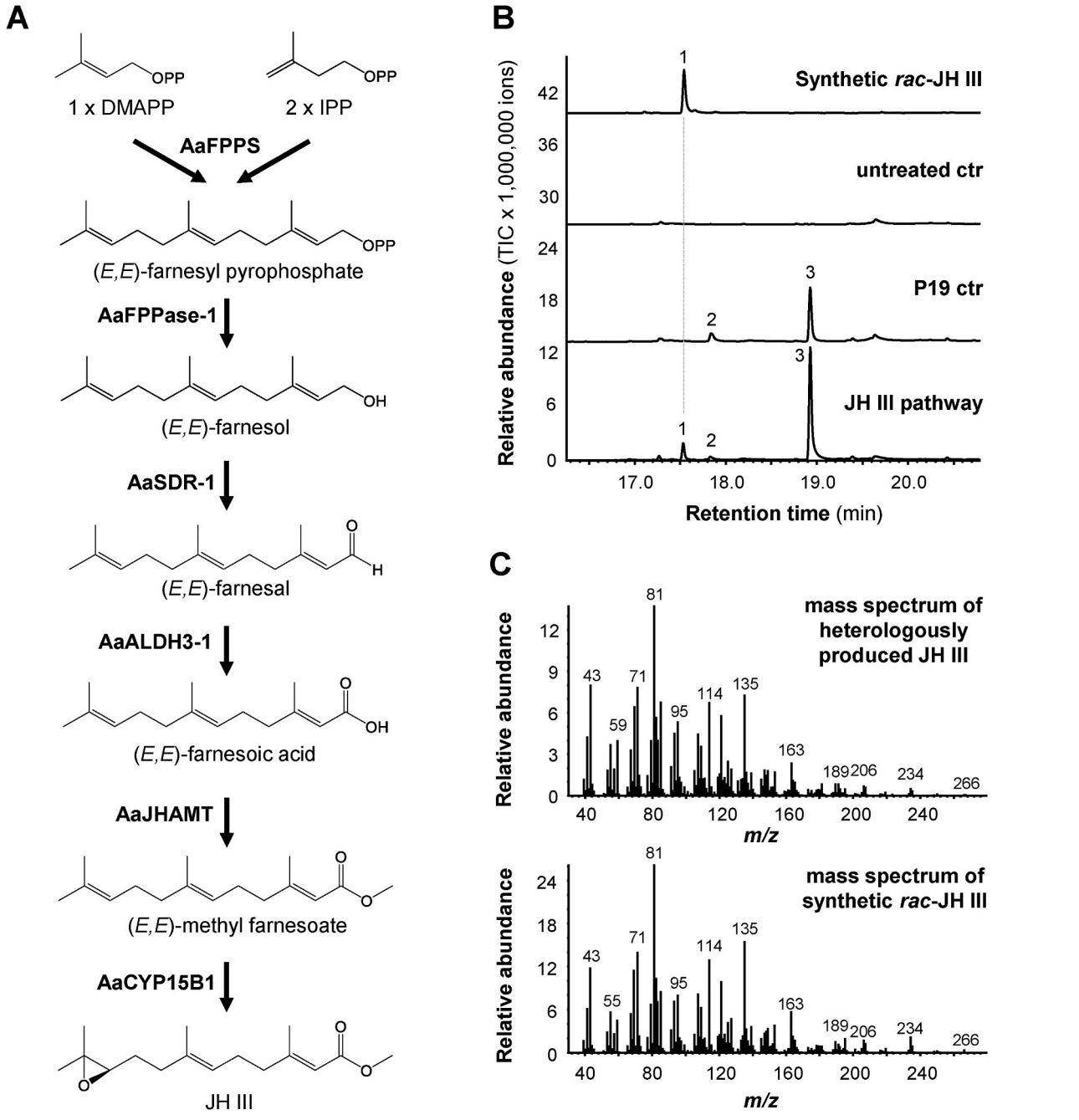

Fig. 1. Engineering of the juvenile hormone pathway from *Aedes aegypti* in *Nicotiana benthamiana*. **(A)** JH III biosynthetic pathway in *Aedes aegypti*. DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate. **(B)** Expression of the JH III pathway in *N. benthamiana* led to the formation of JH III. *AaFPPS* (farnesyl pyrophosphate synthase), *AaFPPase-1* (farnesyl diphosphate pyrophosphatase), *AaSDR-1* (farnesol dehydrogenase), *AaALDH3-1* (farnesal dehydrogenase), *AaJHAMT* (farnesoic acid *O*-methyltransferase), *AaCYP15B1* (methylfarnesoate epoxidase), the P450-reductase gene *AaRed1*, and the silencing suppressor gene *P19* were transiently co-expressed in *N. benthamiana* and hexane leaf extracts were analyzed using gas chromatography-mass spectrometry. Total ion current (TIC) chromatograms of extracts from plants expressing the complete JH III pathway, plants expressing only *P19* (P19 ctr), untreated plants (untreated ctr), and synthetic *rac*-JH III are shown. 1, JH III; 2, unidentified sesquiterpenoid; 3, capsidiol 3-acetate. **(C)** Mass spectra of JH III produced *in planta* (upper) and synthetic *rac*-JH III standard (lower).

Attempts to engineer plants to produce insect-derived natural products by heterologous gene expression of insect enzyme cascades are rare. Two previous examples, the successful engineering of fatty acid-derived moth sex pheromones in *Nicotiana benthamiana* by transiently expressing four steps of the biosynthetic pathways (Ding et al., 2014; Xia et al., 2021) and the production of fatty acids as insect pheromone precursors by stable transformation of *Camelina sativa* (Petkevicius et al., 2020), have been reported. Here, we have established *N. benthamiana* as a production platform for insect JHs by introducing the JH III pathway from *A. aegypti* into this plant. Our work demonstrates the feasibility of producing high yields of JH III by transient transformation of *N. benthamiana* leaves with insect-derived JH III biosynthetic genes. This in turn sets a foundation for engineering plants that capitalize on this naturally evolved insect defense mechanism.

2. Materials and methods

2.1. Cloning and transformation of Agrobacterium tumefaciens cells

Genes were synthesized by Twist Biosciences as codon-optimized sequences and amplified using Platinum SuperFi II PCR Master Mix (ThermoFisher Scientific, catalog number 12368050). Gene sequences and primer sequences are given in Table S1. Amplified products were cleaned using Zymoclean Gel DNA Recovery Kit (200/PK) (ZymoResearch, catalog number D4008) and cloned into the vector p3Ω1 (digested with *Bsa*I-HF) using the In-Fusion® Snap Assembly Master Mix (Takara-Bio, catalog number 638948). The heat-shock method was used to transfer the plasmids into *Escherichia coli* Top 10 and the transformed cells were plated on LB agar containing the appropriate antibiotic selection. Single colonies were then inoculated overnight in liquid LB medium with the appropriate antibiotic selection at 37 °C in a shaking incubator at 250 rpm. Plasmid DNA was isolated using Wizard Plus SV Minipreps DNA Purification System kit (Promega, catalog number A1465), and the inserted sequences were fully sequenced. Electrocompetent *Agrobacterium tumefaciens* cells GV3101 (Goldbio) were transformed using the electroporation method with the different constructs and transformed cells were plated on LB agar containing the appropriate selection marker.

2.2. Agrobacterium-mediated transient expression in Nicotiana benthamiana

Single colonies of the transformed *Agrobacterium* strains were inoculated in 10 mL of LB with the appropriate antibiotics overnight at 28 °C in a shaking incubator at 250 rpm. The overnight cultures were pelleted by centrifugation at 4000 rcf for 10 min at 14 °C. Cell pellets were resuspended in infiltration medium (10 mM MES pH = 5.6, 10 mM $MgCl_2$), and the resulting cell suspensions each containing one of the constructs were mixed to a final OD_{600} of 0.3 to obtain co-infiltration solutions. Acetosyringone was added to each co-infiltration mixture at a final concentration of 150 μM, and then the solutions were incubated in a shaking incubator at 28 °C and 250 rpm for 3 h. The co-infiltration solutions were infiltrated in the abaxial area of *N. benthamiana* (3-week-old) leaves with a needless 1 mL syringe.

2.3. Tissue harvesting and metabolite extraction

Five days post-infiltration, four discs from leaves of each individual plant were excised, weighed, and flash-frozen in liquid nitrogen. Leaf discs were then homogenized using 2 mm diameter metallic beads in a Tissue Lyser II (Qiagen) (20 Hz for 1 min). Homogenized leaf material was transferred in glass vials where 200 μL of hexane was added with a glass capillary pipette. Samples were capped, vortexed, and incubated for 1 h at room temperature. Finally, samples were centrifuged for 5 min at top speed and 100 μL of the supernatant were transferred into new glass vials for gas chromatography-mass spectrometry (GCMS) analysis.

2.4. Feeding leaf discs with (E,E)-farnesol

Four days post-infiltration, leaf discs of infiltrated *N. benthamiana* plants were excised and placed individually into 200 μL HEPES buffer (50 mM, pH 7.5) containing 20 μM (*E,E*)-farnesol (stock solution, 2 mM in DMSO) in a 48-well plate. The plate was sealed with parafilm and placed in a growth chamber overnight (16 h of light, 8 h dark, 25 °C, 40–65% relative humidity). Then, leaf discs were collected in 2 mL plastic tubes, flash frozen in liquid nitrogen, and homogenized using metal beads (2 mm diameter) in a Tissue Lyser II (Qiagen) at 20 Hz for 1 min.

2.5. Synthesis of rac-JH III as a standard for compound identification and quantification

A detailed description of the five-step synthesis of *rac*-JH III starting from (*E,E*)-farnesol can be found in Supplemental dataset 1.

2.6. Gas chromatography-mass spectrometry (GC-MS) analysis

Analysis of hexane extracts was conducted using an Agilent 6890 gas chromatograph coupled to an Agilent MS 5977B quadrupole mass selective detector (quadrupole temp, 150 °C; source temp, 230 °C; electron energy, 70 eV). Samples were injected splitless (injection volume, 1 μL) at an initial oven temperature of 60 °C, which was held for 2 min and then increased to 250 °C with a gradient of 10 °C/min, and further increased to 300 °C with a gradient of 100 °C/min and hold for 2 min. Chromatography was performed using a ZB-5 column (Phenomenex; 39.9 m × 0.25 mm × 0.25 μm, fitted with a 10 m guard column) with He as a carrier gas (3 mL/min). Compounds were identified by comparison of mass spectra and retention times to those of authentic standards (*rac*-JH III, (*E,E*)-farnesol, (*E,E*)-methyl farnesoate) or by comparing with reference spectra in the National Institute of Standards and Technology library (capsidiol 3-acetate).

2.7. Quantification of JH III

A *rac*-JH III stock solution was prepared by dissolving 1 mg of *rac*-JH III in 10 mL hexane to achieve a concentration of 100 ng/μL. The stock solution was diluted with hexane to obtain concentrations ranging from 1 to 25 ng/μL and samples were analyzed using GC-MS. Three technical replicates were used for each measurement. The peak area of *rac*-JH III was assessed and used to build a calibration curve using the MSD Chem Station Data Analysis software (Agilent). A calibration curve and respective R^2 and data are shown in Supplemental dataset 1. The concentration of *rac*-JH III in hexane extracts made from plant material was quantified based on the calibration curve.

2.8. Liquid chromatography-time-of-Flight mass spectrometry analysis

Ground leaf tissue samples (50 mg ± 5%) were extracted with 500 μL methanol, vortexed, and sonicated for 15 min. The samples were then centrifuged at top speed using a tabletop centrifuge for 10 min. The supernatant was filtered with a 0.22 μm PTFE filter and then diluted 1:10 in methanol for LC-MS analysis. For untargeted metabolomics analysis, leaf methanolic extracts (n = 3 biological replicates) were analyzed using a Thermo Scientific UltiMate 3000 ultra-high performance liquid chromatography (UHPLC) system coupled to an Impact II UHR-Q-ToF (Ultra-High-Resolution Quadrupole-Time-of-Flight) mass spectrometer (Bruker Daltonics). Compounds were separated using reverse phase HPLC using a Phenomenex Kinetex XB-C18 column (100 × 2.1 mm, 2.6 μm; 100 Å). For the LC method, the mobile phase consisted of solvent A (10 mM ammonium acetate) and solvent B (methanol) at flow rate of 0.3 mL/min with an injection volume of 2 μL. The gradient used for the chromatographic separation was as follows: 5% B for 1 min, gradual increase from 5% to 90% over the next 4 min, hold at

90% for 2 min and returning to the initial 5% over 3 min. Mass spectrometry analysis was carried out in positive ionization mode. A capillary voltage of 3500 V and an end plate offset of 500 V were used. A nebulizer pressure of 2.2 bar was used, with nitrogen as the drying gas at 250 °C and a flow of 10 L/min. Acquisition was performed at 12 Hz in the mass range from *m/z* 80 to 1000. For collision energy, the stepping option model (from 20 to 50 eV) was used. Sodium formate solution in isopropanol was injected at the beginning of each run for calibration, and the *m/z* values were recalibrated using the expected calibrant ion values. Processing and generation of features was performed using Metaboscape (Bruker) and then exported to the GNPS2 online platform (https://gnps2.org/homepage) using the feature-based molecular network (FBMN) workflow, and SIRIUS software (https://bio.informatik.uni-jena.de/sirius/). The CANOPUS algorithm of SIRIUS was additionally applied for compound class predictions. Finally, molecular networks were exported to Cytoscape software (version 3.10.2) for visualization and to merge the results of GNPS2 and SIRIUS. Features that accumulated in at least one of the treatment groups but not in the P19 control (fold change ≥3) with a mean peak area intensity >10.000 were selected for analysis.

3. Results and discussion

3.1. Reconstitution of the JH III pathway from Aedes aegypti in Nicotiana benthamiana

To engineer the formation of JH III in *Nicotiana benthamiana*, full-length codon-optimized genes encoding farnesyl pyrophosphate synthase (AaFPPS) (Rivera-Perez et al., 2015), farnesyl pyrophosphatase (AaFPPase-1) (Nyati et al., 2013), farnesol dehydrogenase (AaSDR-1) (Mayoral et al., 2009a), farnesal dehydrogenase (AaALDH3-1) (Rivera-Perez et al., 2013), juvenile hormone methyltransferase (AaJHAMT) (Mayoral et al., 2009b; van Ekert et al., 2014), and methyl farnesoate epoxidase (AaCYP15B1) (Nouzova et al., 2021), all from *Aedes aegypti* (Fig. 1a, Table S1), were cloned into binary expression vectors under control of a *ubiquitin-10* promoter and transiently expressed in *N. benthamiana* leaves. Since AaCYP15B1 belongs to the cytochrome P450 family, we co-expressed the P450 reductase (AaRed1) from *A. aegypti* to support electron transfer from NADPH to the P450 enzyme (Vermilion et al., 1981). In addition, P19, which suppresses gene silencing *in planta* and thus improves the efficiency of transient expression (Silhavy, 2002), was co-expressed. Gas chromatography-mass spectrometry (GC-MS) analysis of a hexane extract taken from transformed *N. benthamiana* leaves revealed a compound with a mass of *m/z* 266, which was identified as JH III by comparing its mass spectrum and retention time with those of a synthetic *rac*-JH III standard prepared as described in detail in Supplemental dataset 1 (Fig. 1). Notably, pathway intermediates such as farnesol, farnesal, farnesoic acid, and methyl farnesoate could not be detected. A control plant expressing only P19 as well as an untreated plant showed no accumulation of JH III (Fig. 1).

In addition to JH III, two other abundant sesquiterpenoids were found in the hexane extracts of the leaves. However, these compounds were also observed in the extracts of plants expressing only P19 and in plants infiltrated with the wild-type *Agrobacterium tumefaciens* strain, though were not observed in untreated plants (Fig. 1, Fig. S1). This suggests that these compounds are specifically induced by the bacterial infection and could thus represent potential phytoalexins. Indeed, one of these compounds was tentatively identified based on mass spectra comparisons as capsidiol-3-acetate (Fig. S1), a sesquiterpenoid phytoalexin recently described in *N. benthamiana* and other Solanaceae species (Li et al., 2015; Maurizio Camagna and Takemoto, 2020). The second compound, although structurally related to capsidiol-3-acetate based on the mass spectrum, could not be identified and did not appear to be a biosynthetic intermediate of capsidiol-3-acetate (Greenhagen et al., 2003). More extensive experiments are required to determine whether these sesquiterpene phytoalexins affect the efficiency of *Agrobacterium*-mediated gene expression or if the production of these compounds reduce the available pools of FPP precursor required for production of JH III.

3.2. Enhancing the flux through the mevalonate pathway increased the yield of JH III

Using a calibration curve of synthetic *rac*-JH III, the amount of JH III produced in *N. benthamiana* expressing all of the *A. aegypti* JH III pathway genes was calculated to be 11 ± 4.6 μg/g fresh weight (Fig. 2a). To increase the JH III titer, we first replaced the farnesyl pyrophosphate synthase AaFPPS with the maize (*Zea mays*) FPP synthase (ZmFPPS3-B73) (Seidl-Adams et al., 2015), assuming that a plant FPPS might function more efficiently in the plant host and thus generate more FPP substrate for the JH III pathway. However, *N. benthamiana* plants expressing ZmFPPS3-B73 together with AaFPPase-1, AaSDR-1, AaALDH3-1, AaJHAMT, AaCYP15B1, and AaRed1 produced 18.2 ± 0.5 μg JH III per g fresh weight, which was not significantly different from the JH III concentration found in plants expressing only the *A. aegypti* pathway genes (Fig. 2a). Furthermore, we did not detect any JH III pathway intermediates, suggesting that the availability of IPP and DMAPP as substrates for FPP synthase, rather than the limited efficiency of FPPS or another downstream enzyme, determines the amount of JH III produced.

In plants, IPP and DMAPP that are used as precursors for sesquiterpenes are produced by the mevalonate pathway (Penuelas and Munnebosch, 2005), with the rate-limiting enzyme being 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) (Friesen and Rodwell, 2004). It has been shown that an N-terminal truncated HMGR, which lacks the amino acids involved in feedback regulation and is therefore feedback-insensitive, can be used to increase production of the mevalonate pathway products IPP and DMAPP (Wang et al., 2019). Therefore, we co-expressed a truncated version of HMGR from *Arabidopsis thaliana* together with the genes of the JH III pathway from *A. aegypti* in *N. benthamiana*. Quantification of JH III revealed a concentration of 89.5 ± 21.9 μg JH III per g fresh weight, which corresponds to an eight-fold increase when compared with plants that lacked this HMGR gene (Fig. 2a).

We then tested whether feeding leaf discs with an intermediate of the JH III pathway, specifically (*E,E*)-farnesol, could further increase the yield of JH III. To this end, leaves co-expressing the entire JH III pathway together with AttHMGR were excised and fed with (*E,E*)-farnesol for 24 h. While leaves fed with (*E,E*)-farnesol produced 95.8 ± 8.4 μg JH III per g fresh weight, 62.2 ± 2.9 μg JH III per g fresh weight could be detected in plants overexpressing the full pathway but not provided with the substrate (Fig. 2b). Thus, substrate feeding provided only a modest increase in product titers.

In insects, the titers of juvenile hormones exhibit considerable variation across species and life stages. For the majority of species, a range of 0.1–100 ng/g fresh weight has been reported, although concentrations of up to 1 μg/g fresh weight have been reported (Schooley et al., 1984). The JH III concentrations obtained in *N. benthamiana* in our study were found to be significantly higher than those previously reported for insects. However, these levels are comparable to concentrations reported for the plant *C. iria* (ca. 50–200 μg/g fresh weight), which produces JH III as a natural insecticide (Bede and Tobe, 2000; Schwartz et al., 1998; Toong et al., 1988). Therefore, our results show that reconstructing the JH III pathway in a plant can, in principle, lead to JH III concentrations that can be toxic or lethal to insects.

3.3. The endogenous metabolism of Nicotiana benthamiana can produce the JH III biosynthetic intermediate farnesoic acid

N. benthamiana is well known as a heterologous platform for the production of plant natural products; however, *N. benthamiana* has been

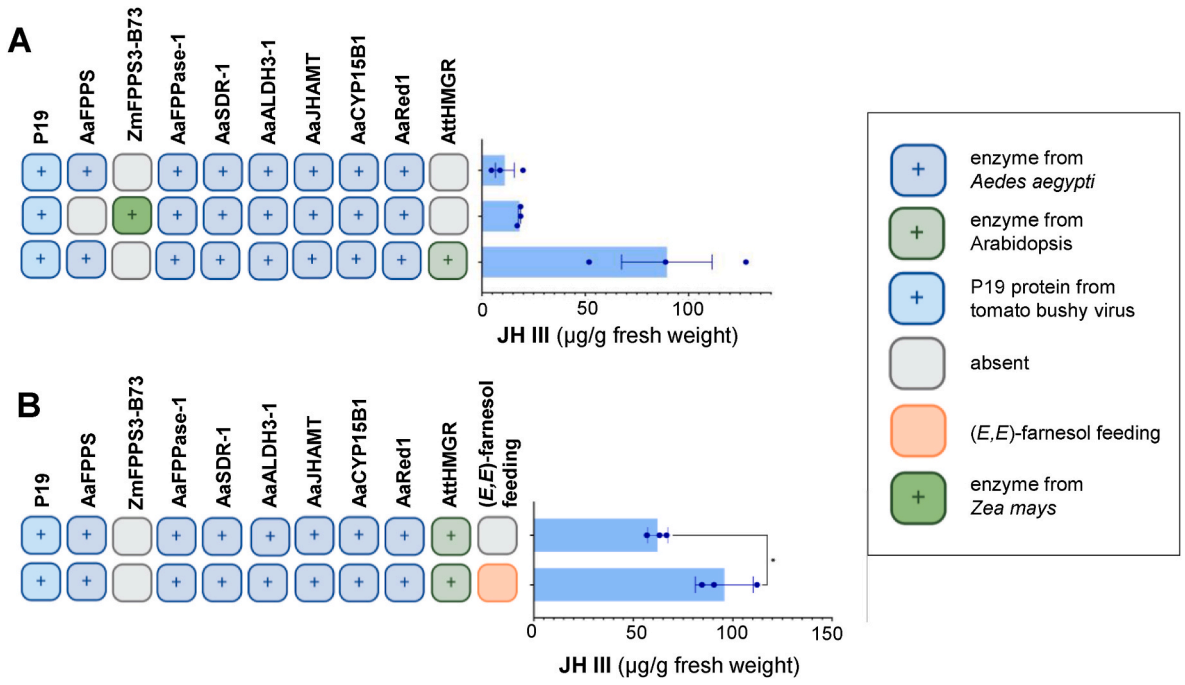

Fig. 2. Optimization of JH III production in *Nicotiana benthamiana*. **(A)** Genes were co-expressed in *N. benthamiana*, leaves were extracted with hexane, and JH III was quantified by gas chromatography-mass spectrometry (GC-MS) following calibration with a synthetic *rac*-JH III standard. AttHMGR, N-terminal truncated and thus feedback-insensitive version of *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl-coenzyme A reductase; ZmFPPS3-B73, farnesyl pyrophosphate synthase from *Zea mays*. **(B)** Genes were co-expressed in *N. benthamiana* and leaf discs were incubated in buffer containing the JH III pathway intermediate (*E,E*)-farnesol. JH III in hexane extracts made from the leaf discs was analyzed by GC-MS and quantified using a calibration curve of synthetic *rac*-JH III. Mean and standard error values are shown ($n = 3$ biological replicates). The asterisk indicates a significant difference (p-value <0.05) between the two groups (paired *t*-test). Created with BioRender.com.

shown to modify heterologously produced products due to the extensive endogenous metabolic activity found in this plant (Liu et al., 2014; Dudley et al., 2022). To assess whether the endogenous enzymes of *N. benthamiana* can catalyze any of the individual steps of the JH III pathway, we performed a dropout experiment in which each enzyme was individually excluded from the reconstructed pathway. The results presented in Fig. 3a show that *N. benthamiana* indeed possesses endogenous enzymes that can catalyze the first four steps of the pathway. The titers of JH III suggest that these endogenous enzymes fully compensate for the corresponding insect enzymes. Moreover, expression of only the last two enzymes of the JH III pathway (AaJHAMT and AaCYP15B1), together with AaRed1 and AttHMGR, resulted in JH III levels comparable to those in plants expressing the entire pathway (Fig. 3b). This clearly indicates that the individual endogenous activities of *N. benthamiana* form an efficient pathway to farnesoic acid.

While we did not detect any endogenous farnesoic acid methyltransferase activity, omission of the P450 enzyme AaCYP15B1 from the JH III pathway resulted in the accumulation of small but detectable amounts of JH III (Fig. 3a, Fig. S2). This shows that even the epoxidation of methyl farnesoate can be catalyzed to a minor extent by an endogenous *N. benthamiana* P450. However, the efficiency of this endogenous activity was low, as evidenced not only by the small amount of JH III produced, but also by the observation of the pathway intermediate methyl farnesoate (Fig. S3). Notably, neither the presence nor absence of the P450 reductase AaRed1 had a significant effect on JH III accumulation, which indicates that the P450 reductase of *N. benthamiana* can also interact with the insect P450 AaCYP15B1.

Untargeted liquid chromatography-Time of Flight mass spectrometry (LC-qTOF) analysis of methanolic plant extracts revealed different metabolic profiles for plants expressing either the entire JH III pathway together with AttHMGR, expressing only AaFPPS, AaFPPase-1, and AttHMGR, or expressing AaFPPS, AaFPPase-1, AaSDR-1, AaALDH3-1, and AttHMGR compared to plants expressing only P19 as a control. Among the up-regulated compounds, several were predicted to be sesquiterpenoids, which also shows that *N. benthamiana* has background activities that may act on metabolic intermediates of the JH III pathway such as FPP as a substrate for endogenous terpene synthases (TPS) (Table S2).

While insects often use pyrophosphatases to produce farnesol or geraniol from their respective pyrophosphates (Cao et al., 2009; Nyati et al., 2013; Köllner et al., 2022), plants usually possess terpene synthases that catalyze these reactions (Degenhardt et al., 2009). StTPS18 from potato (*Solanum tuberosum*), for example, has been shown to convert FPP to (*E,E*)-farnesol (Dwivedi et al., 2022). Since *N. benthamiana* belongs to the same plant family as *S. tuberosum*, it seems likely that the *N. benthamiana* StTPS18 ortholog could be responsible for production of the JH III biosynthetic intermediate (*E,E*)-farnesol. Farnesol dehydrogenases have also been reported in plants from different families, including *A. thaliana* (Bhandari et al., 2010), *Ipomoea batatas* (Inoue et al., 1984), *Polygonum minus* (Ahmad-Sohdi et al., 2015), and *Theobroma cacao* (Satyaveanthan et al., 2021), suggesting that these enzymes are widely distributed in plants. Although a plant-derived farnesal dehydrogenase has been functionally characterized (*P. minus*), the physiological role of the product, (*E,E*)-farnesoic acid, is unclear in these plants (Seman-Kamarulzaman et al., 2016). Overall, these reports and our results suggest that many plants may have evolved the metabolic machinery to produce the JH III precursor (*E,E*)-farnesoic acid.

Interestingly, a farnesoic acid carboxyl methyltransferase (FAMT) that can methylate farnesoic acid has been reported in *A. thaliana*, even though methyl farnesoate has not yet been identified in this plant (Yang et al., 2006). Methyl farnesoate has, however, been reported in other plants (e.g. *C. iria*), but none of the responsible enzymes have been identified (Toong et al., 1988). Our reconstitution experiments indicate that *N. benthamiana* does not have an FAMT and thus heterologous expression of an FAMT from an insect or another plant is required for *in planta* production of JH III.

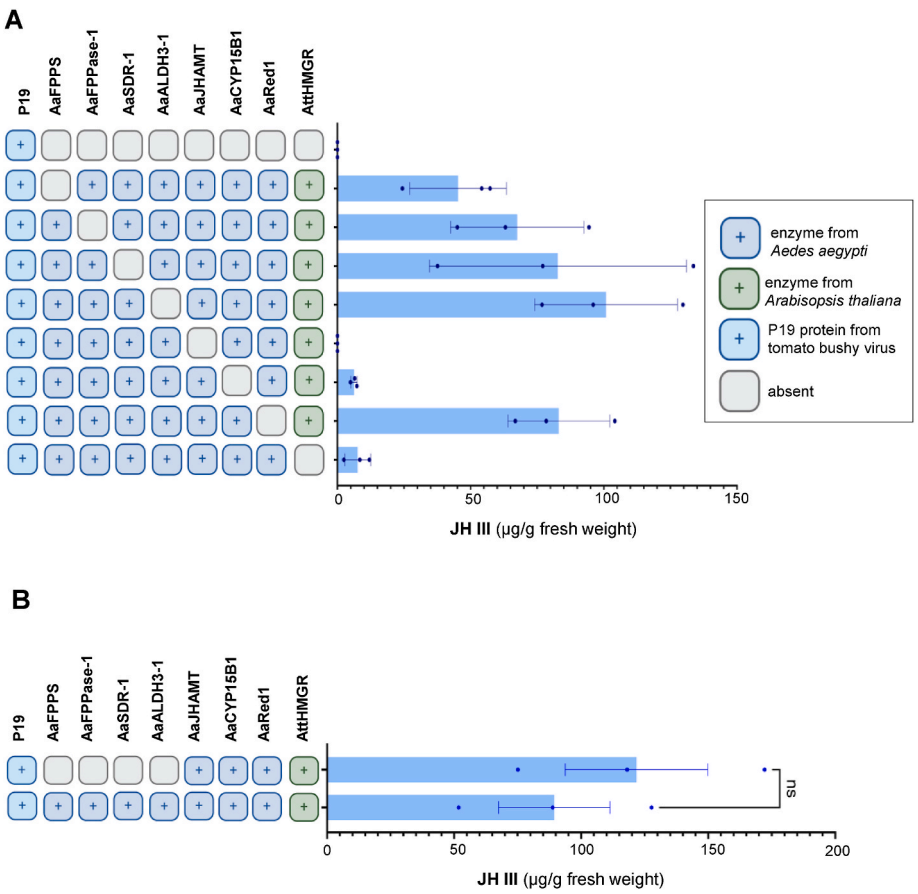

Fig. 3. ***Nicotiana benthamiana*** **possesses background activity for the first four steps of the JH III pathway**. Genes were co-expressed in *Nicotiana benthamiana*, leaves were extracted with hexane, and JH III was analyzed by gas chromatography-mass spectrometry (GCMS) and quantified using a calibration curve of synthetic *rac*-JH III. AttHMGR, N-terminal truncated version of *Arabidopsis thaliana* 3-hydroxy-3-methylglutaryl-coenzyme A reductase **(A)** Dropout analysis of JH III pathway genes in which each of the individual biosynthetic genes are omitted. Means and standard errors are shown ($n = 3$ biological replicates). Abbreviations ns: not significant (p-value >0.05) **(B)** Comparison of JH III titers between plants that express genes encoding for the entire biosynthetic pathway and plants that express only *AaJHAMT* and *AaCYP15B1*. Means and standard errors are shown ($n = 3$ biological replicates). Abbreviations ns: not significant (p-value >0.05).

4. Conclusion

Here, we show that *N. benthamiana* is a well-suited platform for the production of large amounts of JH III by transient expression of insect-derived biosynthetic genes. We have also shown that *N. benthamiana* has a rich metabolic background that can contribute to efficient JH III production. Thus, these engineering efforts also suggest that the sporadic distribution of JH III in the plant kingdom is a result of an evolutionary scenario in which pre-existing enzyme activities could be rapidly assembled into a functional complete or partial JH III pathway. In *N. benthamiana*, only 3 genes (AaJHAMT and AaCYP15B1, along with overexpression of the mevalonic acid pathway enzyme HMGR) need to be expressed to provide titers high enough to confer insecticidal activity. While transient transformation, as performed in this work, is suitable for proof-of-concept studies, long-term or large-scale applications require the generation of stably transformed plants. Stable transformation of crop plants could in principle be achieved by using a multigene construct carrying all genes necessary for JH III production (e.g., AaJHAMT, AaCYP15B1, and AttHMGR in the case of *N. benthamiana*). Since different plant species may have different metabolic backgrounds, initial studies should investigate the presence or absence of endogenous enzymatic activities for each step of the pathway in the target species. Overexpression of the JH III pathway under the control of strong and constitutive promoters, as done in this study, could lead to a significant metabolic burden on the plant, especially considering that JH III production directly competes with the formation of other terpenes, including higher ones such as triterpenoids and tetraterpenoids, which are essential for the plant. However, by fine-tuning the expression of the transgenes using appropriate promoters (Dey et al., 2015), the expression could be adjusted so that the overall fitness of the plant is not compromised by the introduced metabolic pathway. For example, the use of a herbivore-inducible or wound-inducible promoter would ensure that the expression of the pathway genes and thus the formation of JH III only occurs upon wounding and that the metabolic stress on the plant is limited. Furthermore, if JH III is only produced in response to herbivory, it is very unlikely that beneficial insects that do not feed on these plants will come into contact with JH III and suffer any damage. However, further studies, including plant-insect assays and plant fitness tests, are needed to investigate the effects of plant JH III production on beneficial insects and potential metabolic stresses on the plant. Overall, the results presented in this paper pave the way for the development of insect resistance in economically important crops.

CRediT authorship contribution statement

Angeliki Stathaki: Writing – original draft, Investigation, Conceptualization. **Ryan M. Alam:** Writing – review & editing, Investigation. **Tobias G. Köllner:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Sarah E. O'Connor:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Competing interest statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2024.12.005.

Data availability

Data will be made available on request.

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