

This is a repository copy of *Silencing amorpha-4,11-diene synthase genes in Artemisia annua leads to FPP accumulation.*

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/130277/>

Version: Accepted Version

Article:

Catania, Theresa May orcid.org/0000-0002-9882-3878, Branigan, Caroline Anna, Stawniak, Natalia et al. (5 more authors) (2018) Silencing amorpha-4,11-diene synthase genes in Artemisia annua leads to FPP accumulation. *Frontiers in Plant Science*. 547. ISSN 1664-462X

<https://doi.org/10.3389/fpls.2018.00547>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

Silencing amorpha-4,11-diene synthase genes in *Artemisia annua* leads to FPP accumulation with little effect on endogenous terpenes

Theresa M. Catania¹, Caroline Branigan¹, Natalia Stawniak¹, Jennifer Hodson¹, David Harvey¹, Tony R. Larson¹, Tomasz Czechowski¹, Ian A. Graham^{1*}

¹Centre for Novel Agricultural Products, Department of Biology, University of York, United Kingdom

Submitted to Journal:
Frontiers in Plant Science

Specialty Section:
Plant Biotechnology

Article type:
Original Research Article

Manuscript ID:
359955

Received on:
02 Feb 2018

Revised on:
28 Mar 2018

Frontiers website link:
www.frontiersin.org

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

TC, TMC and IAG designed the experiments; TC, TMC, CB, NS, JH and DH performed the experiments; TMC, TC, CB, DH and TL analysed the data; TMC, TC, TL and IAG wrote the manuscript; and all authors revised and approved the manuscript.

Keywords

trichomes, *Artemisia annua*, Artemisinin, Farnesyl diphosphate, amorpha-4,11-diene, Sesquiterpenes, transformation

Abstract

Word count: 307

Artemisia annua is established as an efficient crop for the production of the anti-malarial compound artemisinin, a sesquiterpene lactone synthesised and stored in Glandular Secretory Trichomes (GSTs) located on the leaves and inflorescences. Amorpha-4,11-diene synthase (AMS) catalyses the conversion of farnesyl pyrophosphate (FPP) to amorpha-4,11-diene and diphosphate, which is the first committed step in the synthesis of artemisinin. FPP is the precursor for sesquiterpene and sterol biosynthesis in the plant.

This work aimed to investigate the effect of blocking the synthesis of artemisinin in the GSTs of a high artemisinin yielding line, *Artemis*, by down regulating AMS. We determined that there are up to 12 AMS gene copies in *Artemis*, all expressed in GSTs. We used sequence homology to design an RNAi construct under the control of a GST specific promoter that was predicted to be effective against all 12 of these genes. Stable transformation of *Artemis* with this construct resulted in over 95% reduction in the content of artemisinin and related products, and a significant increase in the FPP pool.

The *Artemis* AMS silenced lines showed no morphological alterations, and metabolomic and gene expression analysis did not detect any changes in the levels of other major sesquiterpene compounds or sesquiterpene synthase genes in leaf material. FPP also acts as a precursor for squalene and sterol biosynthesis but levels of these compounds were also not altered in the AMS silenced lines. Four unknown oxygenated sesquiterpenes were produced in these lines, but at extremely low levels compared to *Artemis* non-transformed controls.

This study finds that engineering *A. annua* GSTs in an *Artemis* background results in endogenous terpenes related to artemisinin being depleted with the precursor FPP actually accumulating rather than being utilised by other endogenous enzymes. The challenge now is to establish if this precursor pool can act as substrate for production of alternative sesquiterpenes in *A. annua*.

Funding statement

We acknowledge financial support for this project from The Bill and Melinda Gates Foundation as well as from The Garfield Weston Foundation for the Centre for Novel Agricultural Products.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

1 **Silencing *amorpha-4,11-diene synthase* genes in *Artemisia annua*** 2 **leads to FPP accumulation with little effect on endogenous terpenes**

3
4 Theresa M. Catania¹, Caroline Branigan¹, Natalia Stawniak¹, Jennifer Hodson¹, David Harvey¹,
5 Tony R. Larson¹, Tomasz Czechowski¹ and Ian A. Graham^{1*}

6 ¹Centre for Novel Agricultural Products, Department of Biology, University of York,
7 Heslington, York YO10 5DD, United Kingdom

8 Address for correspondence to ian.graham@york.ac.uk

9 Keywords: trichomes, *Artemisia*, Artemisinin, Farnesyl diphosphate, *Amorpha-4,11-diene*,
10 sesquiterpene, transformation

11 **Abstract**

12 *Artemisia annua* is established as an efficient crop for the production of the anti-malarial
13 compound artemisinin, a sesquiterpene lactone synthesised and stored in Glandular Secretory
14 Trichomes (GSTs) located on the leaves and inflorescences. *Amorpha-4,11-diene synthase*
15 (*AMS*) catalyses the conversion of farnesyl pyrophosphate (FPP) to amorpha-4,11-diene and
16 diphosphate, which is the first committed step in the synthesis of artemisinin. FPP is the
17 precursor for sesquiterpene and sterol biosynthesis in the plant.

18 This work aimed to investigate the effect of blocking the synthesis of artemisinin in the GSTs
19 of a high artemisinin yielding line, *Artemis*, by down regulating *AMS*. We determined that
20 there are up to 12 *AMS* gene copies in *Artemis*, all expressed in GSTs. We used sequence
21 homology to design an RNAi construct under the control of a GST specific promoter that was
22 predicted to be effective against all 12 of these genes. Stable transformation of *Artemis* with
23 this construct resulted in over 95% reduction in the content of artemisinin and related products,
24 and a significant increase in the FPP pool.

25 The *Artemis AMS* silenced lines showed no morphological alterations, and metabolomic and
26 gene expression analysis did not detect any changes in the levels of other major sesquiterpene
27 compounds or sesquiterpene synthase genes in leaf material. FPP also acts as a precursor for
28 squalene and sterol biosynthesis but levels of these compounds were also not altered in the
29 *AMS* silenced lines. Four unknown oxygenated sesquiterpenes were produced in these lines,
30 but at extremely low levels compared to *Artemis* non-transformed controls.

31 This study finds that engineering *A. annua* GSTs in an *Artemis* background results in
32 endogenous terpenes related to artemisinin being depleted with the precursor FPP actually
33 accumulating rather than being utilised by other endogenous enzymes. The challenge now is
34 to establish if this precursor pool can act as substrate for production of alternative
35 sesquiterpenes in *A. annua*.

36

37 1 Introduction

38 *Artemisia annua* (*A. annua*) is a herbaceous plant from the Asteraceae family native to Asia,
39 known to synthesise the leading antimalarial compound artemisinin a sesquiterpene lactone,
40 within its glandular secretory trichomes (GSTs) (Duke et al. 1994; Olsson et al. 2009). The
41 GSTs of *A.annua* are biseriate glandular trichomes made up of 10 cells topped with a secretory
42 sac. The secretory sac is bounded by a cuticle proximal to the three apical pairs of cells. This
43 arrangement allows phytotoxic compounds such as artemisinin to be sequestered away from
44 the plant, preventing autotoxicity (Duke and Paul 1993; Duke et al. 1994; Ferreira and Janick,
45 1995). The distribution of *A. annua* GSTs across leaves, stems, and inflorescences, combined
46 with the relative ease of artemisinin extraction using organic solvents, has made feasible the
47 commercial scale growth and processing of whole plants for production of the compound as an
48 active pharmaceutical ingredient in Artemisinin Combination Therapies (ACTs). The
49 efficiency of the *A. annua* production system, which can yield artemisinin at greater than 1%
50 lead dry weight and 41.3 Kg per Ha (Ferreira et al. 2005) has meant that it persists as the most
51 efficient and economically feasible platform for production of artemisinin today. With *A.*
52 *annua* as the sole source of artemisinin, demands for the drug have influenced farmers on
53 whether to grow the crop or not, leading to large market price fluctuations (highs of \$1,100 in
54 2005 to less than \$250 per kilogram in 2007 and again in 2015 (Peplow 2016; Noorden 2010).
55 A desire to both stabilize and reduce costs in the supply chain has driven research into yield
56 improvement through modern marker assisted plant breeding and genetic engineering methods
57 and through engineering artemisinin (or precursor) synthesis in heterologous hosts (Ferreira et
58 al. 2005; Han et al. 2006; Graham et al. 2010; Zhang et al. 2011; Paddon and Keasling 2014;
59 Tang et al. 2014; Pulice et al., 2016)

60 The commercial importance of artemisinin synthesis has stimulated ongoing research into the
61 biosynthetic pathways and metabolic capabilities of *A. annua* GSTs. Transcriptomic analysis
62 of GSTs from *A. annua* has identified multiple genes including cytochrome P450s and terpene
63 synthases, which have been subsequently characterised in detail and their trichome-specific
64 expression patterns confirmed (Wang et al. 2009; Olsson et al. 2009; Graham et al. 2010;
65 Olofsson et al. 2011; Olofsson et al. 2012; Soetaert et al. 2013). Metabolomic analysis in *A.*
66 *annua* has identified almost six hundred secondary and/or specialized metabolites, whose
67 production can be linked to the expression of the identified synthases (Brown 2010). This
68 suggests that the GSTs of *A. annua* are highly evolved terpenoid-producing factories, with the
69 potential for producing and storing a diverse range of compounds.

70 The biosynthesis of terpenoids including artemisinin in *A. annua* starts with the biosynthetic
71 precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP),
72 which are in turn products of the methyl erythritol phosphate (MEP) and mevalonate (MVA)
73 pathways (Croteau et al. 2000; Weathers et al. 2006; Wu et al. 2006). IPP and DMAPP
74 precursors for the synthesis of farnesyl pyrophosphate (FPP), which is in turn the immediate
75 precursor of both sterols and sesquiterpenes including artemisinin (Figure 1). Currently 5
76 sesquiterpene synthases have been cloned from *A. annua*, *amorpha-4,11-diene synthase*
77 (*AMS*), the first step in artemisinin synthesis (Bouwmeester and Wallaart 1999); *caryophyllene*
78 *synthase* (*CPS*) (Cai et al. 2002); *germacrene A synthase* (*GAS*) (Bertea et al. 2006); δ -
79 *epicederol synthase* (*ECS*) (Mercke et al. 1999) and *beta farnesene synthase* (*FS*) (Picaud et
80 al. 2005). The expression of these synthases is shown to be predominantly in GSTs and young
81 leaf tissue (Graham et al. 2010; Olofsson et al. 2011) Other sesquiterpenes such as guaianes,
82 longipinanes and eudesmanes have also been isolated suggesting the expression of other
83 sesquiterpene synthases (Brown 2010; Olofsson et al. 2011). These synthases all compete for
84 the precursor FPP, and engineering of the pathway from this point by overexpression of *AMS*

85 (Ma et al. 2009; Ma et al. 2015; Han et al. 2016) or silencing of CPS, BFS, GAS and ECS
86 (Chen et al. 2011; Lv et al. 2016) has been a strategy for increasing artemisinin production.
87 FPP is also utilized by *squalene synthase (SQS)* in the first committed step to sterol synthesis.
88 Silencing of this synthase is shown to remove the sink on FPP from squalene and sterol
89 production resulting in increased artemisinin yield (Yang et al. 2008; Zhang et al. 2009). In
90 these studies flux is altered in the artemisinin pathway leading to increased artemisinin yields
91 when compared to wild type, Ma et al. (2015) and Lv et al. (2016) also show that by
92 manipulating the pathway other endogenous terpenes were also affected.

93 Czechowski et al. (2016) showed a mutation disrupting the amorpha-4,11-diene C-12 oxidase
94 (CYP71AV1) in the high artemisinin yielding cultivar Artemis, produced a novel sesquiterpene
95 epoxide derivative at levels similar to artemisinin (arteannuin X; 0.3 - 0.5% of leaf dry weight).
96 This discovery demonstrates the possibility of engineering *A. annua* GSTs to produce
97 alternative, potentially useful, sesquiterpenes at commercially viable levels. GSTs are targeted
98 for engineering based on their ability to synthesis and store specialized metabolites
99 (Huchelmann et al. 2017). Efforts to engineer GSTs reported in the literature include examples
100 in tobacco, and tomato. Tissier et al. (2013) engineered tobacco GSTs to successfully produce
101 casbene and taxadiene, although production was at levels lower than that for endogenous
102 diterpenoids. The engineering of tomato trichomes has also been carried out by expressing
103 sesquiterpene synthases from wild relatives to confer pest resistance (Bleeker et al. 2012; Yu
104 and Pichersky 2014). Kortbeek et al. (2016) have also used the GSTs of tomato as a platform
105 for engineering sesquiterpenoid production by the overexpression of an avian FPS (farnesyl
106 diphosphate synthase), to increase FPP availability for sesquiterpene production. Engineering
107 of *A. annua* trichomes has been mainly centred on enhancing artemisinin production by
108 constitutively expressing upstream enzymes, artemisinin biosynthetic genes and transcription
109 factors (Tang et al. 2014; Xie et al. 2016; Ikram and Simonsen 2017). More complex pathway
110 regulation has also been attempted - a patent by Tang et al. (2011) describes a method for using
111 the trichome specific *cyp71av1* promoter to drive both the expression of an *ADS (amorpha-*
112 *4,11- diene synthase /AMS)* silencing construct and the patchouli alcohol biosynthesis enzyme,
113 to allow the production of patchouli alcohol in *A. annua*.

114 The objective of the current work was to silence AMS in the GSTs of Artemis a high
115 artemisinin yielding cultivar. Silencing of AMS in this background allowed us to investigate
116 how carbon flux through FPP is affected, in contrast to previous studies performed in low-ART
117 yielding systems where alternative products accumulate (Ma et al. 2015; Lv et al. 2016). We
118 demonstrate that removing artemisinin and related compounds elevates the FPP pool with only
119 minor increases in alternative endogenous metabolites. We conclude that such manipulations,
120 done in a carefully selected genetic background, have the potential to provide a clean chemical
121 background for pathway engineering, without detrimental effects on plant growth and
122 development.

123

124 **2 Methods**

125 **2.1 Plant material**

126 The *A. annua* cultivar Artemis, an F1 hybrid from Mediplant (Conthey, Switzerland) (described
127 in Graham et al. 2010) was used to generate stably transformed material.

128 **2.2 AMS gene copy determination by qPCR**

129 DNA extraction was carried out on 30-50 mg of fresh leaf material harvested from plants
130 growing in the glasshouse and prepared following the methods as described in Graham et al.
131 (2010) and Czechowski et al. (2016).

132 Three technical replications of a 10 µl reaction containing 1 ng of leaf genomic DNA from
133 single plants, 200 nM gene-specific primers in 1x Power SYBER Green PCR Master Mix (Life
134 Technologies Ltd.), were run on a ViiA7 Real-Time PCR system (Life Technologies Ltd.) The
135 gene specific primers were as follows:

136 *AMS_3'*endF: TCTACTCGTTTATCCTATGAGTATATGACTACC

137 *AMS_3'*endR: GGCTATGCACGAAGGATTGGT

138 *AMS_5'*endF: TTACCGAAATACAACGGGCAC

139 *AMS_5'*endR: TTGGCAACCTTTTCCAAAGG

140 Amplification conditions and data normalisation were as described in Czechowski et al. (2016).

141 **2.3 RNA isolation and cDNA synthesis**

142 Fresh young leaf material (leaves 1 - 5) (30-50 mg) from 12-week-old glasshouse grown
143 cuttings was harvested and flash frozen in liquid nitrogen for RNA extraction. The extraction
144 was carried out using the Qiagen RNAeasy kit following the manufacturer's plant protocol
145 including the on column Qiagen DNase treatment. Extracted RNA was quantified
146 spectrophotometrically using the NanoDrop-8000 (NanoDrop products). cDNA was
147 synthesised from 3 µg of the extracted RNA using Invitrogen superscript II reverse
148 transcriptase kit (Thermo Fischer Scientific) using the oligo (dT) primer following the
149 manufacturers protocol.

150 **2.4 Construction of hpRNA vector targeting the AMS gene**

151 Two sections of the *AMS* gene (AF138959) from bases 96 - 192 and 1485 - 1615 were selected
152 and joined to create a 227 bp sequence which was checked for its specificity to the *AMS* target
153 relative to other sesquiterpene synthases from *A. annua* (Cai et al. 2002; Berteau et al. 2006;
154 Mercke et al. 1999; Picaud et al. 2005) (supplemental figure 1). This sequence was then placed
155 in a forward and reverse direction either side of the Chalcone synthase A intron (*petunia*
156 *hybrida*) to create a hairpin construct (Watson et al. 2005), this was driven by the trichome
157 specific promoter *cypav171* (Wang et al. 2011). The full construct was synthesised by
158 GENEART Thermo life technologies. The 3.8 kb construct was cloned into the pRSC2 binary
159 vector and transformed into stratagene solopack gold competent cells. The resulting colonies
160 were tested by PCR using Promega Gotaq and primers designed for the pRSC2 vector.

161 AP1435 pRSC2_activ TAACATCCAACGTCGCTTTTCAG

162 AP1436 pRSC2_RB_in GCCAATATATCCTGTCAAACAC

163 Positive colonies were confirmed by sequencing and the binary vector was then transferred into
164 *Agrobacterium tumefaciens* (LBA4404) by electroporation and 100 µl glycerol stocks set up
165 for subsequent transformations. 48 hours prior to transformation the agrobacterium pre-
166 cultures were set up from glycerol stocks in Luria-Bertani broth (LB) including antibiotic
167 selection (50 mg/L rifampicin, spectomycin and streptomycin). After 24 hours, a 50 ml main
168 culture was set up and allowed to grow overnight to an optical density (OD) of between 0.3
169 and 0.8. At this stage the cultures were spun down and resuspended in co-cultivation media

170 (Murashige and Skoog medium (MS) with 3% sucrose and 100uM acetosyringone) to an OD
171 of 0.2. The culture was then left to shake at 28 °C for 2 hours.

172 **2.5 *Artemisia annua* transformation**

173 Artemis seed were surface sterilised for 1 hour using chlorine vapour (3% HCL in water + one
174 presept tablet (Advanced sterilisation products)) in a sealed box. Seeds were sown into sterile
175 glass jars on MS basal media containing 3% sucrose, 1x MS vitamins and 0.8% plant agar.
176 After sowing the jars were closed and sealed with parafilm and transferred to a growth room
177 16 hours daylength at 29 °C to germinate and grow for 2.5 weeks.

178 The first true leaves of 2.5-week-old seedlings were excised and immersed in petri dishes into
179 either an agrobacterium suspension, or for non-transformed controls (NTC), co-cultivation
180 media without agrobacterium, and placed on a rotary platform. After 15 minutes, the explants
181 were blotted on sterile filter paper and transferred to labelled co-cultivation plates (MS with
182 3% sucrose and 100 µM acetosyringone +0.8% plant agar) the plates were wrapped in foil and
183 stored in the growth room at 25 °C. After 48h the explants were transferred to selection plates
184 (MS medium with 3% sucrose, 0.5 mg/L 6-benzylaminopurine (BAP) and 0.05 mg/L α -
185 naphthalene acetic acid (NAA), 0.8% agar, 500 mg/L carbenicillin and 15 mg/L kanamycin.
186 NTC explants were plated out without kanamycin selection. Explants were transferred to fresh
187 plates after a week and thereafter every 2 weeks. Shoots were excised from the plates as they
188 emerged and placed onto shooting medium in jars (MS medium with 3% sucrose, 0.5 mg/L
189 BAP and 0.05 mg/L NAA, 0.8% plant agar, 500 mg/L carbenicillin, 15 mg/L kanamycin), and
190 transferred on every 3 weeks. NTC shoots were placed into shooting medium containing no
191 antibiotic. Once the shoots were well-established they were transferred to rooting medium (1/2
192 MS medium, 1% sucrose, 0.6% plant agar, 500 mg/L carb, 15 mg/L kanamycin, (NTCs with
193 no antibiotics). Once roots had begun to appear, the shoots were transferred to F2+S compost
194 in P40s and kept in green propagator trays with lids on to maintain humidity. Once well rooted
195 the plants were hardened off and transferred to 4-inch pots in F2 compost and grown in
196 glasshouse facilities at the University of York under long day conditions maintained with
197 supplemental lighting and temperature between 22-25 °C. Plants were propagated via cuttings
198 and grown in triplicate for 12 weeks to provide material for DNA and RNA extractions and for
199 metabolite profiling by UPLC-MS and GC-MS

200 **2.6 Quantitative RT-PCR**

201 Expression levels of *amorpha-4,11-diene synthase* (AMS), *squalene synthase* (SQS),
202 *germacrene A synthase* (GAS); *δ -epicederol synthase* (ECS); *beta farnesene synthase* (BFS);
203 and *caryophyllene synthase* (CPS) relative to ubiquitin (UBI; Genbank accession: GQ901904)
204 were determined by quantitative RT-PCR. Expression levels of each gene were determined for
205 cDNA from NTC and transformed young leaf material prepared as described above (section
206 2.2). Gene-specific primers used were:

207 AMS_For 5'- GGGAGATCAGTTTCTCATCTATGAA- 3'
208 AMS_Rev 5'- CTTTTAGTAGTTGCCGCACTTCTT-3'
209 CPS_For 5'-CAACGATGTAGAAGGCTTGCTTGA-3'
210 CPS_Rev 5'-GTAGATAGTGTGGGTTGGTGTGA-3'
211 ECS_For 5'-GCAACAAGCCTACGAATCACTCAA-3'
212 ECS_Rev 5'-CGTGAAAAATTAAGGACCCTCATAG-3'
213 GAS_For 5'-CTCGTTACTCCTTGGCAAGAATCAT-3'
214 GAS_Rev 5'-GCTCCATAGCACTAATATCCCCTT-3'
215 SQS_For 5'-GACCAGTTCCACCATGTTTCTACT-3'

216 *SQS_Rev* 5'-GCTTTGACAACCCTATTCCAACAAG-3'

217 *FS_For* 5'-GCAAAAGAGTTGGTTCGCAATTAC-3'

218 *FS_Rev* 5'-GTACCCCTCTTTTAGCCATCTGG-3'

219 *UBI_For* 5'-TGATTGGCGTCGTCTTCGA-3'

220 *UBI_Rev* 5'-CCCATCCTCCATTTCTAGCTCAT-3'

221 Amplification conditions and data analysis were as described in Graham et al. 2010 and

222 Czechowski et al. 2016.

223 **2.7 Metabolite analysis by UPLC-MS and GC-MS**

224 Three replicate cuttings from the NTC and each transformed line were grown in 4-inch pots
225 under 16-h days for 12 wk. Metabolite profiles were generated from 50 mg fresh weight pooled
226 samples of leaves at young (first emerging leaf to leaf 6) or mature (the tips of leaves 11-13)
227 developmental stages the fresh leaf samples collected were stored at -80°C . Dry leaf material
228 was obtained from 18-week-old plants, cut just above the zone of senescing leaves, and dried
229 for 14 d at 40°C . Leaves were stripped from the plants, and leaf material was sieved through
230 5-mm mesh to remove small stems. Trichome-specific metabolites (Supplemental figure 2)
231 were extracted and analysed as previously described (Graham et al. 2010, Czechowski et al.
232 2016).

233 **2.8 Architecture and leaf traits and trichome density**

234 Height, leaf area and trichome density were also measured on the NTC and transformed lines
235 as described in Graham et al. (2010).

236 **2.9 FPP quantification**

237 FPP quantification was carried out on isolated GSTs and young leaf material using pooled leaf
238 tips (meristem to leaf 6) collected from the apical meristem and each axillary branch counting
239 down to the axillary branch at leaf position 20. Glandular trichomes were isolated as described
240 in Graham et al (2010). The young leaf material was ground under liquid nitrogen and 1 gram
241 weighed out for extraction. Both the isolated trichomes and the ground leaf were extracted in
242 methanol:water (7:3, v/v), including a total of $0.3\ \mu\text{g}$ farnesyl S-thiolodiphosphate (FSPP;
243 Echelon Biosciences) added as an internal standard. Extracts were processed according to
244 Nagel et al. (2014). Briefly, each extract was passed through a Chromabond HX RA column
245 (150 mg packing), which had first been conditioned with 5 ml methanol and 5 ml of water, and
246 compounds eluted under gravity with 3 ml of 1 M ammonium formate in methanol. The eluate
247 was evaporated under a stream of nitrogen to dryness, dissolved in $250\ \mu\text{L}$ of water:methanol
248 (1:1 v/v), and a $2\ \mu\text{L}$ aliquot injected on a Waters Acquity I-Class UPLC system interfaced to
249 a Thermo Orbitrap Fusion Tribrid mass spectrometer under Xcalibur 4.0 control. Compounds
250 were eluted on a Waters Acquity C18 BEH column (2.1 mm x 100 mm, $1.7\ \mu\text{m}$) at 50°C using
251 the following binary gradient program: solvent A = 20 mM ammonium bicarbonate + 0.1%
252 triethylamine; solvent B = 4:1 acetonitrile:water + 0.1% triethylamine; flowrate 0.4 ml/min;
253 0- 100% B linear gradient over 4 minutes. Post column, compounds were ionized using a
254 heated electrospray source (vaporizer = 358°C ; N_2 flows for sheath/aux/sweep = 45/13/1
255 arbitrary units; source = 2.5 kV; ion transfer tube = $-30\ \text{V}$ and 342°C ; tube lens = $-40\ \text{V}$). Data
256 was acquired in full scan mode with the following settings: orbitrap resolution = 15 k, 100-
257 500 m/z range, max ion time 100 ms, 1 microscan, AGC target = 200000, S-Lens RF Level =
258 60. FPP eluted at ~ 2.4 min and the internal standard (FSPP) at ~ 2.5 min. The deprotonated
259 pseudomolecular ions ($[\text{M}-\text{H}]^-$) of 381.1227 and 397.0998 for FPP and FSPP, respectively,
260 were used for quantification (± 5 ppm window) against a 0.1-100 μM linear FPP/FSPP
261 response ratio calibration curve ($R^2 = 0.99$), using Xcalibur 4.0 software (Thermo). For less

262 complex trichome-only samples, a Thermo LTQ Orbitrap Classic instrument was used in ion
263 trap mode.

264 **2.10 Sterol quantification**

265 200 mg samples of pooled leaf material from the NTC and transformed lines were ground and
266 extracted by sonication in dichloromethane as described by Zhang *et al.*,(2009). Extracts were
267 centrifuged, the upper phase collected and a 1 uL aliquot analysed by GC-MS as described in
268 Czechowski et al. (2016), except that the final GC oven temperature and hold time were
269 increased to 350 °C and 8 min, respectively, to ensure elution of sterols and squalene.
270 ChromaTof 4.0 software (Leco) was used for spectral processing, to produce deconvoluted
271 spectra for identification against the NIST 2014 database and authentic standards. ChromaTof-
272 selected unique masses were used to generate and integrate peak areas under selected ion traces
273 for quantification against authentic sterol and squalene standards.

274 **2.11 Data analysis**

275 Peak lists for UPLC-MS and GC-MS data were obtained and processed using bespoke R scripts
276 as described in Czechowski et al. (2016). Data from GC-MS and UPLC-MS for the young
277 mature and dried leaf were analysed by ANOVAs using GENSTAT software (VSN
278 international) with the Bonferroni post hoc test ($p < 0.05$) to compare between NTC and
279 transformed lines.

280

281 **3 Results**

282 **3.1 Silencing the first committed step in artemisinin production results in accumulation** 283 **of the sesquiterpene precursor FPP in glandular secretory trichomes of *A. annua*.**

284 The *Amorpha-4,11-diene synthase (AMS)* enzyme responsible for catalysing the first
285 committed step in artemisinin production is encoded by a small gene family averaging 12
286 copies in the Artemis F1 hybrid variety (Figure 2). We built a hairpin-based gene silencing
287 construct that included regions showing the least amount of sequence variation to maximise
288 the sequence homology and thus silencing effect across all the members of the gene family
289 (supplemental figure 1 of *AMS* ORF consensus sequence). The trichome specific promoter of
290 the *cyp7av1* gene (Wang et al. 2011) was used to drive expression of the *AMS* gene silencing
291 construct in-planta. *Agrobacterium tumefaciens* based transformation was used to generate
292 three independent transgenic lines expressing the *cyp7lav1::AMS_RNAi* construct in Artemis.
293 Phenotypically the *AMS* silenced lines showed no significant differences when compared to
294 non-transformed controls (NTCs) in terms of height, branch number and leaf total dry weight
295 (supplemental figure 2). Presence of the transgene was determined by PCR using primers
296 designed to detect the *NPTII* selectable marker gene (Figure 3A). Q-RT-PCR revealed that
297 there is a major reduction in steady state levels of *AMS* mRNA in all of the *AMS* silenced lines
298 carrying the gene silencing construct (Figure 3B). This was mirrored by a dramatic decrease in
299 artemisinin concentration in young, mature and dry leaves of the *AMS* silenced lines compared
300 to the NTCs (Figure 3C). Amorpha 4-11-diene levels were found to be higher in young leaf
301 tissue when compared to the mature and dry leaf material in both the NTC and *AMS* silenced
302 lines with two of the lines showing a significant increase compared to the NTC control (Figure
303 4A). There was a significant reduction in all other intermediates downstream of amorpha 4-11-
304 diene in the *AMS* silenced lines compared to NTC (Figure 4).

305 Quantification of farnesyl diphosphate (FPP) in methanolic extracts from ground young leaf
306 tips revealed a significant increase in *AMS* silenced lines compared to the NTCs (Figure 5A).
307 This increase was also confirmed in isolated trichomes (Figure 5B).

308 **3.2 The consequence of FPP increases on known sesquiterpene synthase and squalene** 309 **synthase gene expression**

310 The effect of silencing *AMS* on the expression of the other known sesquiterpene synthases
311 and squalene synthase (detailed in figure 1) was investigated by carrying out qRT-PCR on
312 young leaf material (Figure 6). In the NTC the expression levels of *SQS*, *GAS*, *ECS*, *CPS* and
313 *FS* was found to be ~3- times lower than *AMS*. In the *AMS*-silenced lines *SQS* and *GAS*
314 become the most highly expressed synthases although in comparison to the NTC they were
315 not significantly increased. Comparison of expression of *SQS*, *GAS*, *ECS*, *CPS* and *FS*
316 between the NTC and *AMS*-silenced lines showed they are all lower in the latter except for
317 *GAS* expression in the *AMS* silenced line, *AMS*_RNAi_1 and *ECS* expression in the *AMS*
318 silenced *AMS*_RNAi_3. However, these slight differences in gene expression between NTC
319 and the *AMS* silenced lines were not found to be significant.

320 **3.3 The downstream effect of increased FPP levels on sterol and sesquiterpene synthesis** 321 **in *Artemis* as quantified by GC-MS and UPLC**

322 In *A. annua* FPP is a precursor for not only artemisinin and other sesquiterpenes but also
323 squalene and sterols and these could all therefore be additional sinks for FPP that does not flux
324 into the artemisinin pathway via *AMS* (Figure 1). To determine if the silencing of *AMS* led to
325 a redirection of FPP flux, squalene and sterol levels were quantified from dried leaf material
326 by GC-MS. Squalene, stigmasterol, β -sitosterol and campesterol were identified in both the
327 NTC and transformed lines (Figure 7). Stigmasterol and β -sitosterol were present at higher
328 levels in comparison to squalene and campesterol but overall no significant differences were
329 found between the NTC and the *AMS* silenced lines.

330 To determine if *AMS* silencing led to an increase in sesquiterpenes other than artemisinin, GC-
331 and UPLC-MS analysis was carried out on fresh, young, and mature leaf material, and pooled
332 dried leaf material. From the GC-MS analysis of NTC and transformed lines, 105 compounds
333 were identified, 30 of which were sesquiterpenes. Comparisons between the leaf material
334 sampled (young/mature/dried) revealed the level of sesquiterpenes to be higher in the young
335 leaf samples in comparison to the mature and dried leaf material (supplemental table 1). Further
336 statistical analysis to investigate differences between the 3 *AMS* silenced lines and the NTC
337 found that for 17 of the sesquiterpene compounds levels were significantly higher in the NTC.
338 Significant increases in the *AMS* silenced lines were found for only 6 sesquiterpene compounds
339 (supplemental table 1). In young leaf material these were: beta-farnescene and germacrene; in
340 mature leaf there were increases in 2 unknown compounds with putative $C_{15}H_{24}$ formulae, and
341 in dried leaf material germacrene D and ledene oxide were significantly higher (Figure 8). For
342 the other 7 sesquiterpene compounds no differences were observed.

343 As well as artemisinin and its associated compounds derived from the artemisinin pathway, the
344 UPLC-MS analysis also identified four putative novel oxygenated sesquiterpene ($C_{15}H_{24}O$)
345 compounds in the *AMS* silenced lines. These compounds were identified as being significantly
346 increased although the levels at which they were present was very low, ranging from 0.03- to
347 0.4 $\mu\text{g}/\text{mg}$ DW which is 100 to 10 times (respectively) lower than artemisinin levels (Figure
348 9). Putative sesquiterpenes: M255.1946T53 M239.2007T65 and M239.2005T78 were all
349 found to be significantly increased in young leaf tissue in the transformed lines in comparison

350 to the NTC. M345.1205T24 was found to be significantly increased in only the dried leaf tissue
351 of the *AMS* silenced lines in comparison to the NTC.

352 **4 Discussion**

353 **4.1 Silencing *AMS* leads to accumulation of the sesquiterpene precursor FPP in *A. annua*** 354 **GSTs**

355 In *A. annua* the first committed step in the artemisinin pathway converting FPP to amorpho-
356 4,11-diene is *AMS*. It was hypothesised that by blocking this step FPP would either accumulate
357 or be channelled into the production of known or novel sesquiterpenes. Previous work had
358 shown that *AMS* was not only highly expressed in the Artemis cultivar, but that recovered *AMS*
359 gene sequences were polymorphic (Graham et al. 2010). This indicated that multiple copies of
360 the gene could exist which we confirmed by qPCR (Figure 2). The high copy numbers for *AMS*
361 present in the *A. annua* cv. Artemis could be linked to its high artemisinin yield, and its success
362 as an elite hybrid for commercial production of artemisinin. (Delabays et al.2001). To
363 effectively silence all the *AMS* copies two separate sections of the sequence were selected and
364 joined to create an *AMS* specific sequence, this was driven by the *cyp71av1* trichome specific
365 promoter (Wang et al.2011). Stably transformed lines expressing the construct were achieved,
366 with *AMS* expression reduced to less than 4% of the NTC. Artemisinin content was reduced
367 by 95% alongside a reduction in all artemisinin-related compounds downstream of the *AMS*-
368 catalysed step.

369 One exception was amorpho-4,11-diene where levels were found to be significantly higher in
370 young leaf tissue of the *AMS* silenced lines compared to the NTC (Figure 4A). No such increase
371 was present in mature or dry leaves. The levels of amorpho-4,11-diene in *A. annua* are reported
372 to be low as a consequence of artemisinin biosynthesis (Bouwmeester and Wallaart, 1999).
373 Detection of this early step precursor in young leaves of NTC is consistent with previous
374 findings (Czechowski et al. 2016) which suggest the pathway to artemisinin only becomes
375 active as leaves mature. The increase in the *AMS* silenced lines is unexpected and the reason
376 not obvious - but could relate to the metabolic sink being somehow further compromised as a
377 result of the decreased *AMS*.

378 To establish the impact of silencing *AMS* on FPP levels we adapted a protocol from Nagel et
379 al. (2014) that allowed us to quantify this important precursor for the first time in *A. annua*.
380 We found that silencing of *AMS* led to a significant accumulation of FPP in young leaf tissue
381 and this increase was also confirmed as being trichome specific by carrying out the same
382 extraction on young leaf isolated GSTs (Figure 5 A and B).

383 **4.2 The downstream effect of increased FPP levels on sterol and sesquiterpene synthesis** 384 **in Artemis as quantified by UPLC-MS and GC-MS**

385 Increasing FPP by knocking down *AMS* had no effect on the expression of any of the other
386 known sesquiterpene synthases genes or squalene synthase known to be expressed in either the
387 GSTs or young leaf tissue (Figure 6). UPLC-MS and GC-MS analysis of leaf material was
388 carried out to determine if the FPP accumulating in the *AMS* silenced lines was being
389 redirected into sterol or sesquiterpene production. GC-MS analysis found no significant
390 differences in squalene and sterol levels between the NTC and the *AMS* silenced lines (Figure
391 7) despite this pathway being considered the main competitor for FPP after artemisinin (Zhang
392 et al. 2009). The GC-MS analysis also revealed very few changes in volatiles in the *AMS*
393 silenced lines (supplemental Table 1). Where significant differences were observed the
394 magnitude, changes were very low (Figure 8). These results differ to the findings of Ma et al.

395 (2015) who silenced *AMS* in a low artemisinin background (artemisinin yields of 0.025µg/mg).
396 Alongside reporting a decrease in artemisinin, they also found a significant increase in the
397 levels of caryophyllene and copaene in their *AMS* silenced plants. The increase in these
398 sesquiterpene compounds could be linked to the low artemisinin cultivar used for
399 transformation having other active endogenous sesquiterpene synthases. In *Artemis* a high
400 yielding cultivar the endogenous synthases would appear not to be as active in their ability to
401 utilise the FPP made available away from artemisinin being silenced.

402 Although no differences were seen in known sesquiterpene levels in the *AMS* silenced lines,
403 putative novel sesquiterpene compounds were detected and characterised by UPLC-MS.
404 Significant differences were seen between *Artemis* NTCs and the *AMS* silenced lines for 4
405 putative sesquiterpene compounds although the concentrations were around 30 times lower
406 than artemisinin. The compounds were also mainly identified in young leaf tissue suggesting
407 that these compounds are not end products but rather are further converted as the leaf matures.
408 Compound M345.1205T24 was an exception to the other 3 as it was found in dried leaf only.
409 The very low concentrations of these novel compounds in available plant material ruled out
410 structural determination attempts by NMR.

411 The lack of diversion of the accumulated FPP to other sesquiterpenes or sterols in the *AMS*
412 silenced lines is somewhat surprising. One possible explanation for this is that the *Artemis*
413 hybrid has been selected for high yield artemisinin and the flux of FPP may already be
414 optimised to flow towards artemisinin production.

415 **4.4 Trichomes with elevated FPP as a potential production platform for high value** 416 **sesquiterpenes**

417 *A. annua* is already established as a very efficient crop plant for artemisinin production, with
418 the potential to produce this high value chemical at a relatively low cost of less than \$250 per
419 kilogram. Disruption of *cyp71av1*, leading to novel arteannuin X accumulation demonstrated
420 the plasticity of GST metabolism in *A. annua*, suggesting their potential as factories for new
421 compound production (Czechowski et al. 2016). The GSTs provide an optimal environment
422 for the synthesis of many natural products based on the availability of precursors, co-enzymes,
423 mRNA and protein processing. In *A. annua* the problem of toxicity of some of these compounds
424 is overcome as the GSTs can sequester them in the extracellular cavities of the trichome
425 secretory cells. This coupled with the location of the GSTs on the surface of leaves is
426 advantageous as the compounds are both contained and readily extractable.

427 By silencing *AMS* in a high artemisinin yielding *A. annua* cultivar we have significantly
428 decreased the amount of artemisinin and related compounds produced in the GSTs. As a further
429 result of the silencing we also show that the precursor FPP is accumulated in the GSTs and not
430 catalysed by endogenous synthases. The lack of production of novel compounds at significant
431 amounts suggests that the elevated pool of GST localised FPP is either not available to or not
432 utilised by other sesquiterpene, squalene synthase enzymes. Consequently, the *AMS* silenced
433 lines may represent a platform for production of other high value compounds that require FPP
434 as a precursor and for which genes encoding biosynthetic enzymes are known.

435 **Author contributions**

436 TC, TMC and IAG designed the experiments; TC, TMC, CB, NS, JH and DH performed the
437 experiments; TMC, TC, CB, DH and TL analysed the data; TMC, TC, TL and IAG wrote the
438 manuscript; and all authors revised and approved the manuscript.

439 **Funding**

440 We acknowledge financial support for this project from The Bill and Melinda Gates Foundation
441 as well as from The Garfield Weston Foundation for the Centre for Novel Agricultural
442 Products.

443 **Acknowledgments**

444 We thank the University of York horticulture team for horticultural assistance; C. Calvert, W.
445 Lawley, for project management; we thank X. Simonnet and Médiplant for access to the
446 Artemis pedigree. Mass spectrometry analysis was in part supported by instrumentation within
447 the York Centre of Excellence in Mass Spectrometry (CoEMS). The CoEMS was created
448 thanks to a major capital investment through Science City York, supported by Yorkshire
449 Forward with funds from the Northern Way Initiative, and subsequent support from EPSRC
450 (EP/K039660/1; EP/M028127/1).

451 **Conflict of Interest**

452 The authors declare that the research was conducted in the absence of any commercial or
453 financial relationships that could be construed as a potential conflict of interest.

454 **References**

- 455 Berteà, Cinzia M., Alessandra Voster, Francel W A Verstappen, Massimo Maffei, Jules
456 Beekwilder, and Harro J. Bouwmeester. 2006. "Isoprenoid Biosynthesis in *Artemisia*
457 *Annua*: Cloning and Heterologous Expression of a Germacrene A Synthase from a
458 Glandular Trichome cDNA Library." *Archives of Biochemistry and Biophysics* 448 (1–
459 2): 3–12. <https://doi.org/10.1016/j.abb.2006.02.026>.
- 460 Bleeker, Petra M, Rossana Mirabella, Paul J Diergaarde, Arjen VanDoorn, Alain Tissier,
461 Merijn R Kant, Marcel Prins, Martin de Vos, Michel a Haring, and Robert C Schuurink.
462 2012. "Improved Herbivore Resistance in Cultivated Tomato with the Sesquiterpene
463 Biosynthetic Pathway from a Wild Relative." *Proceedings of the National Academy of*
464 *Sciences of the United States of America* 109: 20124–29.
465 <https://doi.org/10.1073/pnas.1208756109>.
- 466 Bouwmeester, HJ, and Te Wallaart. 1999. "Amorpha-4, 11-Diene Synthase Catalyses the
467 First Probable Step in Artemisinin Biosynthesis." *Phytochemistry* 52: 843–54.
468 [https://doi.org/10.1016/S0031-9422\(99\)00206-X](https://doi.org/10.1016/S0031-9422(99)00206-X).
- 469 Brown, Geoffrey D. 2010. "The Biosynthesis of Artemisinin (Qinghaosu) and the
470 Phytochemistry of *Artemisia Annua* L. (Qinghao)." *Molecules*.
471 <https://doi.org/10.3390/molecules15117603>.
- 472 Cai, Yu, Jun-Wei Jia, John Crock, Zhi-Xin Lin, Xiao-Ya Chen, and Rodney Croteau. 2002.
473 "A cDNA Clone for Beta-Caryophyllene Synthase from *Artemisia Annua*." *Phytochemistry*
474 61 (5): 523–29. <https://doi.org/S0031942202002650> [pii].
- 475 Chen, Jian Lin, Hua Ming Fang, Yun Peng Ji, Gao Bin Pu, Yan Wu Guo, Li Li Huang, Zhi
476 Gao Du, et al. 2011. "Artemisinin Biosynthesis Enhancement in Transgenic *Artemisia*
477 *Annua* Plants by Downregulation of The β -Caryophyllene Synthase Gene." *Planta*
478 *Medica* 77 (15): 1759–65. <https://doi.org/10.1055/s-0030-1271038>.

- 479 Croteau, Rodney, Toni M Kutchan, and Norman G Lewis. 2000. "Secondary Metabolites."
480 *Biochemistry Molecular Biology of Plants* 7 (7): 1250–1318.
481 <https://doi.org/10.1016/j.phytochem.2011.10.011>.
- 482 Czechowski, Tomasz, Tony R. Larson, Theresa M. Catania, David Harvey, Geoffrey D.
483 Brown, and Ian A. Graham. 2016. "Artemisia Annuum Mutant Impaired in Artemisinin
484 Synthesis Demonstrates Importance of Nonenzymatic Conversion in Terpenoid
485 Metabolism." *Proceedings of the National Academy of Sciences* 113 (52): 15150–55.
486 <https://doi.org/10.1073/pnas.1611567113>.
- 487 Delabays, N, X Simonnet, and M Gaudin. 2001. "The Genetics of Artemisinin Content in
488 Artemisia Annuum L. and the Breeding of High Yielding Cultivars." *Current Medicinal
489 Chemistry* 8 (15): 1795–1801. <https://doi.org/10.2174/0929867013371635>.
- 490 Duke, Mary V., Rex N. Paul, Hala N. Elsohly, George Sturtz, and Stephen O. Duke. 1994.
491 "Localization of Artemisinin and Artemisitene in Foliar Tissues of Glanded and
492 Glandless Biotypes of Artemisia Annuum L." *International Journal of Plant Sciences* 155
493 (3): 365–72. <https://doi.org/10.1086/297173>.
- 494 Duke, Stephen O., and Rex N. Paul. 1993. "Development and Fine Structure of the Glandular
495 Trichomes of Artemisia Annuum L." *International Journal of Plant Sciences* 154 (1):
496 107–18. <https://doi.org/10.1086/297096>.
- 497 Ferreira, J.F.S., J. C. Laughlin, N. Delabays, and P.M. de Magalhães. 2005. "Cultivation and
498 Genetics of Artemisia Annuum L. for Increased Production of the Antimalarial
499 Artemisinin." *Plant Genetic Resources: Characterization and Utilization* 3 (2): 206–29.
500 <https://doi.org/10.1079/PGR200585>.
- 501 Ferreira, Jorge F. S., and Jules Janick. 1995. "Floral Morphology of Artemisia Annuum with
502 Special Reference to Trichomes." *International Journal of Plant Sciences* 156 (6): 807–
503 15. <https://doi.org/10.1086/297304>.
- 504 Graham, I. A., K. Besser, S. Blumer, C. A. Branigan, T. Czechowski, L. Elias, I. Guterman,
505 et al. 2010. "The Genetic Map of Artemisia Annuum L. Identifies Loci Affecting Yield of
506 the Antimalarial Drug Artemisinin." *Science* 327 (5963): 328–31.
507 <https://doi.org/10.1126/science.1182612>.
- 508 Han, Jun Li, Ben Ye Liu, He Chun Ye, Hong Wang, Zhen Qiu Li, and Guo Feng Li. 2006.
509 "Effects of Overexpression of the Endogenous Farnesyl Diphosphate Synthase on the
510 Artemisinin Content in Artemisia Annuum L." *Journal of Integrative Plant Biology* 48
511 (4): 482–87. <https://doi.org/10.1111/j.1744-7909.2006.00208.x>.
- 512 Han, Junli, Hongzhen Wang, Selvaraju Kanagarajan, Mengshu Hao, Anneli Lundgren, and
513 Peter E E. Brodelius. 2016. "Promoting Artemisinin Biosynthesis in Artemisia Annuum
514 Plants by Substrate Channeling." *Molecular Plant*.
515 <https://doi.org/10.1016/j.molp.2016.03.004>.
- 516 Huchelmann, Alexandre, Marc Boutry, and Charles Hachez. 2017. "Plant Glandular
517 Trichomes: Natural Cell Factories of High Biotechnological Interest." *Plant Physiology*,
518 pp.00727.2017. <https://doi.org/10.1104/pp.17.00727>.
- 519 Ikram, Nur K. B. K., and Henrik T. Simonsen. 2017. "A Review of Biotechnological
520 Artemisinin Production in Plants." *Frontiers in Plant Science* 8.

- 521 <https://doi.org/10.3389/fpls.2017.01966>.
- 522 Kortbeek, R. W.J., J. Xu, A. Ramirez, E. Spyropoulou, P. Diergaarde, I. Otten-Bruggeman,
523 M. de Both, et al. 2016. "Engineering of Tomato Glandular Trichomes for the
524 Production of Specialized Metabolites." *Methods in Enzymology*, 2016.
525 <https://doi.org/10.1016/bs.mie.2016.02.014>.
- 526 Lv, Zongyou, Fangyuan Zhang, Qifang Pan, Xueqing Fu, Weimin Jiang, Qian Shen,
527 Tingxiang Yan, et al. 2016. "Branch Pathway Blocking in *Artemisia Annua* Is a Useful
528 Method for Obtaining High Yield Artemisinin." *Plant and Cell Physiology* 57 (3): 588–
529 602. <https://doi.org/10.1093/pcp/pcw014>.
- 530 Ma, Chenfei, Huahong Wang, Xin Lu, Hong Wang, Guowang Xu, and Benye Liu. 2009.
531 "Terpenoid Metabolic Profiling Analysis of Transgenic *Artemisia Annua* L. by
532 Comprehensive Two-Dimensional Gas Chromatography Time-of-Flight Mass
533 Spectrometry." *Metabolomics* 5 (4): 497–506. [https://doi.org/10.1007/s11306-009-0170-](https://doi.org/10.1007/s11306-009-0170-6)
534 6.
- 535 Ma, Dong Ming, Zhilong Wang, Liangjiang Wang, Fatima Alejos-Gonzales, Ming An Sun,
536 and De Yu Xie. 2015. "A Genome-Wide Scenario of Terpene Pathways in Self-
537 Pollinated *Artemisia Annua*." *Molecular Plant* 8 (11): 1580–98.
538 <https://doi.org/10.1016/j.molp.2015.07.004>.
- 539 Mercke, Per, John Crock, Rodney Croteau, and Peter E. Brodelius. 1999. "Cloning,
540 Expression, and Characterization of Epi-Cedrol Synthase, a Sesquiterpene Cyclase from
541 *Artemisia Annua* L." *Archives of Biochemistry and Biophysics* 369 (2): 213–22.
542 <https://doi.org/10.1006/abbi.1999.1358>.
- 543 Nagel, R., A. Berasategui, C. Paetz, J. Gershenzon, and A. Schmidt. 2014. "Overexpression
544 of an Isoprenyl Diphosphate Synthase in Spruce Leads to Unexpected Terpene
545 Diversion Products That Function in Plant Defense." *PLANT PHYSIOLOGY* 164 (2):
546 555–69. <https://doi.org/10.1104/pp.113.228940>.
- 547 Noorden, Richard Van. 2010. "Demand for Malaria Drug Soars." *Nature*.
548 <https://doi.org/10.1038/466672a>.
- 549 Olofsson, Linda, Alexander Engström, Anneli Lundgren, and Peter E Brodelius. 2011.
550 "Relative Expression of Genes of Terpene Metabolism in Different Tissues of *Artemisia*
551 *Annua* L." *BMC Plant Biology* 11 (1): 45. <https://doi.org/10.1186/1471-2229-11-45>.
- 552 Olofsson, Linda, Anneli Lundgren, and Peter E. Brodelius. 2012. "Trichome Isolation with
553 and without Fixation Using Laser Microdissection and Pressure Catapulting Followed
554 by RNA Amplification: Expression of Genes of Terpene Metabolism in Apical and Sub-
555 Apical Trichome Cells of *Artemisia Annua* L." *Plant Science* 183: 9–13.
556 <https://doi.org/10.1016/j.plantsci.2011.10.019>.
- 557 Olsson, Mikael E., Linda M. Olofsson, Ann Louise Lindahl, Anneli Lundgren, Maria
558 Brodelius, and Peter E. Brodelius. 2009. "Localization of Enzymes of Artemisinin
559 Biosynthesis to the Apical Cells of Glandular Secretory Trichomes of *Artemisia Annua*
560 L." *Phytochemistry* 70 (9): 1123–28. <https://doi.org/10.1016/j.phytochem.2009.07.009>.
- 561 Paddon, C.J., and J.D. Keasling. 2014. "Semi-Synthetic Artemisinin: A Model for the Use of
562 Synthetic Biology in Pharmaceutical Development." *Nature Reviews Microbiology* 12

- 563 (5): 355–67. <https://doi.org/10.1038/nrmicro3240>.
- 564 Peplow, Mark. 2016. “Synthetic Biology’s First Malaria Drug Meets Market Resistance.”
565 *Nature*, 2016. <https://doi.org/10.1038/530390a>.
- 566 Picaud, S, M Brodelius, and P E Brodelius. 2005. “Expression, Purification and
567 Characterization of Recombinant (E)-Beta-Farnesene Synthase from *Artemisia Annua*.”
568 *Phytochemistry* 66 (9): 961–67. [https://doi.org/S0031-9422\(05\)00142-1](https://doi.org/S0031-9422(05)00142-1)
569 [pii]r10.1016/j.phytochem.2005.03.027.
- 570 Pulice, Giuseppe, Soraya Pelaz, and Luis Matías-Hernández. 2016. “Molecular Farming in
571 *Artemisia Annua*, a Promising Approach to Improve Anti-Malarial Drug Production.”
572 *Frontiers in Plant Science* 7. <https://doi.org/10.3389/fpls.2016.00329>.
- 573 Soetaert, Sandra S.A., Christophe M.F. Van Neste, Mado L. Vandewoestyne, Steven R.
574 Head, Alain Goossens, Filip C.W. Van Nieuwerburgh, and Dieter L.D. Deforce. 2013.
575 “Differential Transcriptome Analysis of Glandular and Filamentous Trichomes in
576 *Artemisia Annua*.” *BMC Plant Biology* 13 (1). [https://doi.org/10.1186/1471-2229-13-](https://doi.org/10.1186/1471-2229-13-220)
577 220.
- 578 Tang, Ke Xuan Shanghai (CN), Wang, Yue Yue Shanghai (CN), Tang, Yue Li Shanghai
579 (CN), Chen Dong-Fang Shanghai (CN). 2011. METHOD OF UTILIZING THE PTS
580 GENE AND RNA INTERFERENCE OF THE ADS GENE TO INCREASE
581 PATCHOULI ALCOHOL CONTENT IN ARTEMISIA ANNUA L. (76), issued 2011.
- 582 Tang, Kexuan, Qian Shen, Tingxiang Yan, and Xueqing Fu. 2014. “Transgenic Approach to
583 Increase Artemisinin Content in *Artemisia Annua* L.” *Plant Cell Reports*.
584 <https://doi.org/10.1007/s00299-014-1566-y>.
- 585 Tissier, Alain, Christophe Sallaud, and Denis Rontein. 2013. “Tobacco Trichomes as a
586 Platform for Terpenoid Biosynthesis Engineering.” In *Isoprenoid Synthesis in Plants*
587 *and Microorganisms: New Concepts and Experimental Approaches*, 271–83.
588 https://doi.org/10.1007/978-1-4614-4063-5_18.
- 589 Wang, Wei, Yejun Wang, Qing Zhang, Yan Qi, and Dianjing Guo. 2009. “Global
590 Characterization of *Artemisia Annua* Glandular Trichome Transcriptome Using 454
591 Pyrosequencing.” *BMC Genomics* 10 (1): 465. [https://doi.org/10.1186/1471-2164-10-](https://doi.org/10.1186/1471-2164-10-465)
592 465.
- 593 Wang, Yueyue, Ke Yang, Fuyuan Jing, Meiya Li, Ting Deng, Runze Huang, Boshi Wang,
594 Guofeng Wang, Xiaofen Sun, and Ke-Xuan Tang. 2011. “Cloning and Characterization
595 of Trichome-Specific Promoter of *cpr71av1* Gene Involved in Artemisinin Biosynthesis
596 in *Artemisia Annua* L.” *Molecular Biology* 45 (5): 751–58.
597 <https://doi.org/10.1134/S0026893311040145>.
- 598 Watson, John M., Adriana F. Fusaro, MingBo Wang, and Peter M. Waterhouse. 2005. “RNA
599 Silencing Platforms in Plants.” *FEBS Letters*.
600 <https://doi.org/10.1016/j.febslet.2005.08.014>.
- 601 Weathers, Pamela J., Shereen Elkholy, and Kristin K. Wobbe. 2006. “Artemisinin: The
602 Biosynthetic Pathway and Its Regulation in *Artemisia Annua*, a Terpenoid-Rich
603 Species.” *In Vitro Cellular & Developmental Biology - Plant* 42 (4): 309–17.
604 <https://doi.org/10.1079/IVP2006782>.

- 605 Wu, Shuiqin, Michel Schalk, Anthony Clark, R Brandon Miles, Robert Coates, and Joe
606 Chappell. 2006. "Redirection of Cytosolic or Plastidic Isoprenoid Precursors Elevates
607 Terpene Production in Plants." *Nature Biotechnology* 24 (11): 1441–47.
608 <https://doi.org/10.1038/nbt1251>.
- 609 Xie, De Yu, Dong Ming Ma, Rika Judd, and Ashley Loray Jones. 2016. "Artemisinin
610 Biosynthesis in *Artemisia Annua* and Metabolic Engineering: Questions, Challenges,
611 and Perspectives." *Phytochemistry Reviews*. <https://doi.org/10.1007/s11101-016-9480-2>.
- 612 Yang, Rui Yi, Li Ling Feng, Xue Qin Yang, Lu Lu Yin, Xiao Ling Xu, and Qing Ping Zeng.
613 2008. "Quantitative Transcript Profiling Reveals down-Regulation of a Sterol Pathway
614 Relevant Gene and Overexpression of Artemisinin Biogenetic Genes in Transgenic
615 *Artemisia Annua* Plants." *Planta Medica* 74 (12): 1510–16. [https://doi.org/10.1055/s-](https://doi.org/10.1055/s-2008-1081333)
616 [2008-1081333](https://doi.org/10.1055/s-2008-1081333).
- 617 Yu, G., and E. Pichersky. 2014. "Heterologous Expression of Methylketone Synthase1 and
618 Methylketone Synthase2 Leads to Production of Methylketones and Myristic Acid in
619 Transgenic Plants." *Plant Physiology* 164 (2): 612–22.
620 <https://doi.org/10.1104/pp.113.228502>.
- 621 Zhang, Ling, Fuyuan Jing, Fupeng Li, Meiya Li, Yuliang Wang, Guofeng Wang, Xiaofen
622 Sun, and Kexuan Tang. 2009. "Development of Transgenic *Artemisia Annua* (Chinese
623 Wormwood) Plants with an Enhanced Content of Artemisinin, an Effective Anti-
624 Malarial Drug, by Hairpin-RNA-Mediated Gene Silencing." *Biotechnology and Applied
625 Biochemistry* 52 (Pt 3): 199–207. <https://doi.org/10.1042/BA20080068>.
- 626 Zhang, Yansheng, Goska Nowak, Darwin W. Reed, and Patrick S. Covello. 2011. "The
627 Production of Artemisinin Precursors in Tobacco." *Plant Biotechnology Journal* 9 (4):
628 445–54. <https://doi.org/10.1111/j.1467-7652.2010.00556.x>.
- 629

Figures

Figure 1 Summary of sesquiterpene production in *Artemisia annua* via the mevalonate (MVA) pathway in the cytosol FPP is highlighted as the key precursor not only for artemisinin synthesis but also for Squalene and other sesquiterpenes. The plastidial methyl erythritol phosphate (MEP) pathway that leads to monoterpene production is also included. Enzymes in red have been cloned from *A. annua* and the sesquiterpene synthases and squalene synthases also cloned from *A. annua* are in blue (DMAPP- dimethylallyl diphosphate, GGPP-geranyl geranyl diphosphate, GPP-Geranyl pyrophosphate, GGPS-Geranylgeranyl pyrophosphate synthase, IPP-isopentenyl diphosphate, IDI- Isopentenyl Diphosphate Isomerase, ABA- Abscisic acid, *FPS-farnesyl diphosphate synthase*, FPP-farnesyl pyrophosphate, *AMS-amorpha-4,11-diene synthase*, *SQS-squalene synthase*, *GAS-germacrene A synthase*, *ECS- δ -epicederol synthase*, *CPS-caryophyllene synthase*, *FS- beta farnesene synthase*).

Figure 2. *AMS* gene copy number in 15 individual lines of the *A. annua* cultivar Artemis estimated by qPCR. Error bars represent standard error (n=4). Sd prefixed numbers represent individual Artemis plants

Figure 3 **A** - PCR results for *NPTII* primers, **B** - Q-RT-PCR of *AMS* gene expression and **C** - Artemisinin concentration ($\mu\text{g}/\text{mg}$ extracted dry weight) in young and mature leaves and dried pooled leaf material for the NTC and *AMS* silenced lines. Error bars \pm SD (NTC – n=6, *AMS*_RNAi lines – n=3). Letters represent Bonferroni test results after ANOVA, groups not sharing letters indicate statistically significant differences ($p < 0.05$).

Figure 4 The concentration ($\mu\text{g}/\text{mg}$ extracted dry weight) of known artemisinin pathway compounds in young leaves, mature leaves and dried pooled leaf material for NTC and *AMS*_RNAi lines. **A** – amorpha-4,11-diene, **B** – artemisinic acid, **C**- dihydroartemisinic acid, **D** – dihydroepideoxyarteannuin B, **E** – arteannuin B, **F** – deoxyartemisinin. Error bars \pm SD (NTC – n=6, *AMS*_RNAi lines – n=3). Letters represent Bonferroni test results after ANOVA, groups not sharing letters indicate statistically significant differences ($p < 0.05$).

Figure 5 **A** - FPP concentrations in $\mu\text{mol}/\text{g}$ for ground young leaf material for the Artemis NTC and Artemis *AMS* silenced lines (concentrations were measured on Thermo Orbitrap Fusion Tribrid mass spectrometer). Error bars \pm SD (NTC – n=6, *AMS*_RNAi lines – n=3). **B** On column FPP concentrations in μmol measured from isolated trichome extracts for Artemis NTC and Artemis *AMS* silenced lines (concentrations were measured on a Thermo LTQ Orbitrap Classic instrument, used in ion trap mode). Error bars \pm SD (NTC – n=6, *AMS*_RNAi lines – n=3). Letters represent Bonferroni test results after ANOVA, groups not sharing letters indicate statistically significant differences ($p < 0.05$).

Figure 6 Expression of functionally characterised sesquiterpene synthase (*AMS*, *GAS*, *ECS*, *CPS* and *FS*) and *SQUALENE SYNTHASE* (*SQS*) genes in young leaf tissue from NTC and *AMS* silenced lines. Error bars \pm SD (NTC – n=6, *AMS*_RNAi lines – n=3).

Figure 7 Average peak area from GC-MS for squalene and sterol compounds identified from dried pooled leaf material for NTC and *AMS* silenced lines. Error bars \pm SD (NTC – n=6, *AMS*_RNAi lines – n=3). Letters represent Bonferroni test results after ANOVA, groups not sharing letters indicate statistically significant differences ($p < 0.05$).

Figure 8 Internal standard (IS) and dry weight normalised peak area averages for sesquiterpene levels in **A**-young leaf, **B**-mature leaf and **C**-pooled dried leaf material in NTC and *AMS* silenced lines. Error bars +/- SD (NTC – n=6, *AMS*_RNAi – n=3). Letters represent Bonferroni test results after ANOVA, groups not sharing letters indicate statistically significant differences ($p < 0.05$).

Figure 9 The concentration ($\mu\text{g}/\text{mg}$ extracted dry weight) of putative novel sesquiterpene compounds in young and mature leaves and dried pooled leaf material for the *AMS* silenced lines and NTCs. **A** – sesquiterpene M255.1946T53, **B** – sesquiterpene M345.1205T24, **C** – M239.2007T65 and **D** – sesquiterpene M239.2005T78 Error bars +/- SD (NTC – n=6, *AMS*_RNAi lines – n=3). Letters represent Bonferroni test results after ANOVA, groups not sharing letters indicate statistically significant differences ($p < 0.05$).

In review

In review

Figure 1.JPEG

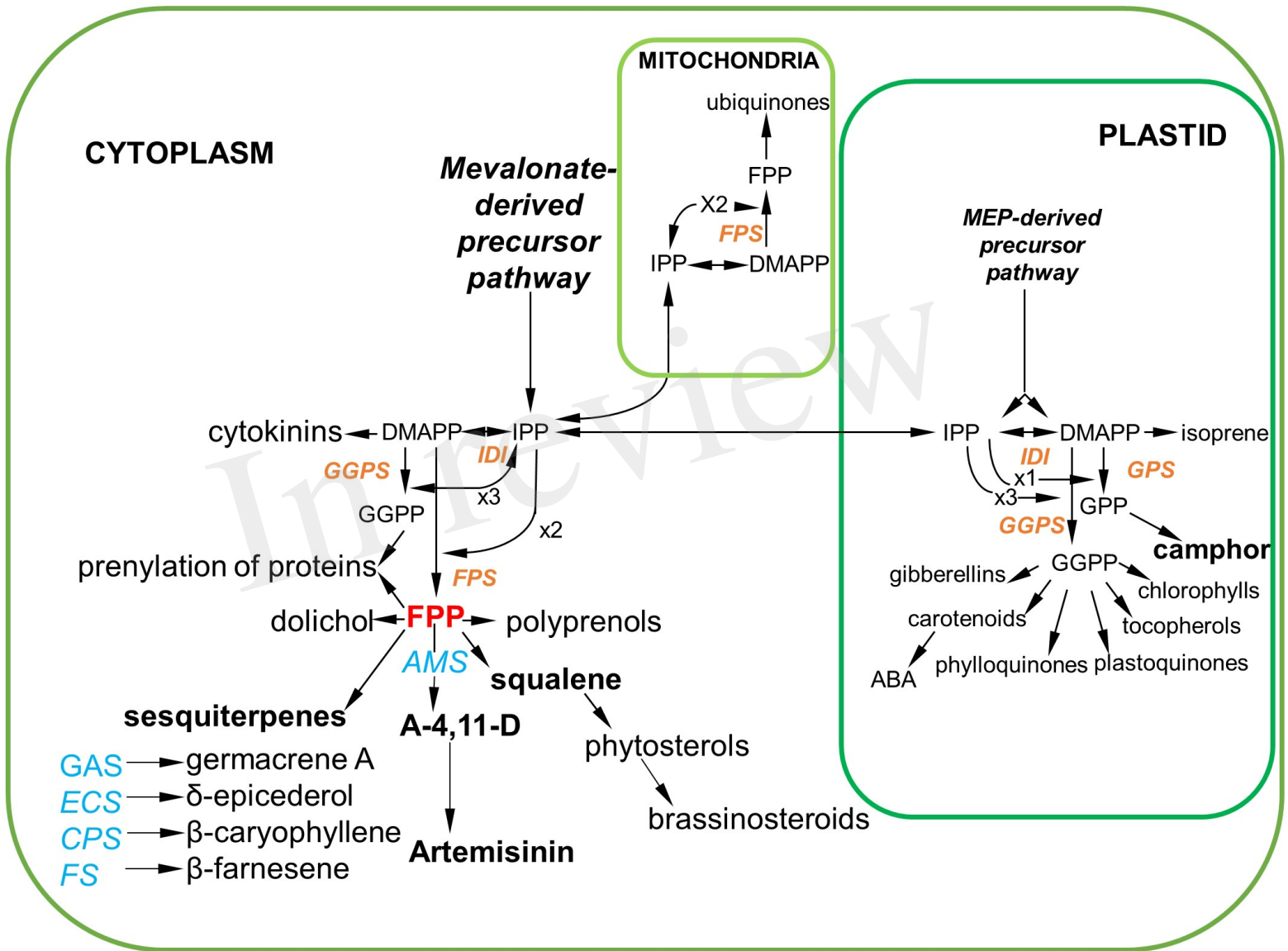
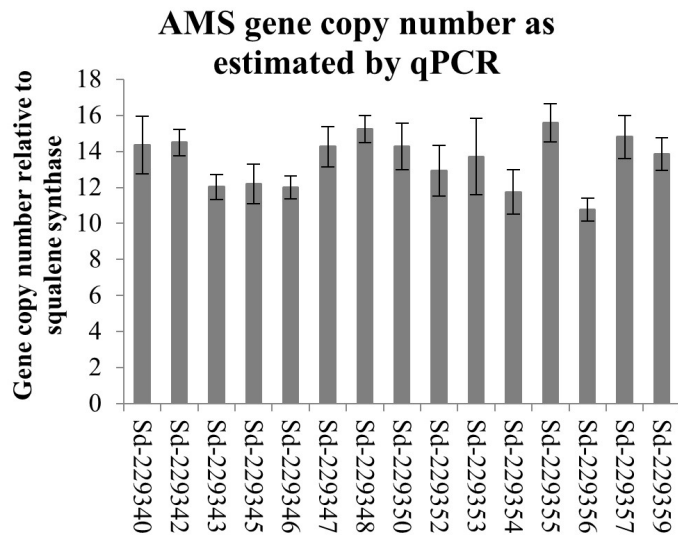


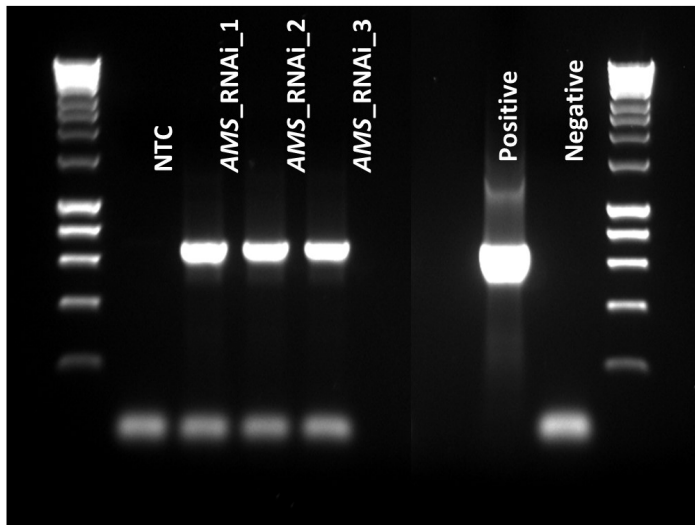
Figure 2.JPEG



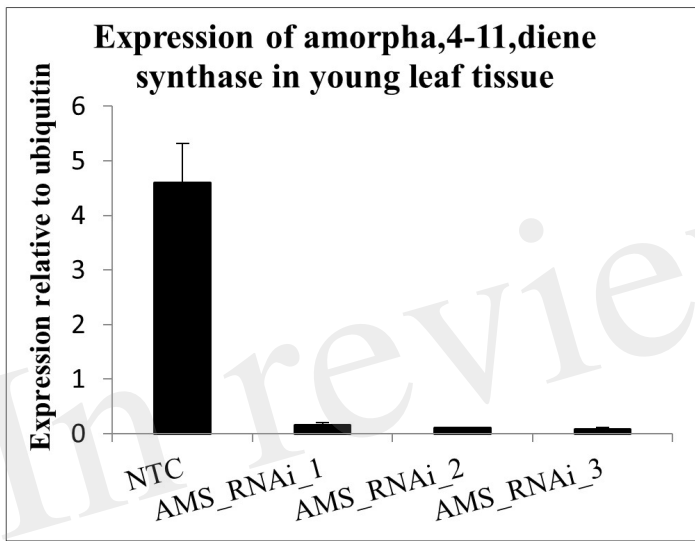
In review

Figure 3.JPEG

A



B



C

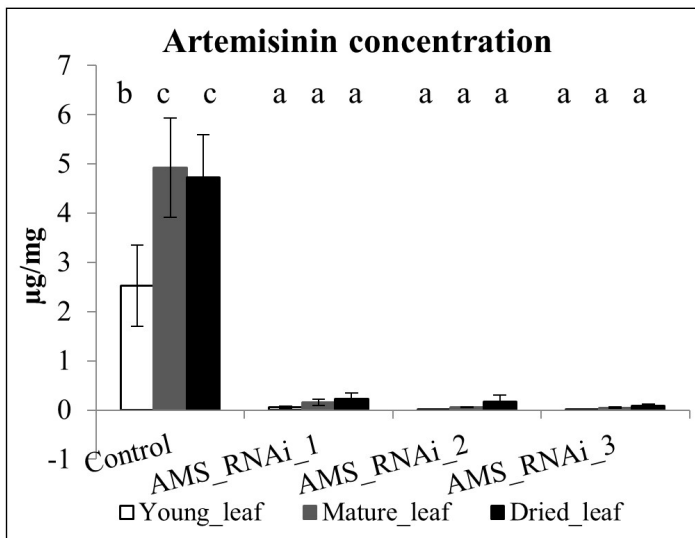
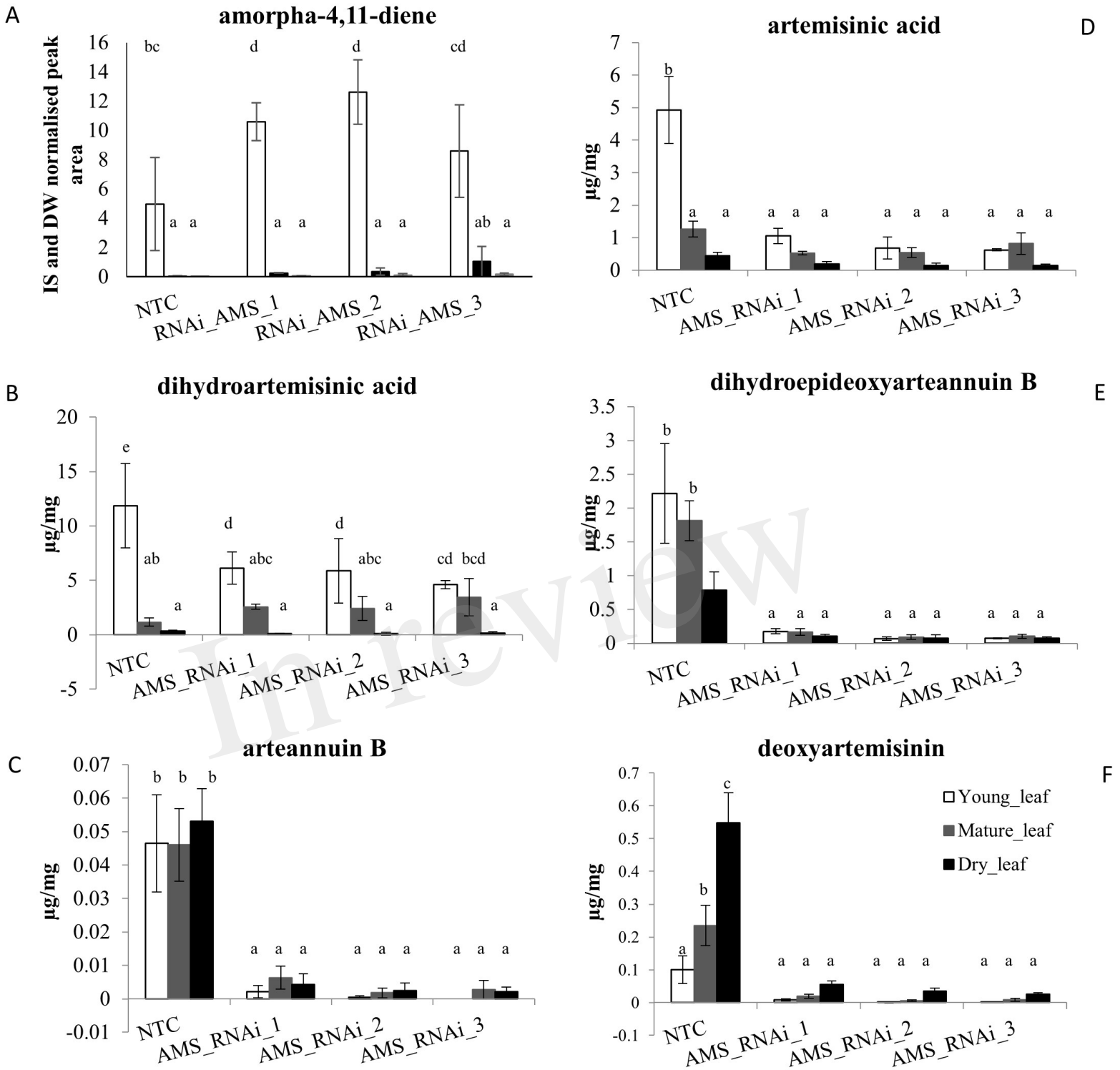


Figure 4.JPEG



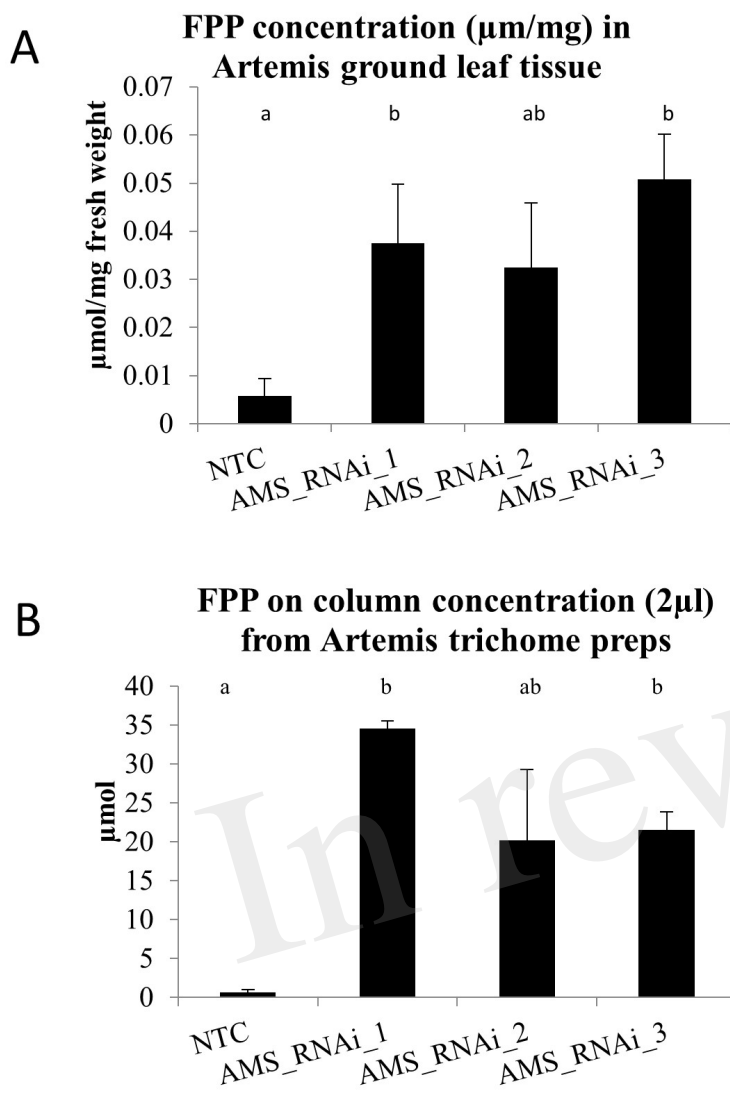


Figure 6.JPEG

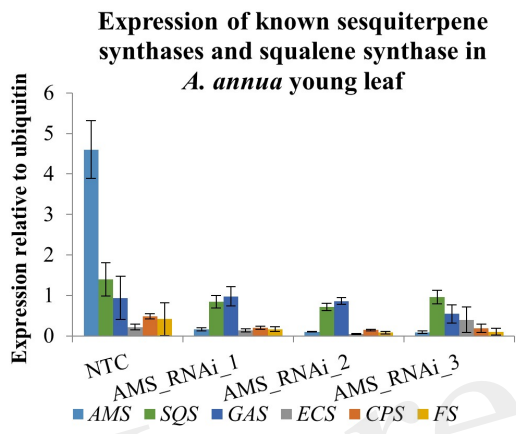
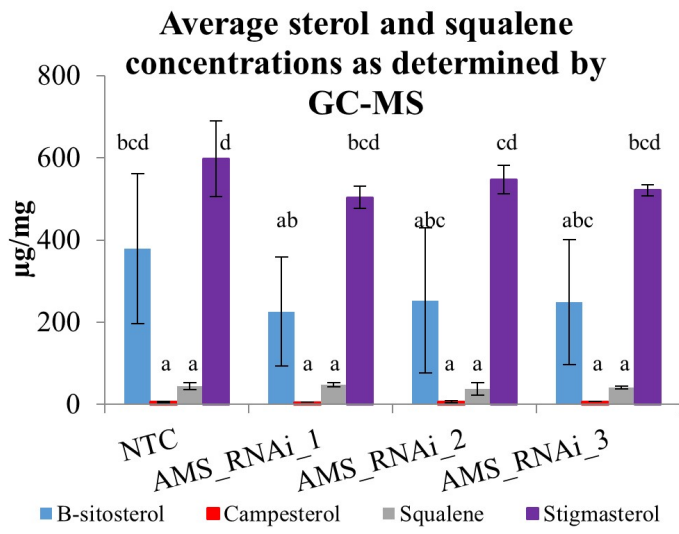


Figure 7.JPEG



In review

Figure 8.JPEG

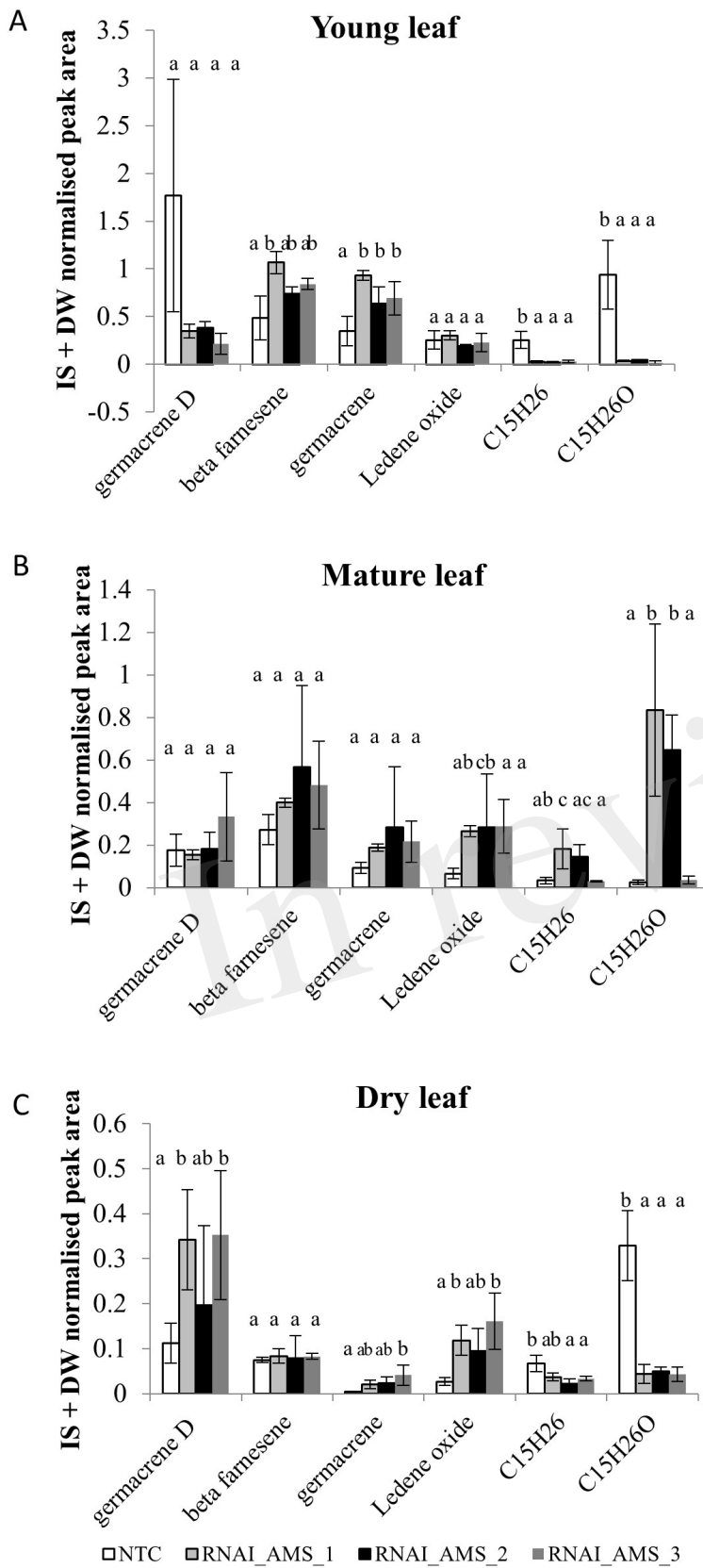


Figure 9.JPEG

