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

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### ***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*.

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(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**

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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);  $\delta$ -  
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; Bertea 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  $\alpha$ -  
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)*;  *$\delta$ -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'-GTAGATAGTGTTGGGTTGGTGTGA-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  $-80^{\circ}\text{C}$ . 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  $40^{\circ}\text{C}$ . 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  $50^{\circ}\text{C}$  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 =  $358^{\circ}\text{C}$ ;  $\text{N}_2$  flows for sheath/aux/sweep = 45/13/1  
255 arbitrary units; source = 2.5 kV; ion transfer tube =  $-30\ \text{V}$  and  $342^{\circ}\text{C}$ ; 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  $\sim 2.4$  min and the internal standard (FSPP) at  $\sim 2.5$  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 ( $\pm 5$  ppm window) against a 0.1-100  $\mu\text{M}$  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,  $\beta$ -sitosterol and campesterol were identified in both the  
327 NTC and transformed lines (Figure 7). Stigmasterol and  $\beta$ -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 amorpha-  
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 the  
360 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 amorpha-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 amorpha-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  
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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.

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## 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- $\delta$ -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 +/- 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 +/- 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 +/- SD (NTC – n=6, *AMS*\_RNAi lines – n=3). **B** On column FPP concentrations in  $\mu\text{mol}$  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 +/- 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 +/- 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 +/- 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

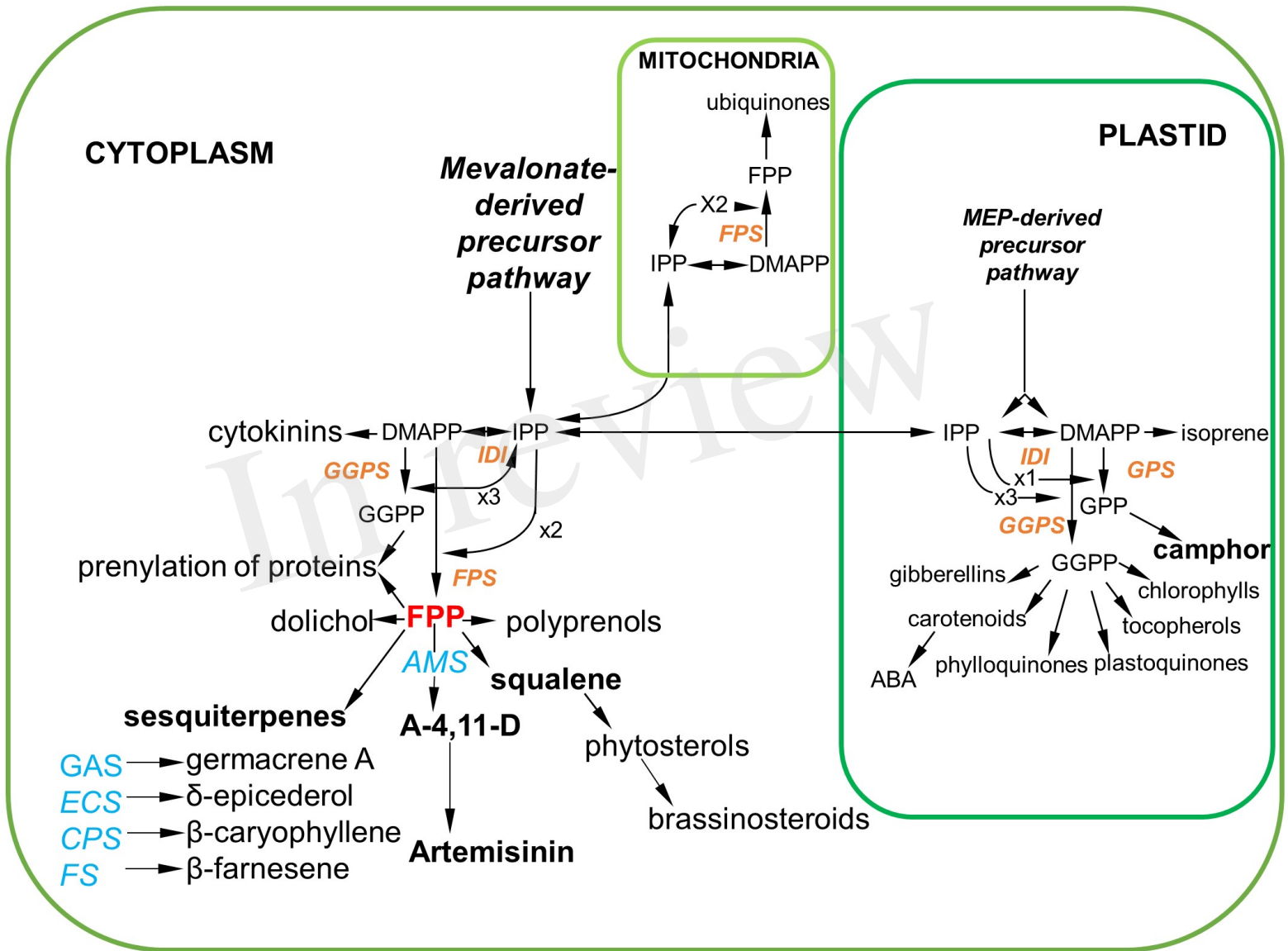
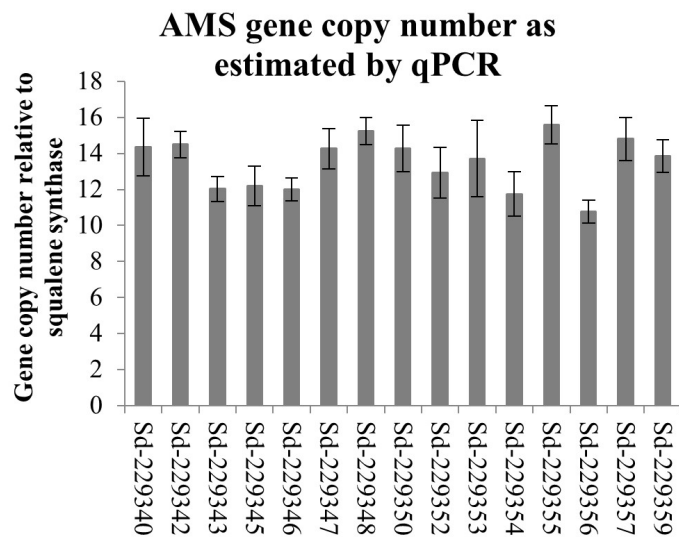


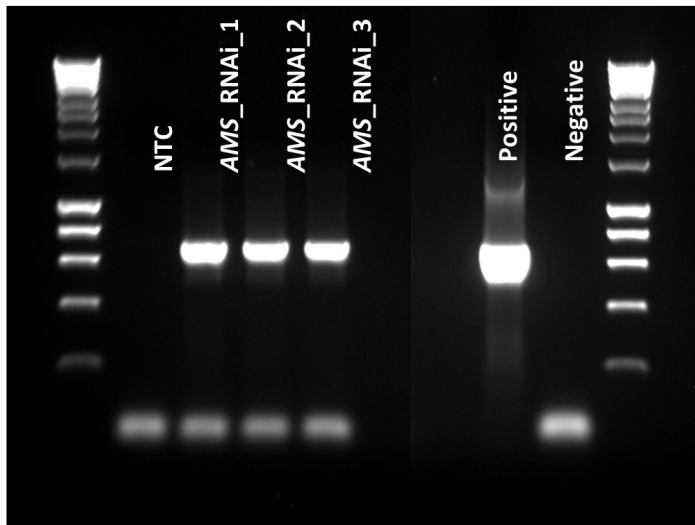
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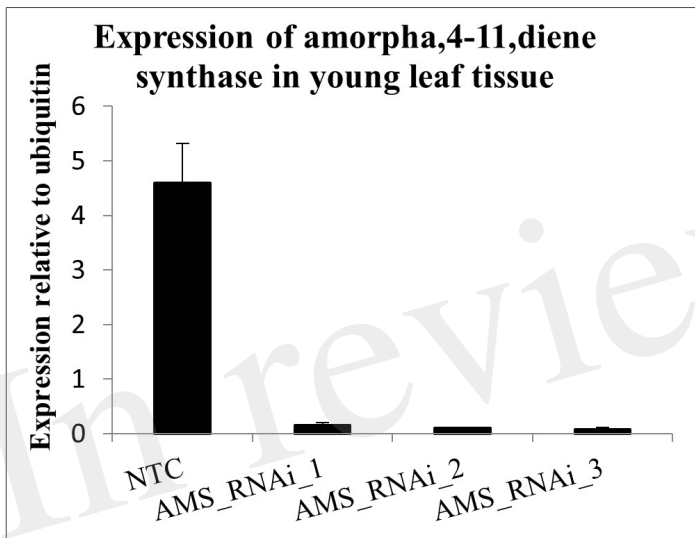
In review

Figure 3.JPEG

A



B



C

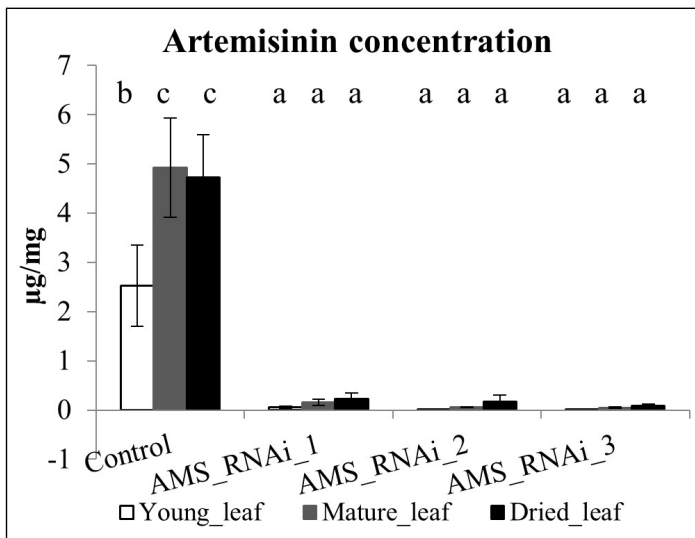
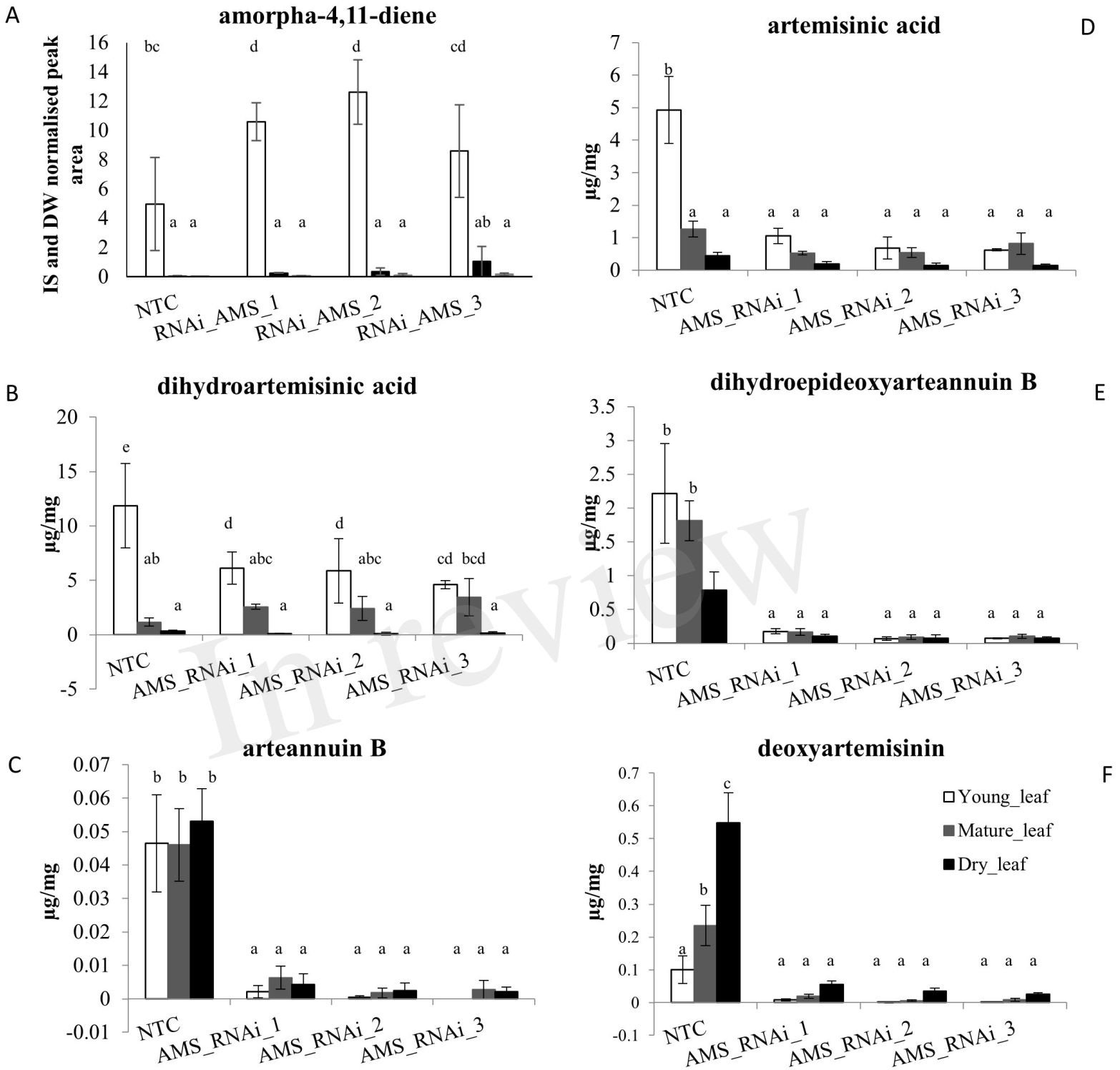


Figure 4.JPEG



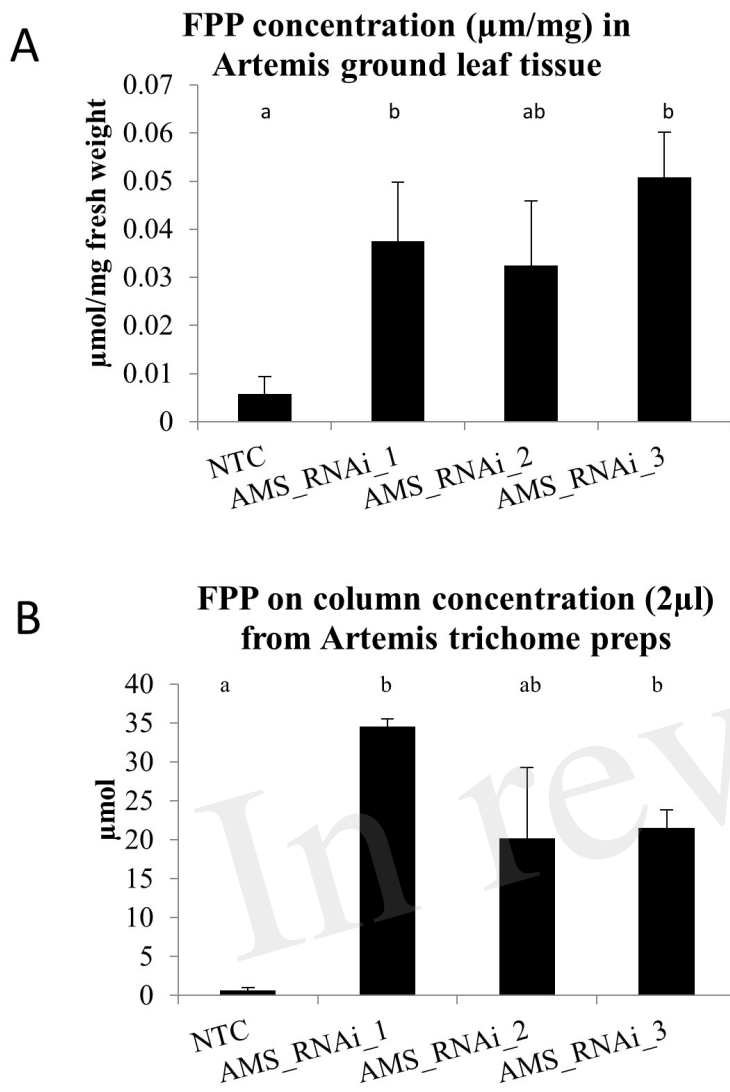


Figure 6.JPEG

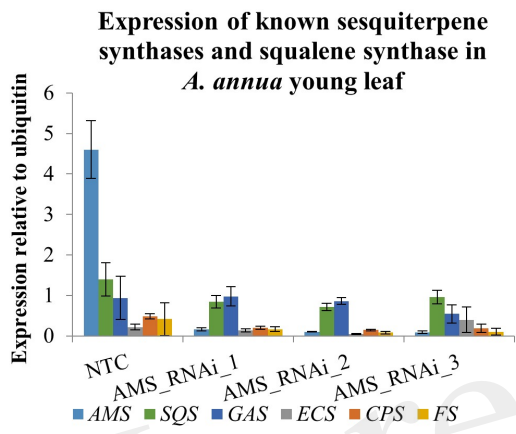
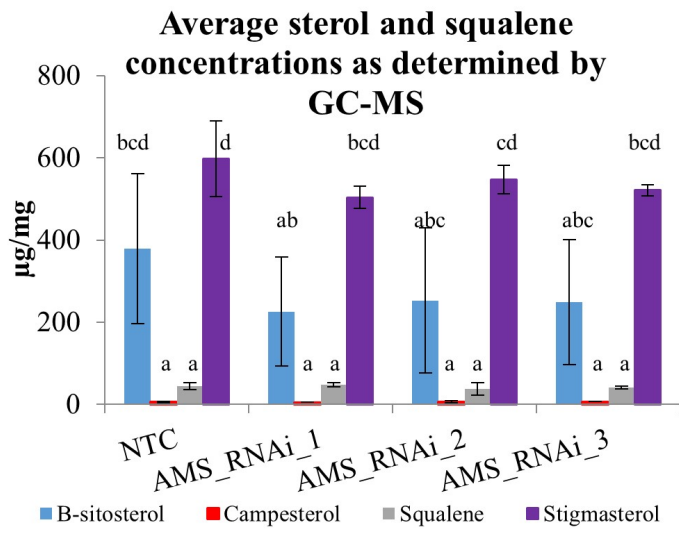


Figure 7.JPEG



In review

Figure 8.JPEG

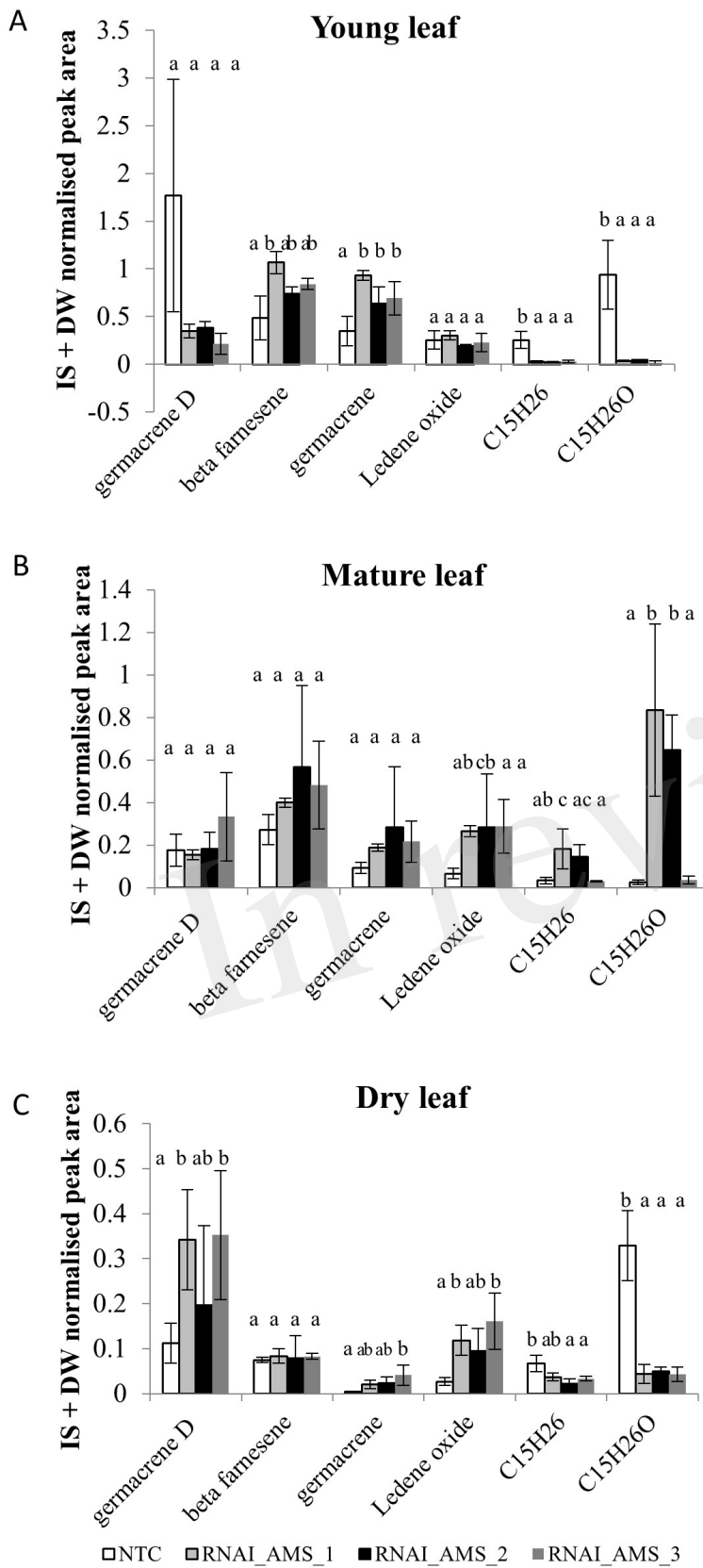


Figure 9.JPEG

