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1 ***Artemisia annua* L. plants lacking *Bornyl diPhosphate Synthase***  
2 **reallocate carbon from monoterpenes to sesquiterpenes except**  
3 **artemisinin**

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19 **Running title:** Camphor null *Artemisia annua*

20 **Keywords:** *Artemisia annua*, artemisinin biosynthesis, camphor biosynthesis, *Bornyl*  
21 *diPhosphate Synthase*, Glandular Secretory Trichomes,

22 **Abstract**

23 The monoterpene camphor is produced in glandular secretory trichomes of the medicinal plant  
24 *Artemisia annua*, which also produces the antimalarial drug artemisinin. We have found that,  
25 depending on growth conditions, camphor can accumulate at levels ranging from 1- 10% leaf dry  
26 weight (LDW) in the Artemis F1 hybrid, which has been developed for commercial production of  
27 artemisinin at up to 1% LDW. We discovered that a camphor null (camphor-0) phenotype segregates  
28 in the progeny of self-pollinated Artemis material. Camphor-0 plants also show reduced levels of other  
29 less abundant monoterpenes and increased levels of the sesquiterpene precursor farnesyl  
30 pyrophosphate plus sesquiterpenes, including enzymatically derived artemisinin pathway  
31 intermediates but not artemisinin. One possible explanation for this is that high camphor concentrations  
32 in the glandular secretory trichomes play an important role in generating the hydrophobic conditions  
33 required for the non-enzymatic conversion of dihydroartemisinic acid tertiary hydroperoxide to  
34 artemisinin. We established that the camphor-0 phenotype associates with a genomic deletion that  
35 results in loss of a *Bornyl diPhosphate Synthase* (*AaBPS*) gene candidate. Functional characterization  
36 of the corresponding enzyme *in vitro* confirmed it can catalyse the first committed step in not only  
37 camphor biosynthesis but also in a number of other monoterpenes, accounting for over 60% of total  
38 volatiles in *A. annua* leaves. This *in vitro* analysis is consistent with loss of monoterpenes in camphor-  
39 0 plants. The *AaBPS* promoter drives high reporter gene expression in *A. annua* glandular secretory

40 trichomes of juvenile leaves with expression shifting to non-glandular trichomes in mature leaves,  
41 which is consistent with *AaBPS* transcript abundance.

## 42 1 Introduction

43 Malaria still poses a global threat, with 229 million cases occurring worldwide and 409,000 deaths in  
44 2020 (2020 WHO Malaria report). Artemisinin, the main component in the WHO recommended  
45 treatment for malaria, is produced in glandular secretory trichomes (GSTs), specialised 10-cell  
46 structures found on the surface of the leaves, stems and flower buds of *Artemisia annua* L. More recent  
47 work suggests that the non-glandular trichome cells also express artemisinin biosynthetic pathway  
48 genes and produce artemisinin (Judd et al., 2019). The demand for the plant-sourced drug has been  
49 responded to by breeding efforts to improve yields, including the development of F1 hybrids such as  
50 Artemis (Delabays *et al.*, 2001). Recently, over eighty additional natural products have been NMR-  
51 characterised from *A. annua*, including monoterpenes, sesquiterpenes, diterpenes, triterpenes / sterols,  
52 phenylpropanoids, flavonoids, aliphatic hydrocarbons, aromatic and aliphatic alcohols, aldehydes,  
53 ketones and acids (Czechowski *et al.*, 2018). *A. annua* essential oil is synthesised in GSTs and has been  
54 the subject of numerous studies reporting antibacterial and antifungal activities, but chemical  
55 composition varies widely depending on the phylogeographic origin of the plants. Generally, the five  
56 major constituents in essential oil across *A. annua* varieties are artemisia ketone (2-68%), camphor (3-  
57 48%), 1,8-cineole (5-31.5%), germacrene D (0.3-21.2%) and borneol (7-20%) (Bilia *et al.*, 2014).  
58 Camphor, traditionally obtained through the distillation of the wood of the camphor tree (*Cinnamomum*  
59 *camphora*), is a major essential oil component of many aromatic plant species. In addition to its use as  
60 a skin penetration enhancer (Chen *et al.*, 2013), camphor also exhibits insecticidal, antimicrobial,  
61 antiviral, anticoccidial, anti-nociceptive, anticancer and antitussive activities (Chen *et al.*, 2013). The  
62 camphor biosynthetic pathway begins with the cyclisation of geranyl diphosphate (GPP) by the enzyme  
63 (+)-bornyl diphosphate synthase (BPS), yielding (+)-bornyl diphosphate, which is then hydrolysed to  
64 (+)-borneol through the action of bornyl-diphosphate diphosphatase. The last step is catalysed by (+)-  
65 borneol dehydrogenase (BDH) as it oxidises (+)-borneol to (+)-camphor (Croteau and Karp, 1976,  
66 Croteau and Karp, 1979b, Croteau *et al.*, 1981). (+)-bornyl diphosphate synthases have been  
67 functionally characterised from sage, lavender and *Lippia dulcis* (Wise *et al.*, 1998, Despinasse *et al.*,  
68 2017, Hurd *et al.*, 2017, Singh *et al.*, 2020). Sage Bornyl diPhosphate Synthase (SoBPS) has been  
69 cloned and structurally characterised revealing the exact molecular mechanism of GPP cyclisation to  
70 bornyl –diphosphate (Wise *et al.*, 1998, Whittington *et al.*, 2002). Interestingly, recombinant SoBPS is  
71 also able to produce significant amounts of other monoterpenes including camphene, limonene, a-  
72 pinene, terpinolene and myrcene (Wise *et al.*, 1998). <sup>13</sup>C isotope labelling studies have shown the GPP  
73 used for biosynthesis of camphor is produced through the non-mevalonate (MEP) pathway by  
74 combination of the C5 isoprenoid units, isopentenyl pyrophosphate (IPP) and dimethylallyl  
75 pyrophosphate (DMAPP) (Croteau *et al.*, 1981). The pool of isoprenoid precursors is also required for  
76 the production of artemisinin, where IPP and DMAPP are of mixed biosynthetic origin, coming from  
77 both the cytosolic mevalonate and plastidial MEP pathways (Schramek *et al.*, 2010). Camphor  
78 biosynthesis may therefore represent one of the major sinks for the plastidial pool of IPP and DMAPP  
79 in *A. annua* GSTs and if this is the case then a block in camphor synthesis could lead to increased  
80 production of artemisinin.

81 Detailed metabolite profiling of leaf material from an *A. annua* cv. Artemis F1 hybrid identified  
82 camphor as the most abundant volatile compound. Screening genetic variation led to the discovery of  
83 a camphor-0 phenotype in both Artemis F2 material and Artemis M2 material, that had been derived  
84 from selfed ethyl methanesulfonate (EMS) mutagenised Artemis M1 material. This allowed us to  
85 assess the impact of the removal of camphor, and the majority of monoterpenes, on the accumulation

86 of artemisinin. Contrary to our expectations and despite a significant increase in farnesyl  
87 pyrophosphate (FPP, the precursor of the artemisinin biosynthetic pathway), and a number of pathway  
88 intermediates, artemisinin levels were not changed. We propose a model to explain these results and  
89 establish the genetic and biochemical basis of the camphor-0 phenotype.

## 90 **2 Materials and Methods**

### 91 **2.1 Plant material.**

92 Artemis is an F1 hybrid variety developed by Mediplant (Conthey, Switzerland), produced by crossing  
93 C4 and C1 parental material of East Asian origin (Delabays *et al.* 2001). Its artemisinin content has  
94 been reported to reach 1.4% of the leaf dry weight when grown in the field (Townsend *et al.* 2013).  
95 Artemis F1 population and M2 populations were created and grown from cuttings at Mediplant,  
96 Conthey, Switzerland and Yorkshire, UK field trial sites as described previously (Graham *et al.*, 2010,  
97 Townsend *et al.* 2013). An F2 family-based pedigree population containing 662 individuals, created by  
98 selfing 84 randomly selected F1 Artemis individuals, was grown for 12 weeks in the glasshouse, under  
99 long- day conditions (16 hrs day / 8 hrs night) at 22°C max / 17°C min in P40 trays using Levington  
100 F2 seed and modular compost.

### 101 **2.2 Plant crossing.**

102 Plant crosses between camphor-0 M2 individuals grown from cuttings were created as described  
103 previously (Czechowski *et al.* 2016). Cuttings from parental genotypes were maintained in 10 cm  
104 diameter pots under 16-hour days for 12 weeks. Plants were then transferred to 12-hour days to induce  
105 flowering. Flowering was identified as the point at which the first ray florets were visible. Once  
106 flowering commenced, bags were placed over two plants to enable hybrid production. These bags were  
107 shaken every two days to encourage pollination. Once all flowers had died back the bags were removed  
108 and the flower heads allowed to dry out under glass for a further 6 weeks before harvesting.

### 109 **2.3 Metabolite analysis by Gas- (GC-) and Ultra-High Performance Liquid Chromatography** 110 **– Mass Spectrometry (UPLC-MS)**

111 Metabolite analysis by GC- and UPLC-MS was performed as described previously (Czechowski *et al.*,  
112 2016). Eighteen plants from Artemis F1 and from progenies of one selected camphor-0 sibling cross  
113 were grown in glasshouse conditions from seeds in 4-inch pots for 12 weeks under long- day conditions  
114 as described above. Metabolite profiles were generated from 50mg FW pooled samples of leaves at  
115 different developmental stages: 1-5 (counted from the apical meristem) representing the juvenile stage;  
116 leaves 7-9 representing the young, expanding stage; and leaves 11-13 representing the mature,  
117 expanded stage. Fresh leaf samples were stored at -80°C. Trichome-specific metabolites were extracted  
118 as described previously (Czechowski *et al.* 2016) with minor modifications. Briefly, 50 mg of fresh  
119 material was extracted by gentle shaking in 500 µl chloroform for 1 h.

120 For UPLC-MS analysis of sesquiterpenes, a diluted (1:5 (v/v) extract:ethanol) 2µL aliquot was injected  
121 on an Acquity UPLC system (Waters, Elstree, UK) fitted with a Luna 50×2 mm 2.5 µm HST column  
122 (Phenomenex, Macclesfield, UK). Metabolites were eluted at 0.6 mL/min and 40°C using a linear  
123 gradient from 40% to 100% acetonitrile containing 0.1% (v/v) formic acid over 2.5 min.  
124 Pseudomolecular [M+H]<sup>+</sup> ions were detected using a Thermo Fisher LTQ-Orbitrap (ThermoFisher,  
125 Hemel Hempstead, UK) mass spectrometer fitted with an atmospheric pressure chemical ionisation  
126 source operating in positive ionisation mode under the control of Xcalibur 2.1 software. Data were  
127 acquired over the m/z range 100 - 1000 in FTMS centroid mode with resolution set to 7500 FWHM at

128 m/z 400. Data extraction and analysis was performed using packages and custom scripts in R 3.2.2  
129 (<https://www.R-project.org/>). XCMS (Smith et al., 2006) incorporating the centWave algorithm  
130 ((Tautenhahn et al., 2008)) was used for untargeted peak extraction. Deisotoping, fragment, and adduct  
131 removal were performed using CAMERA (Kuhl et al., 2012) Artemisinin was quantified using the  
132 standard curve of the response ratio of artemisinin (Sigma, Poole, UK) to internal standard ( $\beta$ -  
133 artemether; Hallochem Pharmaceutical, Hong Kong) added to extracts and standards. Metabolites were  
134 identified with reference to authentic standards or NMR-resolved structures and empirical mass  
135 formulae calculated using the R package rcdk (Guha et al., 2007) within 10 ppm error and elemental  
136 constraints of: C = 1–100, H = 1–200, O = 0–20, N = 0–1. Peak concentrations were calculated using  
137 bracketed response curves, where standard curves were run every ~30 samples. Metabolite  
138 concentrations were expressed as a proportion of the residual dry leaf material following extraction.

139 For analysis of monoterpenes and volatile sesquiterpenes, an aliquot of chloroform extract (prior to  
140 dilution with ethanol for UPLC analysis) was taken for GCMS analysis using an Agilent 6890 GC  
141 interfaced to a Leco Pegasus IV TOF MS (Leco, Stockport, UK). A 1  $\mu$ L aliquot was injected into a  
142 CIS4 injector (Gerstel, Mülheim an der Ruhr, Germany) fitted with a 2 mm ID glass liner containing  
143 deactivated glass wool at 10°C. The injector was ramped from 10°C to 300°C at 12°C/s then held at  
144 300°C for 5 min. The carrier gas was He at constant flow of 1 mL/min and the injection split ratio was  
145 1:10. Peaks were eluted using a Restek Rxi-5Sil MS column, 30m x 0.25 mm ID x 0.25  $\mu$ m film  
146 thickness (Thames Restek, Saunderton, UK). The following temperature gradient was used: isothermal  
147 40°C 2 min, ramp at 20°C/min to 320°C then hold for 1 min; total run time ~20 min. The transfer line  
148 was maintained at 250°C and the MS used to collect -70eV EI scans over the m/z range 20–450 at a  
149 scan rate of 20 spectra/second. Acquisition was controlled by ChromaTof 4.5 software (Leco).  
150 ChromaTof was used to identify peaks and deconvolute spectra from each run, assuming a peak width  
151 of 3s and a minimum s/n of 10. Peak areas were exported as deconvoluted total ion traces (DTIC) and  
152 annotated against authentic standards and NIST spectral matches. For semi-quantitative comparisons,  
153 DTIC peak areas were normalized to the added internal standard (tetradecane) and sample dry weight.  
154 A standard curve was created for camphor, to enable absolute concentration comparisons with  
155 artemisinin.

156 R stats base package, nlme, multcomp, and multcompView were used for all statistical data analysis

#### 157 **2.4 Extraction and quantification of isoprenoid diphosphates (GPP, FPP, GGPP)**

158 Extraction and quantification of isoprenoid diphosphates was performed as described previously  
159 (Catania *et al.* 2018). Twelve plants from Artemis F1 and from progenies of one selected camphor-0  
160 M2 cross were grown in glasshouse conditions from seeds in 4-inch pots for 12 weeks in a randomised  
161 way as described above. Juvenile leaves (leaf 1-5) were harvested from main stem and side branches  
162 and pooled from two plants to achieve around 1g of fresh material which was immediately flash frozen  
163 in LN2. The material was ground to a fine powder using a TissueLyser II ball mill fitted with stainless  
164 steel grinding jars (Qiagen, Crawley, UK) operated at 15 Hz for 15 sec with one repeat. Powdered leaf  
165 material was weighed out and extracted three times with 5ml of ice cold methanol:water (7:3, v/v),  
166 including a 0.3  $\mu$ g/ml of each of three internal standards: geranyl-, farnesyl- and geranylgeranyl-S-  
167 thiolodiphosphates (GSPP, FSPP and GGSP; Echelon Biosciences). Extracts were processed  
168 according to Nagel *et al.*, (2014). Total extract volume was brought up to 20 ml with water. Briefly,  
169 each extract was passed through a Chromabond HX RA column (150 mg packing), which had first  
170 been conditioned with 5 ml methanol and 5 ml of water, and compounds eluted under gravity with 3  
171 ml of 1 M ammonium formate in methanol. The eluate was evaporated under a stream of nitrogen to  
172 dryness, dissolved in 250  $\mu$ L of water:methanol (1:1.v/v), and a 2  $\mu$ L aliquot injected on a Waters

173 Acquity I-Class UPLC system interfaced to a Thermo Orbitrap Fusion Tribrid mass spectrometer under  
 174 Xcalibur 4.0 control. Isoprenoid compounds were eluted on a Waters Acquity C18 BEH column  
 175 (2.1mm x 100 mm, 1.7 um) at 50°C using the following binary gradient program: solvent A = 20mM  
 176 ammonium bicarbonate + 0.1% triethylamine; solvent B = 4:1 acetonitrile:water + 0.1% triethylamine;  
 177 flowrate 0.4 ml/min; 0-100% B linear gradient over 4 minutes. Post column, compounds were ionized  
 178 using a heated electrospray source (vaporizer = (250)°C; N2 flows for sheath/aux/sweep = 30/15/10  
 179 arbitrary units; source = 4kV; ion transfer tube = -30V and 275°C; tube lens = -40V). Data was acquired  
 180 in full scan Ion trap mode with the following settings: 100-500 m/z range, max ion time 100ms, 1  
 181 microscan, AGC target = 3.00e+04.

182 No signal could be detected for GPP (elution time 2.1 min) or GGPP (elution time 3 min) in any of the  
 183 biological samples analysed, despite the clear signal observed for the 1-50uM linear GGPP/GSPP  
 184 response ratio calibration curve (R2 = 0.999) and for the 1-50uM linear GPP/GSPP response ratio  
 185 calibration curve (R2 = 0.9913). FPP eluted at ~2.6 min and the internal standard (FSPP) at ~2.7 min.  
 186 The deprotonated pseudomolecular ions ([M-H]<sup>-</sup>) of 381.1519 and 397.1261 for FPP and FSPP,  
 187 respectively, were used for quantification (+/- 5ppm window) against a 1-100uM linear FPP/FSPP  
 188 response ratio calibration curve (R2 = 0.9852), using Xcalibur 4.0 software (Thermo).

## 189 **2.5 RNA extraction, cDNA preparation and gene expression analysis using qRT-PCR.**

190 Total RNA was extracted from the same leaf tissue as subjected to metabolite profiling analysis. Leaf  
 191 tissue from juvenile expanding- and mature-stage leaves sampled as described above was ground to a  
 192 fine powder using Qiagen Retsch MM300 TissueLyser (Qiagen, Hilden, Germany) and total RNA  
 193 extracted using the RNAeasy kit with on-column DNaseI digestion step (Qiagen, Hilden, Germany).  
 194 RNA was quantified using NanoDrop-1000 (NanoDrop products, Wilmington, USA) and its integrity  
 195 was checked on agarose gels. 2 ug of total RNA was reversely transcribed using SuperScript II kit (Life  
 196 Technologies Ltd, Paisley, UK) and Oligo(dT)12-18 Primer (Life Technologies Ltd, Paisley, UK)  
 197 according to manufacturer's instructions. Expression levels of putative *BorenoI diPhosphahte Synthase*  
 198 (*AaBPS*) and its two close homologues: *AaBPS-likeA* and *AaBPS-likeB*; *Farnesyl diPhosphate*  
 199 *Synthase (FPS)*, *amorpho-4,11-diene synthase (AMS)*, *amorpho-4,11-diene C-12 oxidase*  
 200 (*CYP71AV1*), *cytochrome P450 reductase (CPR)*, *artemisinic aldehyde Δ 11 (13) reductase (DBR2)*  
 201 and aldehyde dehydrogenase (*ALDH1*), relative to *ubiquitin (UBQ)* were determined by qPCR as  
 202 described before (Czechowski *et al.* 2018). Reactions were run in 3 technical replicates. Gene-specific  
 203 primers used are detailed in Table S3. Real-time PCR was performed on CFX384 Teal-Rime System  
 204 (Bio-Rad Laboratories) using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad  
 205 Laboratories). Each 10-μL reaction contained 1 μL of a 5-fold dilution of the cDNA synthesis reaction,  
 206 5μL of 2X supermix, and primers at a final concentration of 250 nM. The cycling conditions included  
 207 an initial activation step for 30 s at 98°C followed by 40 cycles of denaturation at 98°C for 10 s and  
 208 annealing/extension at 60°C for 30 s. Fluorescence data were acquired during the annealing/extension  
 209 phase. A melt curve was obtained at the end of the amplification to allow confirmation of product  
 210 specificity. C<sub>T</sub> values were obtained using CFX Manager Software (Bio-Rad laboratories) and  
 211 amplification efficiencies (E) obtained using LinReg PCR (Ruijter *et al.*, 2009). Transcript abundance  
 212 for the gene of interest (GOI) relative to *UBiQuitin* gene (UBQ) was determined using the formula:  
 213  $GOI \text{ expression level} = (E_{GOI})^{\Delta C_T} / (E_{UBQ})^{\Delta C_T}$ .

## 214 **2.6 Genomic DNA extractions**

215 For DNA extraction 30-50 mg of fresh leaf material was harvested from plants growing in the  
 216 glasshouse. DNA was extracted using Qiagen BioSprint 96. Extracted DNA was quantified

217 spectrophotometrically using NanoDrop-8000 (NanoDrop products, Wilmington, USA) and  
218 normalized to 10 ng/ul for genotyping assays, inverse PCR and other PCR analysis.

## 219 **2.7 Genotyping analysis of camphor-0 and Artemis F2 Populations.**

220 Allele specific primers for *AaBPS*, *AaBPS-likeA* and *AaBPS-likeB* genomic DNA sequences were  
221 designed for KASPar and ABI3730xl genotyping assays based on the regions allowing to distinguish  
222 between closely related sequences, as depicted on Figure S5A. Primer sequences are listed in Table  
223 S3.

224 Twenty nanograms of leaf genomic DNA extracted from individual Artemis F2 plants was used for  
225 10ul KASPar assay reaction containing: 1x KASP V4.0 low ROX master mix (LGC Genomics,  
226 Teddington, UK); 167nM of each of the two allele specific primers and 414nM of universal primer  
227 according to the manufacturer's recommendations. Allelic discrimination runs and allelic  
228 discrimination analysis were performed on Viiia7 system (Life Technologies Ltd, Paisley, UK)  
229 according to manufacturer's recommendations.

230 For the ABI3730xl SNP assays, two differentially sized primers specific to each SNP alleles were  
231 designed and used in one PCR reaction with a common, locus specific primer containing M13 tail. A  
232 mismatch base at position -4 or -5 from 3' end of each allele-specific primer was introduced to increase  
233 allele-specificity of the PCR reactions. Universal fluorescent (FAM) labeled M13 primers were  
234 included in the reaction to incorporate FAM dye label to allow visualisation on the capillary apparatus.  
235 PCR amplification was performed in 10 µl total volume, with 2 ng genomic DNA, 1x AmpliTaq Gold®  
236 PCR Master Mix (Applied Biosystems, Foster City, CA) containing 0.25 Units of AmpliTaq Gold, 50  
237 nM forward and reverse primers and 750nM M13 primer. PCR was carried out with 40 cycles using  
238 an annealing temperature of 60°C. PCR reactions were diluted 1:20 in H<sub>2</sub>O and fractionated on an ABI  
239 3730xl capillary sequencer (Applied Biosystems, Foster City, CA). SNPs were analysed and scored  
240 using GeneMarker™ software (Softgenetics, State College, PA).

## 241 **2.8 Inverse PCR of *AaBPS* 5' flanking region.**

242 Inverse PCR was carried out on genomic DNA essentially according to the method of Ochman *et al.*,  
243 (1988) except that a second round of PCR was included giving a linear product containing DNA  
244 flanking the BPS gene. Nested primers were designed around the *AaBPS* sequence as follows: primer  
245 pair 1 (outer) comprised BPS5'\_F1 and BPS5'\_R1; primer pair 2 (inner) comprised BPS5'\_F2 and  
246 BPS5'\_R2. (Primer sequences are listed in Table S3). 250 ng of genomic DNA extracted from C1 and  
247 C4 Artemis parents was digested with *Ball* and diluted 1:10, 1:100 and 1:1000. Inverse PCR was  
248 carried out using QIAGEN Multiplex PCR Kit (Qiagen, Crawley, UK) with primer pair 1 (outer) with  
249 dilutions of *Ball*-digest and PCR conditions were: 95°C for 15min followed by 40 cycles of  
250 denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72 °C for 5 min, which was  
251 followed by final extension at 72 °C for 5 min. Nested PCR was carried out on 1 ul of undiluted inverse  
252 PCR products using inner primer pairs for 5'-flanking ends, QIAGEN Multiplex PCR Kit (Qiagen,  
253 Crawley, UK) and the same cycling conditions as above except annealing was carried out at 61°C.  
254 Nested PCR resulted in 1934bp product which was gel-purified, diluted 1/10 and ligated into  
255 Strataclone vector using Strataclone PCR cloning kit (Beckman Coulter Genomics, Takeley, UK) and  
256 StrataClone solo competent cells transformed by heat shock. Positive clones were sent for Sanger  
257 sequencing using M13 universal primers.

## 258 **2.9 Construction of *AaBPS::Gusi* vector and *A. annua* transformations**

259 The pSAT7a vector (Tzfira *et al.*, 2007) was used to create the *AaBPS promoter::Gusi* reporter fusion  
 260 construct. The Gusi sequence was extracted from the *pBII21::Gusi* using the Sac I and Sal I restriction  
 261 sites and ligated into the *pSAT7a* vector to create a *pSAT7a::Gusi* vector. A 1934bp fragment of the  
 262 BPS promoter was amplified from genomic DNA extracted from Artemis parent C1, using the primers  
 263 tailed with *AgeI* and *NotI* restriction sites, listed in Table S3. Restriction digest was carried out  
 264 following PCR and the digested amplified fragments cloned and verified by sequencing prior to cloning  
 265 into the *pSAT7a::Gusi* vector. The full *AaBPS promoter::Gusi* construct (3357bp) was digested out of  
 266 the *pSAT7a* using *AgeI* and *NotI* restriction sites and the fragment blunted using T4 DNA polymerase.  
 267 The construct was cloned into the *pRSC2* binary vector using the *EcoRV* site and then the resulting  
 268 colonies were verified for orientation and sequence prior to transformation into the binary vector  
 269 pRSC2. The binary vector was then transformed into *Agrobacterium tumefaciens* (LBA4404) by  
 270 electroporation and 100 µl glycerol stocks set up for subsequent plant transformations. Transformation  
 271 of *Artemisia annua* Artemis was carried out following the protocol described by Catania *et al.*, (2018).

## 272 **2.10 Histochemical Gus staining**

273 Gus ( $\beta$ -glucuronidase) staining of transformed material was carried out following the protocol  
 274 described by Jefferson *et al.*, (1987). Briefly plant material for staining was submerged in GUS stain  
 275 and vacuum infiltrated for 20 minutes followed by incubation at 37°C. Samples were incubated for up  
 276 to 24 hours. The reaction was followed by observation with a dissecting microscope and stopped when  
 277 the stain was sufficiently developed. To enable the stain to be more clearly visualised the samples were  
 278 cleared with successive washes in 70 % ethanol at 37°C.

## 279 **2.11 PCR analysis of *AaBPS* locus.**

280 Primers covering the entire *AaBPS* genomic sequence with 5' and 3' flanking sequences obtained from  
 281 inverse PCR were designed as depicted on Figure 5A. Primer sequences are listed in Table S3.  
 282 Genomic DNA was extracted from glasshouse grown fourteen camphor-0 M2 lines, two randomly  
 283 selected Artemis F1 individuals and from C1 and C4 Artemis parents. Twenty nanograms of genomic  
 284 DNA was used in 20 ul PCR reactions containing 500nM of each forward and reverse primer, 1U of  
 285 Phusion® High-Fidelity DNA Polymerase NEB, and 200nM of dNTPs. PCR conditions were as  
 286 follows 98°C - 30 sec, followed by 10 cycles of 98°C – 10 sec 70°C – 30sec Touch down) – decrease  
 287 1°C per cycle, 72°C – 2.5 min, followed by 30 cycles of 98°C – 10 sec, 60°C – 30 sec, 72°C – 2.5 min  
 288 and final extension at 72°C for 5 min. PCR products were ligated into Strataclone vector using  
 289 Strataclone PCR cloning kit (Beckman Coulter Genomics, Takeley, UK) and StrataClone solo  
 290 competent cells transformed by heat shock. Positive clones were sent for Sanger sequencing using M13  
 291 universal primers.

## 292 **2.12 Heterologous expression of *AaBPS* and purification of recombinant protein**

293 In order to confirm the catalytic function of *AaBPS*, we carried out heterologous expression in BL21  
 294 (DE3) *E. coli* strain. ChloroP analysis revealed that has a putative plastid targeting (PT) sequence at  
 295 the 5' end. Three sequences were tested for expression: full length and two truncated forms. ChloroP  
 296 and TargetP analysis predicted the PT sequence cleavage site after residue A34 in the predicted amino  
 297 acid sequence. BPS\_tr1 was truncated to this point, ie: sequence begins at residue C35. Whittington *et*  
 298 *al.*, (2002) reported a longer N-terminal PT region in Sage BPS, beginning the coding region just before  
 299 the active site lid residues. BPS\_tr2 begins at R58, the start of the predicted active site lid residues of  
 300 *AaBPS*. Primers for PCR cloning of the *AaBPS* sequence were designed to incorporate either an *NheI*  
 301 site at the 5' end of the coding sequence, and a *BamHI* site at the 3' end. After sequencing to confirm  
 302 accuracy, products were cloned into either the *NheI*-*BamHI* or sites of pET28a, as appropriate and then



303 sub-cloned into pDONR207 entry vector. The three AaBPS versions (Full length and two truncations)  
304 were then transferred into pH9GW destination vector via the LR reaction and then transformed into  
305 BL21 (DE3) *E. coli* strain for protein expression. Expression was scaled up to 1 L cultures and protein  
306 purified using 2-step large scale purification processes using metal affinity chromatography (1 ml)  
307 coupled to a Superdex 16/600 200pg gel filtration column (120 ml). Between 50 and 300 µg of each  
308 concentrated protein was obtained using this purification method for the subsequent activity assays.

### 309 **2.13 AaBPS activity assay.**

310 We adapted an activity assay that had been used in the lab to assay a prenyl-diphosphate synthase  
311 together with an assay used for the Sage bornyl diphosphate synthase (Wise *et al.*, 1998). The reaction  
312 contained: 1X MTC buffer, 1mM DTT, 10mM MgCl<sub>2</sub>, and 75µM GPP were set up in 2mL glass vial  
313 in total volume of 500µL. Reactions were started by addition of 50 µl protein (0.15 or 0.85 µM final  
314 concentration) and overlaid with 500 µl pentane and incubated at 31°C for 3 hours with slow shaking  
315 (130 rpm). To hydrolyse the pyrophosphate product, 20 units of rAPid alkaline phosphatase (Roche  
316 04898133001) was added to the aqueous layer and gently mixed. Reactions were incubated at 30°C for  
317 2 hours. Vials were vortexed vigorously for 10 seconds, then centrifuged at 1,500 xg for 10 minutes.  
318 2µl of the organic layer was sampled directly out of the vial and injected onto the GC-MS following  
319 the GC-MS method used before (Wise *et al.*, 1998).

## 320 **3 Results**

### 321 **3.1 A single recessive allele is responsible for camphor-0 phenotype in Artemis M2 and F2** 322 **plants**

323 We previously performed EMS mutagenesis on the *A. annua* Artemis F1 hybrid and produced an M2  
324 population (Graham *et al.*, 2010; Czechowski *et al.*, 2016). This population was grown in parallel with  
325 an F1 Artemis mapping population in multiple field trials in the UK and Switzerland and individuals  
326 from each were subjected to phenotyping that included detailed metabolite profiling (Larson *et al.*,  
327 2013, Townsend *et al.*, 2013). Concentrations of artemisinin and camphor in dry leaf material from  
328 these two field grown populations were quantified against standard curves as described in Materials  
329 and Methods (Fig. 1). The concentration of camphor on a leaf dry weight basis (maximum of 1%) was  
330 of a similar order to artemisinin (maximum of 1.4%) in the Artemis F1 field grown material (Fig. 1A).  
331 We performed a glasshouse based screen to select about 10% of plants from the M2 population on the  
332 basis of high artemisinin content as previously described (Graham *et al.*, 2010, Larson *et al.*, 2013,  
333 Townsend *et al.*, 2013). The vast majority of these individuals showed elevated camphor and  
334 artemisinin content reaching 6% and 3% of leaf dry weight, respectively, with camphor concentration  
335 actually exceeding that of artemisinin in most of the lines (Fig. 1B). That camphor and artemisinin  
336 concentrations show a strong positive correlation in individuals from both F1 and M2 populations (Fig.  
337 1A and 1B) suggested that the monoterpene and sesquiterpene pathways are not competing for flux  
338 from the plastidial isoprenoid pathway (Schramek *et al.*, 2010). The analysis revealed that 14 out of  
339 the 233 M2 field-grown lines almost completely lacked camphor but had relatively normal levels of  
340 artemisinin (Fig. 1B) and no morphological alterations (data not shown). All 14 of these camphor-0  
341 M2 lines came from different M1 parents, suggesting that some form of segregation rather than  
342 mutagenesis may be responsible for emergence of the camphor-0 phenotype in the M2 material. The  
343 Artemis F1 hybrid variety is derived from a cross between two heterozygous parents (Delabays *et al.*,  
344 2001; Graham *et al.*, 2010). We selfed 85 randomly chosen Artemis F1 individuals that had not been  
345 subjected to EMS mutagenesis and measured camphor and artemisinin in dry leaves from 662 of the  
346 resulting F2 progeny and found that 126 of these exhibited the camphor-0 phenotype (Fig. S1). That  
347 the camphor-0 phenotype is not present in F1 plants but appears in F2 populations (Fig. 1 and Fig. S1)

348 strongly suggests that the Camphor-0 phenotype is not due to EMS mutagenesis but is instead due to  
349 segregation of a recessive trait that emerges in the F2 generation having been acquired from one or  
350 other of the parental lines which we previously showed contain a high level of heterozygosity (Graham  
351 *et al.*, 2010).

352 We performed test crosses on five of the selected camphor-0 M2 individuals that confirmed the  
353 phenotype was due to a single genetic locus as described in Materials and Methods (Fig. S2). Progeny  
354 of these crosses were all camphor-0 (Fig. S2) further confirming the phenotype being due to a single  
355 recessive allele that had also been fixed in the M2 material. Camphor-0 progeny of test-crosses  
356 displayed the same morphology as Artemis F1 controls when grown under glasshouse conditions (Fig.  
357 S3).

### 358 **3.2 Metabolite and gene expression profiling of developmental stages of camphor-0 leaves**

359 Three distinct leaf developmental stages: young (leaves 1-5), expanding (leaves 7-9) and mature  
360 (leaves 11-13) were harvested from individual plants and subjected to metabolite (GC- and UPLC-MS)  
361 and gene expression (qRT-PCR) profiling as described in the Materials and Methods. This  
362 developmental series captures the major transition points in artemisinin biosynthesis and wider  
363 terpenoid metabolism in leaves of *A. annua* (Czechowski *et al.*, 2016, Czechowski *et al.*, 2018). The  
364 metabolite analysis revealed that camphor is the most abundant terpenoid detected in Artemis F1 leaf  
365 extracts with concentration reaching up to 10% of extracted dry weight in young leaves from  
366 glasshouse grown material (Fig. 2A i). While artemisinin levels remained unaltered in camphor-0  
367 material (Fig. 2A ii), Table S1), other significant changes in artemisinin-pathway metabolites were  
368 detected including an increase in the artemisinin precursors amorpho-4,11-diene (A-4,11-D) and  
369 dihydroartemisinic acid (DHAA) in young and expanding leaves (Fig. 2A iii) and iv), Table S1 and  
370 Table S2). There was also a significant increase in the level of dihydroartemisinic acid tertiary  
371 hydroperoxide (DHAAOOH), a previously described intermediate of non-enzymatic conversion of  
372 DHAA, in camphor-0 expanding leaves (Fig. 2A v) and Table S1). We also observed elevated levels  
373 of two products of the alternative non-enzymatic conversion of DHAA: deoxyartemisinin and dihydro-  
374 epi-deoxyarteannuin B (DHEDB) in mature leaves of camphor-0 lines, when compared with Artemis  
375 F1 (Fig. 2A vi) and vii), Table S1). The significant increases in artemisinin precursor metabolites in  
376 camphor-0 material were not accompanied by changes in the level of transcripts of Artemisinin-  
377 pathway genes, except for a marked increase in *AaDBR2* transcript in camphor-0 mature leaves (Fig.  
378 S4).

379 Other, less abundant monoterpenes were also missing (camphene, *cis*- and *trans*-sabinene hydrate,  
380 pinocarvone, carvone) or strongly reduced ( $\alpha$ -pinene,  $\alpha$ -terpineol) in all leaf types of camphor-0 lines.  
381 (Fig. 2A viii) – xi) and Table S2). Monoterpenes missing in the camphor-0 lines represent around 50%  
382 of the total volatiles measured by GC-MS in Artemis F1 young leaves (Table S2), which is reflected  
383 by almost a 2-fold reduction of total volatile content of camphor-0 young leaves (Fig. 2B). There was,  
384 however, a significant increase in some sesquiterpenes such as  $\alpha$ -bisabolol and spathulol (Fig. 2A xii)  
385 and Table S2), in addition to the artemisinin-pathway metabolites detailed above. To further investigate  
386 the increase in sesquiterpenes unrelated to the artemisinin pathway in camphor-0 material, we  
387 measured the level of the isoprenoid precursors GPP, FPP and GGPP in juvenile leaf material using  
388 previously described protocols (Catania *et al.* 2018, Nagel *et al.* 2014). While GPP and GGPP were  
389 undetectable in all extracts, FPP levels were elevated by approximately 5-fold in camphor-0 juvenile  
390 leaves compared to F1 Artemis (Fig. 2C). FPP synthase gene transcript levels were unchanged in  
391 camphor-0 material (Fig. S4).

392 Metabolite changes in the camphor-0 glandular secretory trichomes (GSTs) are summarised in Fig.  
393 2D.

### 394 **3.3 Absence of *AaBPS* from the *A. annua* genome correlates with the camphor-0 phenotype**

395 BPS catalyses the first committed step in camphor biosynthesis (Fig. 3A). BLAST analysis of an *A.*  
396 *annua* EST library (Graham *et al.*, 2010) had previously revealed several monoterpene synthases that  
397 had been functionally characterised as linalool synthases *QH1* and *QH5* (Jia *et al.*, 1999) and  $\beta$ -pinene  
398 synthase *QH6* (Lu *et al.*, 2002). One candidate monoterpene synthase was designated *BORNYL-*  
399 *DIPHOSPHATE SYNTHASE* (*AaBPS*) based on highest sequence homology to other characterised  
400 plant *BPS* genes. The predicted protein sequence of the *AaBPS* contains a putative plastid targeting  
401 sequence at the 5' terminus. *AaBPS* was found to be preferentially expressed in trichomes or trichome-  
402 containing tissues of *A. annua* at levels higher than any of the other monoterpene synthase candidates  
403 (Graham *et al.*, 2010), and was the only gene from the camphor biosynthetic pathway, annotated in *A.*  
404 *annua* EST library (Graham *et al.*, 2010), therefore a plausible candidate to investigate further.

405 We also identified two other *AaBPS-like* cDNA sequences that we designated as *AaBPS-likeA* and  
406 *AaBPS-likeB*. These both have 93% nucleotide identity with *AaBPS* and predicted amino acid identities  
407 of 87% for *AaBPS-likeA* and 88% for *AaBPS-likeB*. However, these sequences differ from *AaBPS* at  
408 conserved positions in the active- and substrate binding sites (Fig. S5A). The low degree of nucleotide  
409 variation between these three genes led us to develop three gene specific SNP-based molecular markers  
410 which we used to genotype camphor-0 and camphor-containing material using KASPar and ABI3730  
411 platforms as described in Materials and Methods (Fig. S5B). qRT-PCR gene expression analysis of  
412 mRNA isolated from the three leaf developmental stages revealed that, while *AaBPS* is expressed at  
413 high levels in young leaves and lower levels in expanding leaves of Artemis F1 (Fig. 3B), it is not  
414 detected in mRNA from any of the three leaf stages of camphor-0 plants (Fig. 3B). *AaBPS* gene  
415 expression across the different leaf stages of Artemis F1 (Fig. 3B) correlates with camphor levels (Fig.  
416 2A i) and is similar to a number of genes involved in artemisinin biosynthesis including *Amorpha-*  
417 *4,11-diene synthase* (*AaAMS*), *Amorpha-4,11-diene C12 oxidase* (*AaCYP71AV1*), *Artemisinic*  
418 *aldehyde  $\Delta 11(13)$  reductase* (*AaDBR2*) and *Aldehyde dehydrogenase* (*AaALDH1*) (Fig. S4). *AaBPS-*  
419 *likeA* expression follows a similar pattern of expression to *AaBPS*, but at 10-fold lower levels in both  
420 Artemis F1 camphor-containing and camphor-0 material (Fig. 3B). *AaBPS-likeB* transcripts were not  
421 detected in any of the leaf material analysed by qRT-PCR.

422 We used the Iterative Threading ASSEmbly Refinement (I-TASSER) approach (Yang *et al.*, 2015,  
423 Zhang *et al.*, 2017) to perform protein structure predictions on amino acid sequences of *AaBPS*,  
424 *AaBPS-likeA* and *AaBPS-likeB*. While the predicted *AaBPS* protein structure overlaid very well with  
425 the bornyl diphosphate synthase crystal structure from *Salvia officinalis* (SoBPS, Fig. 3C) the *AaBPS-*  
426 *likeA* and *AaBPS-likeB* overlays with SoBPS were both disrupted at the conserved GPP binding site  
427 which would appear to be due to the presence of phenylalanine at position 346 rather than leucine at  
428 position 347 and isoleucine at position 343 in *AaBPS* and SoBPS, respectively (Fig. 3C; Fig. S5C).  
429 The SoBPS active site cavity is considered to be a tight fit for the GPP hydrocarbon chain with an  
430 estimated packing density of the enzyme–substrate complex of around 78% (Whittington *et al.*, 2002).  
431 The presence of a large aromatic amino acid, such as phenylalanine at the GPP binding site of *AaBPS-*  
432 *likeA* and *AaBPS-likeB* could result in steric hindrance and disrupt any monoterpene synthase function  
433 of these *AaBPS* homologues (Fig. S5C).

### 434 **3.4 The *AaBPS* promoter drives high reporter gene expression in glandular secretory** 435 **trichomes and hair-like (T-shape) non-secretory trichomes.**

436 Inverse PCR was performed on genomic DNA isolated from Artemis F1 material and the resulting  
437 1934bp sequence upstream of the *AaBPS* start codon was cloned upstream of the *beta-glucuronidase*  
438 (*GUS*) reporter gene and the resulting construct was used to transform Artemis F1 material, using a  
439 previously described *Agrobacterium tumefaciens* based protocol (Catania *et al.*, 2018) as described  
440 in Materials and Methods). GUS activity staining of various tissues from the T2 generation of  
441 *promBPS::GUSi* transformed plants showed that the *AaBPS* promoter drives high GUS expression in  
442 glandular secretory trichomes of the juvenile leaves (L1-5) and hair-like (T-shape) non-secretory  
443 trichomes present in mature leaves (L11-13), leaf petioles and stems (Fig. 4).

### 444 3.5 Camphor-0 lines lack the entire AaBPS gene locus

445 To further investigate the *AaBPS* locus in camphor-0 material we used Artemis F1 genomic DNA to  
446 PCR amplify a 4.25 kb region that included promoter sequence obtained by inverse PCR and confirmed  
447 by *promoter::GUS* fusions (Fig. 4). Primer pairs were then designed across the 4.25 kb region (Fig.  
448 5A) and used to establish that the *AaBPS* sequence was absent from genomic DNA of 14 camphor-0  
449 M2 individuals but present in genomic DNA from Artemis F1 and the Artemis C1 and C4 parents (Fig.  
450 5B). Genomic DNA from camphor-0 M2 individuals did amplify a 3.97 kb fragment containing  
451 *Amorpha 4,11 diene synthase* gene (Fig. 5B). *AaBPS* locus-related PCR products were cloned and  
452 verified by DNA sequencing. While this PCR analysis does not define the entirety of the genomic  
453 deletion in camphor-0 plants, our analysis does show that both the promoter and the entire coding  
454 region of the *AaBPS* locus are missing.

### 455 3.6 Recombinant AaBPS protein performs the first committed step in camphor biosynthesis

456 We cloned the full length and two 5' truncated forms of the *AaBPS* coding sequence into a plasmid  
457 vector for heterologous expression in *E. coli*. Truncation of the putative plastid targeting (PT) sequence  
458 was carried out to overcome any possible interference with the production of the protein in the  
459 microbial system (Fig. 6A). Analysis of the *AaBPS* gene using software such as ChloroP or TargetP  
460 predicted the 5' plastid targeting (PT) sequence cleavage site after residue A34 in its amino acid  
461 sequence resulting in the truncated AaBPS\_tr1 sequence beginning at residue C35. A longer N-terminal  
462 PT region in Sage BPS, resulting in the coding region just before the active site lid residues has also  
463 been reported (Whittington *et al.*, 2002) and on this basis AaBPS\_tr2 was designed to begin at R58,  
464 the start of the predicted active site lid residues of *A. annua* BPS. Transformation of *E. coli* BL21  
465 (DE3) resulted in very low levels of soluble full-length protein compared to both truncated versions.  
466 All three versions of purified AaBPS protein were subjected to the sage Bornyl diPhosphate Synthase  
467 (SoBPS) activity assay using GPP as a substrate with borneol being detected by GC-MS following  
468 rAPid alkaline phosphatase treatment of the diphosphate product (Wise *et al.*, 1998). The full-length  
469 and truncated forms of the AaBPS protein all produce borneol [1] as the major product (Fig. 6B).  
470 Unreacted GPP substrates can also be seen hydrolysed to geraniol [2] (Fig. 6B). The BPS\_tr1 protein  
471 is the most active form, producing the most soluble protein as well as turning over 95% of the GPP  
472 substrate into borneol and other minor monoterpenes (Fig. 6B). We have observed a number of minor  
473 peaks in the AaBPS-tr1 profile which were absent in the control reaction without AaBPS-tr1 protein  
474 added (Fig. 6B). We used the NIST database to assign these products as described in the Materials and  
475 Methods section. Our analysis shows that AaBPS-tr1 is producing 5.6% camphene [9], 0.2% camphene  
476 hydrate [4], 2.5% limonene [8], 2.2%  $\alpha$ -pinene [10], 1.8% *trans*-sabinene hydrate [5], 0.2% *cis*-  
477 sabinene hydrate [7], 0.3%  $\alpha$ -terpineol [3] and 0.3% terpinolene [6], in addition to borneol as the major  
478 product (86.8%) from GPP substrate (Fig. 6C).

## 479 4 Discussion

480 Our previous work to increase content of the antimalarial drug artemisinin in the medicinal plant  
481 *Artemisia annua* L. resulted in creation of F1 and F2 mapping populations and M2 mutagenised  
482 populations (Graham *et al.*, 2010). Camphor is a monoterpene, described as one of the major essential  
483 oil constituents across *A. annua* varieties. Here we report the discovery of the camphor-null phenotype,  
484 apparent in F2 and M2 populations derived from the Artemis F1 variety. The fact the camphor-0  
485 phenotype is not present in F1 plants but appears in F2 populations (Fig. 1 and Fig. S1), together with  
486 results of test crosses of camphor-0 material derived from the M2 population indicating the phenotype  
487 is due to a single recessive allele, strongly suggests that the Camphor-0 phenotype is a consequence  
488 of segregation of a recessive trait that emerges in the F2 and M2 generations having been acquired  
489 from one or other of the parental lines which we previously showed contain a high level of  
490 heterozygosity (Graham *et al.*, 2010).

491 Our results show that camphor accumulates to high concentrations of up to 10% leaf dry weight in  
492 Artemis F1 material and that the absence of camphor does not result in an increased flux into  
493 artemisinin but rather an increase in artemisinin pathway precursors (Fig. 2A, Table S1). Differential  
494 extraction techniques have shown that artemisinin accumulates in sub-apical cavities of GSTs (Duke  
495 *et al.*, 1994) and accumulation of other sesquiterpene lactones in sub-apical cavities of GSTs have also  
496 been reported across *Artemisia* species (Cappelletti *et al.*, 1986). Although the exact condition in *A.*  
497 *annua* trichomes required for the conversion of DHAA into artemisinin is unknown previous reports  
498 strongly suggest such auto-catalytic conversion requires both light and a non-aqueous environment  
499 whereas spontaneous transformation of DHAA to DHEDB and deoxyartemisinin appears to be  
500 facilitated by a more aqueous environment (Brown and Sy 2004, Czechowski *et al.* 2016). One possible  
501 explanation of the wild type levels of artemisinin observed in Camphor-0 mutants despite strong  
502 increases in FPP and artemisinin precursors is actually that camphor may be an important contributor  
503 to the non-aqueous environment required for the conversion of DHAA to artemisinin (Fig. 2D). In the  
504 absence of camphor, we show that in addition to the accumulation of the enzymatically produced  
505 artemisinin precursors amorpha-4,11-diene and DHAA, there is also an increase in levels of DHEDB  
506 and deoxyartemisinin, possibly as a result of the more aqueous camphor-0 environment favouring non-  
507 enzymatic flux of DHAA into these compounds rather than artemisinin (Fig. 2A, Table S1). That  
508 artemisinin does still accumulate to wild type levels in the Camphor-0 mutant suggests that to a certain  
509 extent the non-aqueous environment generated by other compounds such as non-polar lipids is  
510 sufficient for the spontaneous conversion of DHAA to artemisinin. Comparative metabolite profiling  
511 of sub-apical cavities versus GST cells, using techniques such as single cell sampling followed by  
512 electrospray ionization-mass spectrometry (Nakashima *et al.*, 2016) would shed further light on the *in*  
513 *vivo* chemical environment required for non-enzymatic DHAA conversions and the possible role of  
514 camphor in these processes in *Artemisia annua* L.

515 Detailed metabolite profiling revealed that a number of other lower abundant monoterpenes (relative  
516 to camphor) were also missing or strongly reduced in camphor-0 material when compared to camphor  
517 - containing Artemis F1, amounting to a reduction in total volatile content of camphor-0 young leaves  
518 by 50% (Fig. 2 B, Table S2). Levels of sesquiterpenes unrelated to artemisinin were on the other hand  
519 elevated in camphor-0 leaf material, which was accompanied by a significant increase in the content  
520 of FPP precursor for sesquiterpene synthesis (Fig. 2A and C, Table S1). RNAi-mediated silencing of  
521 *amorpha-4,11-diene synthase* (*AMS*) genes resulted in a similar increase in FPP accumulation in  
522 transgenic *A. annua* (Catania *et al.* 2018). That study showed no impact of an increase in FPP on di-  
523 or triterpene levels in the silenced lines.

524 Bioinformatic analysis of the previously published transcriptomics datasets from *Artemisia annua*  
525 (Graham *et al.*, 2010) identified *Bornyl diPhosphate Synthase* (*AaBPS*) as a candidate involved in

526 catalysing the first committed step in camphor biosynthesis in the Artemis F1 hybrid. *AaBPS* is  
527 expressed highly in GSTs and its expression was not detectable in camphor-0 individuals (Fig. 3B).  
528 PCR analysis of genomic DNA showed that the *AaBPS* sequence is absent from the genomes of all  
529 camphor-0 individuals derived from both F2 and M2 populations but is present in Artemis F1 and the  
530 C4 and C1 Artemis parents. -No other sequences present in the transcriptomics datasets are very closely  
531 related to *AaBPS*. However, *AaBPS-likeA* and *AaBPS-likeB* were present in genomic DNA from all  
532 camphor-0 and camphor-containing material (Fig. S5B) and *AaBPS-likeA* is also expressed in both  
533 camphor-0 and camphor-containing individuals (Fig. 3B). Protein modelling predicts that of the three  
534 homologues only AaBPS is likely to be functional on GPP substrate, which is essential for it to act in  
535 the first committed step in camphor biosynthesis.

536 We cloned and functionally characterised the *AaBPS* promoter using promoter-GUS fusions expressed  
537 in *Artemisia annua* Artemis F1 and obtained expression patterns consistent with the EST and qRT-  
538 PCR analysis of *AaBPS* gene expression (Fig. 3B, Graham *et al.* 2010). It is interesting that the *AaBPS*  
539 promoter shows similar activity in hair-like (T-shape) non-secretory trichomes on mature leaves and  
540 stems (Figure 4C-E) but not on the young leaf where its activity seems to be specific to glandular  
541 secretory trichomes (Figure 4A and B). These results demonstrate the importance of both temporal and  
542 spatial regulation of gene expression underpinning production of this most abundant of monoterpenes  
543 in *A. annua*.

544 Heterologous expression of the *AaBPS* sequence in *E. coli* followed by protein purification and *in vitro*  
545 bioactivity assays confirm the identity of AaBPS as a BORNEOL DIPHOSPHATE SYNTHASE that  
546 converts GPP substrate to borneol (Fig. 6B). The most active version of the protein sequence with  
547 plastid targeting (PT) sequence removed (*AaBPS-tr1*) produced not only borneol (86.8% of the total  
548 activity) but also small amounts of other monoterpenes including camphene (5.6%), camphene hydrate  
549 (0.2%), limonene (2.5%),  $\alpha$ -pinene (2.2%), *trans*-sabinene hydrate (1.8%), *cis*-sabinene hydrate  
550 (0.2%),  $\alpha$ -terpineol (0.3%) and terpinolene (0.3%) (Fig. 6C). AaBPS *in vitro* activity on GPP is  
551 therefore similar to sage BPS, as SoBPS has been shown to produce borneol (75%), (+)- $\alpha$ -pinene  
552 (3.4%), (-)-camphene (9.5%), (+)-camphene (0.5%), (-)-limonene (3.9%), (+)-limonene (3.9%),  
553 terpinolene (2.1%), and myrcene (1.5%) (Wise *et al.* 1998). It is notable that the relative abundance of  
554 the minor AaBPS *in vitro* activity products correlate with the abundance of these compounds in all  
555 three types of *Artemis* leaf tissues analysed by GC-MS (Fig. 6D). It is also noteworthy that some of the  
556 minor monoterpenes produced by AaBPS *in vitro* are absent (camphene, *cis*- and *trans*-sabinene  
557 hydrate) or strongly reduced ( $\alpha$ -pinene) in leaf tissues of camphor-0 line (Fig. 2, Table S2). These  
558 results lead us to conclude that AaBPS is not only responsible for production of camphor but also other  
559 monoterpenes, amounting to over 50% of total volatiles in *A. annua* F1 hybrid *Artemis* leaves (Fig.  
560 6D).

## 561 **5 Conflict of Interest**

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

## 564 **6 Authors contributions**

565 T.C., C.B., A.R., D.R., D.H., T.C., D.Z., M.S. conducted the experiments; T.C., C.B., D.J.B., I.A.G.  
566 and P.O.M. designed experiments, T.C., C.B., T.R.L., Y.L., M.S., P.O.M. analysed the data, T.C. and  
567 I.A.G wrote the article with input from Y.L., T.R.L and P.O.M.

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## 656 10 Figure legends

### 657 Fig. 1 Artemisinin and camphor concentration in *Artemis* F1 mapping- and M2 mutant 658 populations.

659 Camphor and artemisinin concentration in dry leaf material from Artemis F1s (A) and M2 (B)  
660 populations grown in Mediplant (Switzerland) field trials (2008). Leaf material was harvested from  
661 248 F1 and 233 M2 individuals, extracted and analysed by ultra-high performance liquid- and gas  
662 chromatography–mass spectrometry (UPLC- and GC-MS) as described in Materials and Methods.  
663 Camphor and artemisinin levels were quantified against standard curves using authentic standards.

### 664 Fig. 2 Detailed molecular characterization of camphor-0 M3 lines.

665 Levels of artemisinin-related and other selected mono- and sesquiterpenes (A) were quantified by gas  
666 chromatography–mass spectrometry (GC-MS) (i), (iii), (viii) - (xii) and ultra-high performance liquid  
667 chromatography–mass spectrometry (UPLC-MS) (ii), (iv)-(vii) analysis of extracts from fresh leaves  
668 L1-5 (juvenile), L7-9 (expanding), L11-13 (mature) as counted from the apical meristem from 12-  
669 weeks old glasshouse-grown Artemis F1 (blue bars) and selected camphor-0 M3 (orange bars); error  
670 bars – SE (n=5). nd - not detectable. GC-MS data for camphor were converted from internal standard  
671 (IS) and dry weight (DW) normalised peak areas to  $\mu\text{g} / \text{mg}$  extracted dry weight (vii) against a standard  
672 curve run with pure camphor. For both (A) and (B) letters represent Tukey's range test results after one  
673 way ANOVA or REML. Groups not sharing letters indicate statistically significant differences. (B)  
674 Level of total volatiles measured by GC-MS in three types of leaf material as indicated in (A). (C)  
675 Level of FPP measured in extracts from fresh juvenile leaves L1-5 Artemis F1 (blue bars) and selected  
676 camphor-0 M3 (orange bars); FW - fresh weight, error bars – SE (n=3), \*- statistically significant  
677 difference (t-test) between Artemis and camphor-0 at  $p < 0.05$ . (D) Summary of metabolite changes in  
678 the camphor-0 glandular secretory trichomes (GSTs), full arrows – known enzymatic steps, dotted  
679 arrows – potential non enzymatic conversions, full green arrows – metabolite changes (all leaf stages).  
680 Metabolite abbreviations: GPP - geranyl diphosphate, FPP – farnesyl diphosphate, A-4,11-D –  
681 amorpha-4,11-diene, DHAA - dihydroartemisinic acid, DHAAOOH- dihydroartemisinic acid tertiary  
682 hydroperoxide, DHEDB – dihydro-epi-deoxyarteannin B, Enzyme abbreviations: IDI - Isopentenyl  
683 Diphosphate Isomerase, GPS - Geranyl diPhosphate synthase, BPS - Bornyl diPhosphate synthase,  
684 FPS- Farnesyl diPhosphate Synthase. Artemisinin pathway: AMS – amorpha-4,11-diene synthase,  
685 CYP71AV1 - amorpha-4,11-diene C-12 oxidase, CPR – cytochrome P450 reductase, DBR2 -  
686 artemisinic aldehyde  $\Delta$  11 (13) reductase, ALDH1 - aldehyde dehydrogenase. Question marks indicate  
687 putative active transport systems operating in *A. annua* GSTs.

### 688 Fig. 3 Identification of the *AaBPS* coding sequence and *AaBPS* expression .

689 (A) Camphor biosynthesis pathway modified from (Croteau *et al*, 1981). (B) Expression of *AaBPS* and  
690 *AaBPS-likeA* in L1-5 juvenile, L7-9 expanding and L11-13 mature leaves. Error bars – SE (n=12). nd-  
691 transcript not detectable by qRT-PCR. \* - statistically significant difference (t-test) between Artemis

692 and camphor-0 at  $p < 0.05$ . **(C)** Predicted AaBPS protein sequence was modelled (green) using the I-  
 693 TASSER approach (C-score 0.5) and overlaid on sage (purple) Bornyl-diPhosphate Synthase (SoBPS,  
 694 pdb structure 1N1B, 40% amino acid identity) I-TASSER predicted GPP binding residues highlighted  
 695 in yellow, predicted catalytic W326 in red.  $Mg^{2+}$  (green) and  $Hg^{2+}$  (grey) ions from 1N1B structure are  
 696 also shown.

697 **Fig. 4 Characterisation of *AaBPS* promoter using promoter-GUS fusions.**

698 Tissues of 12-weeks old transgenic lines expressing *pAaBPS::GUSi*: **(A)** juvenile leaf (10x) **(B)**  
 699 juvenile leaf (40x) **(C)** mature leaf (10x) **(D)** leaf petiole (10x) **(E)** stem (10x) were stained for *GUS*  
 700 activity as described in Materials and Methods and photographed using bright-field microscopy  
 701 (magnification indicated in the brackets). Scale bars indicated for each picture in red.

702 **Fig. 5 Genomic deletion in camphor-0 lines.**

703 **(A)** Location of primers (P1-P8) designed for various parts of *AaBPS* gene including 5' promoter region  
 704 (Primer sequences in Table S3). **(B)** PCR amplification on genomic DNA isolated from 14 camphor-0  
 705 M2 (lines 1-14), two individual Artemis F1 (lines 15-16), Artemis Parents C4 (line 17) and C1 (line  
 706 18) using primers annotated on panel A. Line 19 – no template control. Line M – Gene Ruler™ 1kb  
 707 DNA ladder (Thermo Fisher). Amplification of full length *AaAMS* gene (GenBank Accession  
 708 AF327527) used as a positive control. Size of PCR amplicons predicted from genomic DNA sequence  
 709 shown.

710 **Fig. 6 Functional characterisation of *AaBPS*.**

711 **(A)** Alignment of predicted amino acid sequence of AaBPS (GenBank accession OL656813) with  
 712 SoBPS (GenBank accession AF051900). Truncation points used to generate truncated versions of  
 713 AaBPS are indicated as BPS-tr1 and BPS-tr2. BPS-fl represents a full length protein. Red bars – active  
 714 site lid, Blue bars - active site, Brown bar – substrate binding site annotated from Whittington *et al.*,  
 715 2002. Identical (Black) and similar (grey) positions highlighted. **(B)** AaBPS *in vitro* protein activity  
 716 assay using gas chromatography–mass spectrometry (GC-MS) based detection of monoterpenes. No  
 717 protein control includes geraniol, the product of GPP hydrolysis (peak 1). Identification of peak 1 and  
 718 2 as geraniol and borneol respectively was assigned using known standards. Identities of peaks 3-10  
 719 were assigned using the NIST database and shown in C. **(C)** Relative abundance of AaBPS *in-vitro*  
 720 products for the most active truncated version of the protein (BPS-tr1). **(D)** Relative abundance of  
 721 selected volatiles in three types of *A. annua* Artemis leaves measured by GC-MS: L1-5 (juvenile), L7-  
 722 9 (expanding), L11-13 (mature). Error bars – SE (n=6).

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