

This is a repository copy of *Artemisia annua* mutant impaired in artemisinin synthesis demonstrates importance of nonenzymatic conversion in terpenoid metabolism.

White Rose Research Online URL for this paper:
<https://eprints.whiterose.ac.uk/112845/>

Version: Accepted Version

Article:

Czechowski, Tomasz, Larson, TR, Catania, Theresa May orcid.org/0000-0002-9882-3878 et al. (3 more authors) (2016) *Artemisia annua* mutant impaired in artemisinin synthesis demonstrates importance of nonenzymatic conversion in terpenoid metabolism. *Proceedings of the National Academy of Sciences of the United States of America*. pp. 15150-15155. ISSN 1091-6490

<https://doi.org/10.1073/pnas.1611567113>

Reuse

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

Takedown

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

An *Artemisia annua* mutant impaired in artemisinin synthesis demonstrates importance of non-enzymatic conversion in terpenoid metabolism

Tomasz Czechowski^a, Tony R. Larson^a, Theresa M. Catania^a, David Harvey^a, Geoffrey D. Brown^b, and Ian A. Graham^{a1}

^aCentre for Novel Agricultural Products, Department of Biology, University of York, Heslington, York YO10 5DD, United Kingdom; ^bDepartment of Chemistry, University of Reading, Reading RG6 6AD, United Kingdom

Submitted to Proceedings of the National Academy of Sciences of the United States of America

Artemisinin, a sesquiterpene lactone produced by *Artemisia annua* glandular secretory trichomes, is the active ingredient in the most effective treatment for malaria currently available. We identified a mutation that disrupts the CYP71AV1 enzyme, responsible for a series of oxidation reactions in the artemisinin biosynthetic pathway. Detailed metabolic studies of *cyp71av1-1* revealed that the consequence of blocking the artemisinin biosynthetic pathway is the redirection of sesquiterpene metabolism to a novel sesquiterpene epoxide, which we designate arteannuin X. This sesquiterpene approaches half the concentration observed for artemisinin in wild type plants, demonstrating high-flux plasticity in *A. annua* glandular trichomes and their potential as factories for the production of novel alternate sesquiterpenes at commercially viable levels. Detailed metabolite profiling of leaf maturation time-series and precursor-feeding experiments revealed that non-enzymatic conversion steps are central to both artemisinin and arteannuin X biosynthesis. In particular, feeding studies using ¹³C-labelled dihydroartemisinic acid (DHAA) provided strong evidence that the final steps in the synthesis of artemisinin are non-enzymatic *in vivo*. Our findings also suggest that the specialised sub-apical cavity of glandular secretory trichomes functions as a location for both the chemical conversion and storage of phytotoxic compounds, including artemisinin. We conclude that metabolic engineering to produce high yields of novel secondary compounds such as sesquiterpenes is feasible in complex glandular trichomes. Such systems offer advantages over single cell microbial hosts for production of toxic natural products.

Artemisinin | p450 oxidase | terpenoid | sesquiterpene | *Artemisia annua*

Introduction

The sesquiterpene lactone, artemisinin is the active ingredient in artemisinin-combination therapies - the most effective treatment for malaria currently available. The production of artemisinin occurs in specialized 10-cell biserial glandular trichomes present on the leaves, stems and inflorescences of *Artemisia annua* (1-3). Artemisinin is phytotoxic (4) and is believed to accumulate in the sub-apical extracellular cavity of glandular trichomes (2). This ability of trichomes to transfer compounds into extracellular cavities (5, 6) overcomes the problem of cellular toxicity. Conveniently, natural products located in these cavities are readily extractable. This is exemplified by artemisinin, which is extracted on a commercial scale by submerging intact dried *A. annua* leaf material in organic solvent with the active ingredient being directly crystallised from the condensed organic fraction (7). There has been much interest in determining the steps involved in the biosynthesis of artemisinin in recent years, largely driven by efforts to produce this compound through a completely biosynthetic microbial-based fermentation route (8, 9). Presently microbial production is at best semi-synthetic, terminating at artemisinic acid (AA), which must then be extracted from culture and chemically converted to artemisinin using photooxidation (8, 10). The

lack of a low cost, scalable conversion process is considered to be a major factor in the failure so far of the semi-synthetic route to sustainably impact the market making it uncompetitive with plant-based production (11)

Although the enzymatic steps involved in production of the non-phytotoxic precursors amorpha-4,11-diene (A-4,11-D) and dihydroartemisinic acid (DHAA) have been elucidated (12-15) and the associated genes have been shown to be highly expressed in both the apical and sub-apical cells of the glandular secretory trichomes (3, 16), the final steps in the conversion of DHAA to artemisinin are considered to be non-enzymatic and may be extracellular (17, 18). Therefore, microbial-based "complete" synthetic biology routes to artemisinin may never be achievable. Meanwhile, modern molecular breeding has succeeded in improving *A. annua* (19), creating hybrids reaching artemisinin yields of 1.4% dry leaf biomass in commercial field trials (20) (<http://www.artemisia1seed.org/>).

The glandular secretory trichomes of *A. annua* produce almost six hundred secondary or specialised metabolites many of which are terpenoids (21). These include a significant number of terpene allylic hydroperoxides and endoperoxides (21). This latter class, of which artemisinin is a member, are typically bioactive and therefore potential targets for development as pharmaceuticals (22). Consistent with their phytochemical complexity, it is known that glandular secretory trichomes express multiple members of gene families, encoding enzymes of specialised metabolism including terpene synthases and cytochrome P450 oxidases (16, 19, 23). Many of these enzymes are considered to

Significance

The anti-malarial, artemisinin, is a sesquiterpene lactone produced by glandular secretory trichomes on the leaves of *Artemisia annua*. Using a mutant impaired in artemisinin synthesis we demonstrate the importance of non-enzymatic conversions in terpenoid metabolism and highlight the ability of *A. annua* glandular secretory trichomes to re-direct flux into a novel sesquiterpene. The research presented offers new insight into the mechanism of the final steps of artemisinin synthesis in *A. annua*, with significant implications for future production of secondary compounds in native vs heterologous host systems.

Reserved for Publication Footnotes

Figure 1

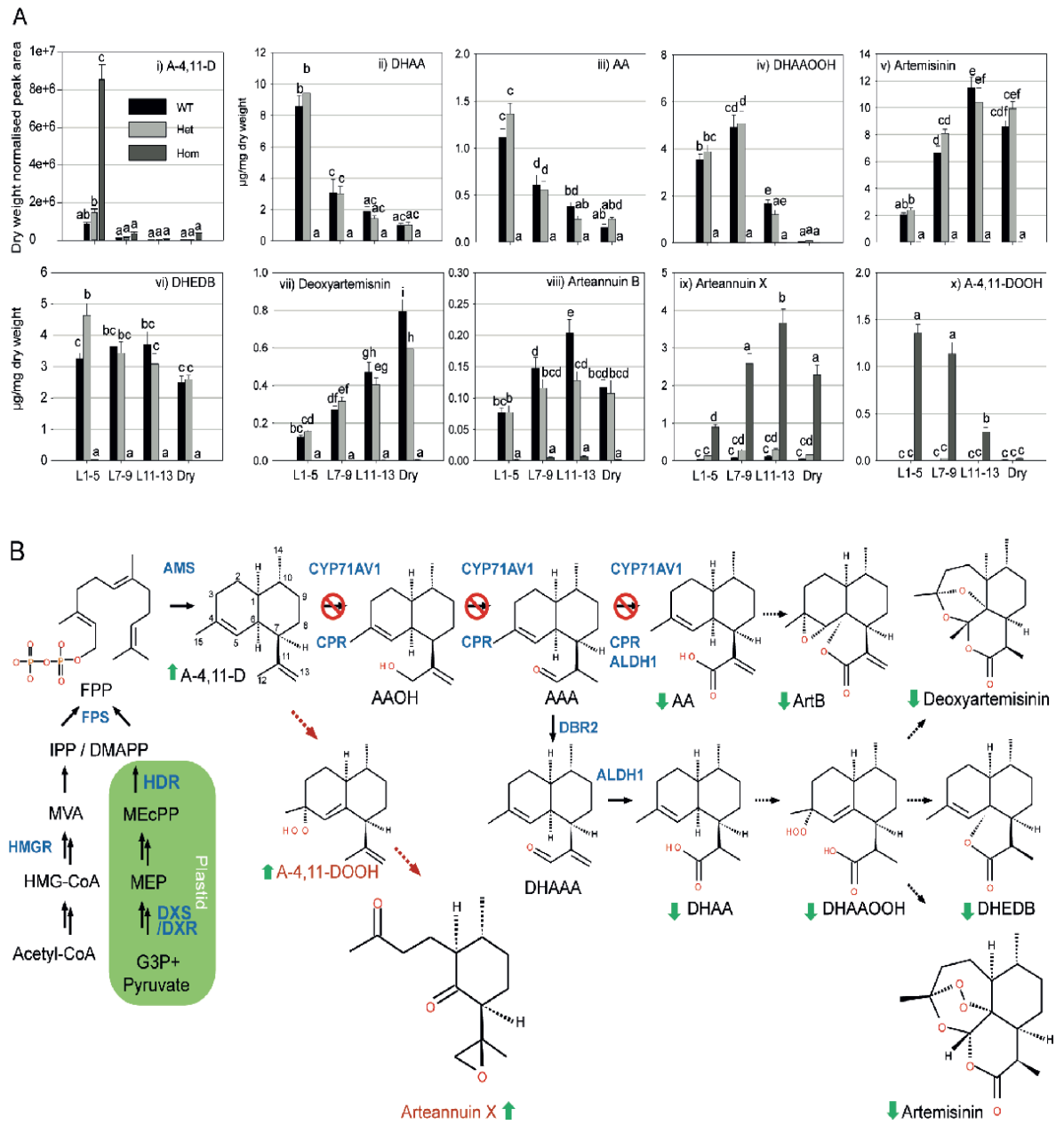


Fig. 1. Effects of *cyp71av1-1* mutation on selected sesquiterpene levels in fresh and dried leaves. **A)** Level of selected sesquiterpenes were quantified by GC-MS (i) and UPLC-MS (ii)-(x) in fresh leaf (L) 1-5 (juvenile), 7-9 (expanding), 11-13 (mature) as counted from the apical meristem, plus oven-dried whole plant-stripped leaves (dry) from 12-weeks old glasshouse-grown homozygous (hom), heterozygous (het) *cyp71av1-1* and segregating wild type (WT) as described in SI; error bars – SEM (n=15 for L1-5, L7-9 and L11-13; n=6 for dry leaf). Letters represent Tukey's range test results after one way ANOVA or REML (see SI Materials and Methods for details). Groups not sharing letters indicate statistically significant differences. **(B)** Summary of the effects of *cyp71av1-1* mutation on the level of selected sesquiterpenes; red cross indicates steps of the pathway targeted by *cyp71av1-1* mutation, full arrows – known enzymatic steps, dotted arrows – potential non enzymatic conversions, brown dotted arrows – novel pathway operating in the *cyp71av1-1* mutant, full green arrows – metabolite changes (all types of leaves). Metabolite abbreviations: G-3-P – glyceraldehyde-3-phosphate; MEP – 2-C-methylerythritol 4-phosphate; MEcPP – 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate. Cytosolic precursors: HMG-CoA – 3-hydroxy-3-methylglutaryl-CoA; MVA – mevalonate, FPP – farnesyl diphosphate, A-4,11-D – amorpha-4,11-diene, AAOH – artemisinic alcohol, AAA – artemisinic aldehyde, AA – artemisinic acid, ArtB – arteannuin B, DHAAA – dihydroartemisinic aldehyde, DHAA – dihydroartemisinic acid, DHAAOOH – dihydroartemisinic acid tertiary hydroperoxide, DHEDB – dihydro-*epi*-deoxyarteannuin B, DeoxyArt- deoxyartemisinin: A-4,11-DOOH- amorpha-4,11-diene tertiary hydroperoxide. Enzyme abbreviations: HMGR- 3-hydroxy-3-methylglutaryl coenzyme A reductase, HDR- 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, DXR-1-deoxy-D-xylulose-5-phosphate reductoisomerase, DXS- 1-deoxy-D-xylulose-5-phosphate synthase. FPP- Farnesyl diphosphate. Artemisinin pathway: AMS – amorpha-4,11-diene synthase, CYP71AV1 – amorpha-4,11-diene C-12 oxidase, CPR – cytochrome P450 reductase, DBR2 – artemisinic aldehyde Δ 11 (13) reductase, ALDH1 – aldehyde dehydrogenase.

be promiscuous (24). We reasoned that this plasticity could be exploited by developing biochemical knock-outs, re-directing flux

to new high-value sesquiterpenes in a proven plant production system.

Figure 2

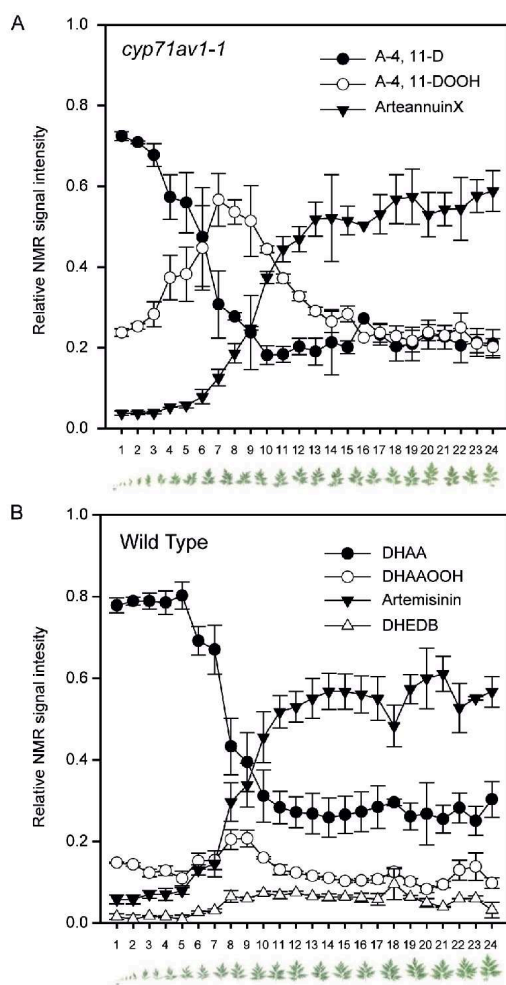


Fig. 2. Developmental patterns of artemisinin and arteannuin X biosynthesis. Leaves 1 to 24 (counting from apical meristem and shown below graphs) detached from the main stem of three *cyp71av1-1* (A) and three wild type plants (B). Chloroform extracts were subjected to NMR analysis (see SI Materials and Methods for details) and abundance for selected metabolites calculated from the integration of distinctive resonances is shown as proportion of the total for each leaf. Abbreviations: A-4,11-D – amorpha-4,11-diene, A-4,11-DOOH – amorpha-4,11-diene tertiary hydroperoxide, DHAA – dihydroartemisinic acid, DHAAOOH – dihydroartemisinic acid tertiary hydroperoxide, DHEDB – dihydroepoxyarteannuin B; error bars – SEM (n=3)

Recent attempts to knock down the amorpha-4,11-diene synthase using RNAi in self-pollinating *A. annua* resulted in only a modest (30-50%) reduction in artemisinin levels (25). We have chosen to target CYP71AV1, which catalyses the three-step conversion of amorpha-4,11-diene to artemisinic acid (13, 26, 27). When we knocked out this enzyme, as expected artemisinin was not produced - however, rather than amorpha-4,11-diene accumulating, it was instead readily converted to a novel sesquiterpene epoxide, arteannuin X. Detailed metabolite analysis revealed that production of this compound paralleled the production of artemisinin during leaf maturation with early steps occurring in young leaves and later steps in older leaves. Our findings confirm the function of the CYP71AV1 enzyme in *planta* and also demonstrate the flexibility of glandular secretory trichome biochemistry, such that it is capable of re-directing the flux of amorpha-4,11-diene into a novel sesquiterpene epoxide at levels similar to artemisinin.

Figure 3

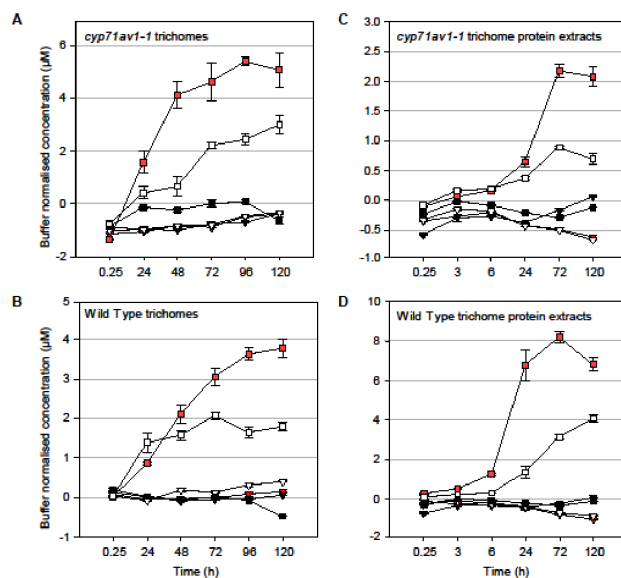


Fig. 3. Feeding intact and protein extracted glandular secretory trichomes (GSTs) with ^{13}C -isotope labelled DHAA. Intact GSTs isolated from young leaves of *cyp71av1-1* (A) or segregating wild type (B) and trichome protein extracts from *cyp71av1-1* (C) or segregating wild type (D) were fed with $[\text{U-}^{13}\text{C}_{15}]$ -DHAA as described in SI materials and methods. The concentration of selected $[\text{U-}^{13}\text{C}_{15}]$ -labelled metabolites was first corrected for the different densities of the GST extracts and then calculated with subtraction of relevant feeding buffer controls, containing no trichomes or no protein extracts. Metabolites are represented by shapes: Artemisinin-triangles, DHEDB – squares, Treatments are represented by colours: white – intact, light incubated GSTs; black- intact, dark incubated GSTs red - boiled, light incubated GSTs. Level of metabolites was monitored by UPLC-MS. See SI Materials and Methods for details. Error bars – SEM (n=3). Labelled substrate ($[\text{U-}^{13}\text{C}_{15}]$ -DHAA) levels started at 30 - 40 μM (off the scale of the graphs) and decreased as expected over the course of the experiments.

Results and Discussion

Disruption of *CYP71AV1* results in the accumulation of a novel sesquiterpene epoxide at the expense of artemisinin

We used the TILLING method (28) to screen for mutations in the single copy (Fig. S1) *CYP71AV1* gene in an F2 population of *A. annua* that had been derived from an ethyl methane sulfonate (EMS)-mutagenized population of the Artemis F1 hybrid as previously described (19). This resulted in an allelic series of 10 mutants, of which just one was non-sense due to a G to A transition in the second exon of *CYP71AV1* (Fig. S2 A). This mutation, which we designate *cyp71av1-1*, gives a predicted conversion of amino acid Trp124 in the polypeptide to a stop codon resulting in a major truncation of the enzyme and loss of most of the putative heme-binding sites, as well as Ser473, which is crucial for catalysing oxidation reactions (Fig. S2 B,C).

Previous work had shown that early stage intermediates in the artemisinin pathway accumulate in young *A. annua* leaves and as they mature artemisinin accumulates (25, 29). In order to investigate the effects of the *cyp71av1-1* mutation on artemisinin biosynthesis we analysed three leaf developmental stages: juvenile (leaves 1-5 as counted down from apical meristem), expanding (leaves 7-9) and mature (leaves 11-13). To generate material for this analysis we backcrossed *cyp71av1-1* to *Artemis* parents, selfed the progeny and performed DNA marker-based selection of wild type (WT), heterozygous and homozygous *cyp71av1-1* individuals from the segregating backcrossed F2 population (Fig. S3). We did not detect any morphological differences between

341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408

wild type and *cyp71av1-1* material (Fig. S4). We also extended the analysis to include oven-dried leaf material stripped from entire plants in order to investigate metabolite conversions occurring post-harvest.

Compared to WT and heterozygous material, the juvenile leaves of *cyp71av1-1* contain significantly elevated levels of the first committed metabolite in the artemisinin pathway, amorpha-4,11-diene (A-4,11-D; Fig. 1A i), Supplemental Tables 2 and 4), consistent with the reported *in vitro* activity of CYP71AV1 (13, 26, 27). Metabolite profiling further revealed a complete loss of all metabolites downstream of A-4,11-D including artemisinin, which typically accumulate in juvenile, expanding and mature wild type leaves (Fig. 1A ii)- 1A viii); 1C; Supplemental tables 1 and 3).

Other classes of secondary metabolites, including aromatic alcohols and ketones, coumarins, monoterpenes and other sesquiterpenes remained largely unchanged in *cyp71av1-1* (Supplemental Tables 1 and 2). However, levels of some minor mono- (eucalyptol, borneol, sabinene) and sesquiterpenes (calarene, α -bisabolol, cedrenol,) were reduced and levels of two minor flavonoids (retusin and artemetin) were increased in *cyp71av1-1* (Supplemental Tables 1 and 2). These and other changes were largely restricted to the juvenile leaves (Supplemental Tables 1 and 2, Fig S5) which are relatively dense in glandular trichomes (29) and exhibit high expression levels of terpene synthases (30). Recent attempts to silence *AMORPHA-4,11-DIENE SYNTHASE* (*AMS*) expression in self-pollinating varieties of *Artemisia annua* resulted in increased levels of two non-amorphadiene sesquiterpenes, caryophyllene and copaene, which may be due to an elevated pool of FPP acting as substrate for other sesquiterpene synthases in the glandular trichomes (25).

Ultra-Performance Liquid-Chromatography tandem Mass Spectrometry (UPLC-MS) analysis revealed that *cyp71av1-1* leaves accumulate large amounts of an oxygenated C₁₄ metabolite (Supplemental Table 1, Peak_ID M221.1535T43). One and two dimensional Nuclear Magnetic Resonance (1D and 2D-NMR) spectroscopic techniques were used to elucidate the structure of this amorphadiene sesquiterpene as (2*S*,3*R*,6*S*)-3-methyl-6-(2*R*-methyloxiran-2-yl)-2-(3-oxobutyl) cyclohexanone, which we refer to as arteannuin X (Fig. 1 A ix) and 1B). At 0.4% leaf dry weight in mature leaves the concentration of arteannuin X in *cyp71av1-1* is almost half that of artemisinin in wild type leaves and the developmental profile for accumulation in expanding and mature leaves is similar for both compounds (Fig. 1A ix); Supplemental table 1). The fact that arteannuin X is present in trace amounts in wild type and heterozygous *cyp71av1-1* material (Fig. 1A ix); Supplemental Table 1) suggests it normally occurs as a by-product of amorpha-4,11-diene oxidation. *In vivo* and *in vitro* formation of low abundance by-products derived from other intermediates of artemisinin synthesis have previously been reported (12, 17, 18).

NMR analysis of *cyp71av1-1* extracts identified a second major compound, amorpha-4,11-diene tertiary allylic hydroperoxide (A-4,11-DOOH; Fig. 1A x); 1B) that had not previously been reported in wild type *A. annua*. The pattern of accumulation of A-4,11-DOOH (Fig. 1A x)) pre-empted that of arteannuin X reaching a concentration of almost 0.15% leaf dry weight in juvenile and expanding leaves of *cyp71av1-1* before decreasing in mature leaves (Fig. 1A x)). Although A4,11-D-OOH was sufficiently stable to survive the chromatographic isolation procedures which were required in order to obtain it in a pure state for analysis by NMR, it was found to be unstable under prolonged storage in deuterated chloroform, where it spontaneously converted to arteannuin X. This provided the first circumstantial evidence that arteannuin X might be biosynthesised from A-4,11-D *via* its tertiary hydroperoxide (A-4,11-DOOH) *in vivo*, in much the same way that DHAA has previously been shown to be

transformed to artemisinin *via* the tertiary allylic hydroperoxide of dihydroartemisinic acid, DHAAOOH (17).

In planta similarities between the synthesis of arteannuin X and artemisinin

Using UPLC-MS we compared the conversion profile of A-4,11-D to arteannuin X in leaves with that of DHAA to artemisinin. Specifically, we also monitored DHAAOOH, the final intermediate in the *in-vivo* production of artemisinin (Fig. 1A iv), 1B). We found that DHAAOOH levels peak in expanding WT leaves at a concentration of 0.5% leaf dry weight (Fig. 1A iv), Supplemental Table 1). Artemisinin levels increase gradually from juvenile to mature leaves, reaching a maximum concentration of 1.2% dry leaf weight and remaining stable during the post-harvest drying process. (Fig. 1A iv), Supplemental Table 1). Previous work has shown that the DHAAOOH intermediate can also give rise to both dihydro-*epi*-deoxyarteannuin B (DHEDB) (31), and (by Hock-cleavage) deoxyartemisinin (17). While DHEDB remains at a concentration 3-fold lower than artemisinin throughout leaf maturation and post-harvest (Fig. 1A vi), Supplemental table 1), the levels of deoxyartemisinin increase during dry leaf storage, accumulating to 0.1% leaf dry weight (Fig. 1A vii), Supplemental Table 1). These data suggest that post-harvest any remaining DHAAOOH is preferentially converted to deoxyartemisinin rather than artemisinin.

We next carried out a more detailed analysis of the progression during leaf maturation of A-4,11-D to either arteannuin X or artemisinin in *cyp71av1-1* and WT, respectively, by performing ¹H NMR analysis on individual extracts from a 24-leaf maturation series (Fig. 2). Determination of the relative amounts of the three most abundant sesquiterpene metabolites associated with *cyp71av1-1* (A-4,11-D, A-4,11-DOOH and arteannuin X) revealed a progressive decline in A-4,11-D, which was matched by an increase in arteannuin X. This analysis also demonstrated that the amount of A-4,11-DOOH reaches a maximum in leaves 7-8 (Fig. 2A). This pattern is entirely consistent with our hypothesis that A-4,11-D is converted to arteannuin X *via* the intermediate A-4,11-DOOH. ¹H NMR analysis of wild type material clearly demonstrated that a decline in DHAA inversely correlates with an increase in artemisinin which reaches a maximum at leaves 14-15, while the DHAAOOH intermediate peaks around leaves 7-8 (Fig. 2B), as for A-4,11-DOOH.

The results of the above experiments revealed clear parallels in the conversion of A-4,11-D to arteannuin X *via* the hydroperoxide intermediate (A-4,11-DOOH) and the final steps in the conversion of DHAA to artemisinin *via* DHAAOOH. There is strong *in vivo* and *in vitro* evidence for a non-enzymatic autoxidation of DHAA to DHAAOOH and subsequent non-enzymatic rearrangement to artemisinin (17). Our data suggest that a similar auto-oxidation operates in *cyp71av1-1* to convert amorpha-4,11-diene to A-4,11-DOOH and on to arteannuin X. The spontaneous conversion of A-4,11-DOOH to arteannuin-X in deuterated chloroform noted above is entirely consistent with this hypothesis.

In vivo evidence for non-enzymatic DHAA conversion in *cyp71av1-1* and WT trichomes.

Previous reports have suggested peroxidase and/or dioxygenase enzymes may be involved in the conversion of DHAA to artemisinin (16, 32). To further investigate DHAA conversion we fed [U-¹³C₁₅]-DHAA to boiled, intact and dark-incubated trichomes that had been isolated from *cyp71av1-1* (which lacks endogenous DHAA) and wild type leaves (see SI Materials and Methods for details). Chloroform extracts of [U-¹³C₁₅]-DHAA fed trichomes and no-trichome controls were subjected to UPLC-MS analysis and the mass spectrum of each sesquiterpene ¹²C monoisotope metabolite was used to predict the mass spectra expected from the corresponding [U-¹³C₁₅]- isotopomer. Metabolite concentrations were first normalised to the trichome density

for a given sample. As DHAA can slowly degrade and convert spontaneously to “downstream” products over time labelled metabolite concentrations in trichome samples were also corrected by subtracting concentrations measured in matched time-equivalent buffer controls. (Fig. 3).

It was found that [U-¹³C₁₅]-artemisinin did not accumulate substantially over the 5-day feeding period in extracts of [U-¹³C₁₅]-DHAA-fed trichomes in either *cyp71av1-1* or WT leaves (Fig. 3A and B white, red and black triangles). Lack of artemisinin accumulation in trichomes fed with [U-¹³C₁₅]-DHAA is perhaps not surprising, as previous results have indicated that DHAAOOH cannot efficiently undergo Hock-cleavage into artemisinin in an aqueous environment (such as trichome extraction buffer), and that it may preferentially form DHEDB (17). We found an accumulation of [U-¹³C₁₅]-DHEDB in extracts from both light-incubated boiled and intact trichomes from *cyp71av1-1* and wild type leaves (Fig. 3A and B, white and red squares). Dark-incubated samples do not show an accumulation of the [U-¹³C₁₅] labelled DHEDB (Fig. 3A and B, black squares) leading us to conclude that DHEDB formation is light-dependent. Both *cyp71av1-1* and wild type trichomes show very similar patterns of [U-¹³C₁₅]-DHEDB accumulation which reached a plateau between 2-3 days after feeding commenced (Fig 3A and B, white and red squares). It is also evident that boiling accelerates the formation of DHEDB in both *cyp71av1-1* and wild type trichomes (Fig 3A and B, red vs. white squares), consistent with the process being non-enzymatic. We did not detect labelled DHAAOOH itself which suggests the experimental conditions favoured rapid conversion of this intermediate through to DHEDB.

In order to investigate if uptake of [U-¹³C₁₅]-DHAA into isolated trichomes is a limiting factor in the feeding experiment we fed the labelled substrate to crude protein extracts from isolated trichomes. The products and temporal pattern of their accumulation was very similar to that obtained for the intact trichome feeding (Fig 3C and D). Notably, there is no accumulation of [U-¹³C₁₅]-artemisinin over the 5 day feeding period (Fig 3C and D red, black and white triangles) while [U-¹³C₁₅]-DHEDB accumulates in both light-incubated boiled and non-boiled protein extracts from *cyp71av1-1* and wild type trichomes (Fig 3C and D red and white squares). It is also evident that boiling accelerates the formation of DHEDB in both *cyp71av1-1* and wild type trichome protein extracts (Fig 3C and D, red vs. white squares), consistent with the process being non-enzymatic.

The intact trichome- and trichome protein extract-feeding experiments demonstrate that in *cyp71av1-1*, a block in artemisinin and DHEDB biosynthesis can be rescued by direct feeding of DHAA, which is expected given the role of CYP71AV1 in the artemisinin biosynthetic pathway (Fig. 1B). The lack of further accumulation of artemisinin over a 5 day period in trichomes maintained in an aqueous media contrasts with the gradual accumulation of artemisinin in trichomes during leaf maturation (Fig 1 and 2). These observations lead us to suggest that the non-aqueous environment present in the intact sub-apical cavity of glandular secretory trichomes is essential for the efficient conversion of DHAA to artemisinin *via* DHAAOOH. In the absence of such an environment, DHAA is instead converted to DHEDB in a light dependent non-enzymatic process.

The hydrophobic nature of amorpho-4,11-diene prevented us from preparing aqueous solutions of [U-¹³C₁₅]-A-4,11-D for

trichome-feeding experiments and performing a similar analysis of arteannuin X in the *cyp71av1-1* mutant.

Given that the final steps in artemisinin biosynthesis appear to be non-enzymatic, the question arises as to how its production during leaf maturation is controlled. It is reasonable to assume that active transport system(s) will be responsible for pumping artemisinin precursors into the sub-apical cavity of glandular secretory trichomes. Transport of DHAA into the sub-apical cavity could be a limiting factor with spatial and temporal expression patterns of relevant transporters controlling the increase in artemisinin during leaf maturation (Fig. 2B).

Conclusion

We have described an *A. annua* CYP71AV1 knock-out mutant which provides the first *in planta* confirmation for the function of this enzyme. The *cyp71av1-1* mutant accumulates high levels of amorpho-4,11-diene, which is converted to arteannuin X, a novel *nor-seco*-amorphane sesquiterpene epoxide. This work clearly demonstrates the plasticity of metabolism in the glandular secretory trichomes of *A. annua*; when one pathway is blocked novel sesquiterpene alternatives are produced, highlighting the potential of trichomes as factories for production of new compounds with potential medicinal and industrial applications.

We found that the *in-vivo* oxidation of amorpho-4,11-diene to arteannuin X parallels that of DHAA to artemisinin during the progression of leaf maturation. We were able to chemically complement *cyp71av1-1* by externally feeding DHAA to intact trichome preparations and trichome protein extracts. This demonstrated that the conversion of DHAA to DHEDB, via a tertiary allylic hydroperoxide, is a non-enzymatic, light-requiring process. The lack of accumulation of artemisinin in these experiments supports the idea that a non-aqueous environment, as provided by the sub-apical cavity of glandular secretory trichomes, is essential for the non-enzymatic production of the endoperoxide containing artemisinin from DHAA. Taken together these findings highlight the importance of non-enzymatic conversions in terpenoid metabolism of *A. annua* glandular secretory trichomes. This, together with the observation that artemisinin is known to be cytotoxic to various cell types (4, 33, 34), suggests a functional requirement for the specialised sub-apical cavity as a location for both chemical conversion and storage. It also highlights the challenges of producing certain types of plant natural products in microbial systems which lack this level of structural complexity, and the need for more research into the compartmentation of metabolic processes in plant production systems

Materials and Methods

Full details of plant material used, plant growth conditions, screening of EMS-mutagenized population, genotyping, metabolomic analyses, 2-D NMR structural characterization, trichome extractions and trichome feeding with ¹³C-labelled substrates are presented in the Supplementary Information.

Acknowledgements

We would like to thank: C. Paddon and K. Monroe (Zagaya) for providing [U-¹³C₁₅]- (uniformly) labelled amorpho-4,11-diene and dihydroartemisinic acid; T. Winzer for preliminary experimental involvement, L. Doucet, D. Vyas, C. Whitehead and B. Kowalik, for laboratory assistance; C. Abbot and A. Fenwick for horticulture assistance; P. Roberts for graphic design; X. Simonnet and Médiplant for access to the Artemis variety. We acknowledge financial support for this project from The Bill and Melinda Gates Foundation. GDB would like to thank the BBSRC for financial support (grant no, BB/G008744/1 “The Biosynthesis of Artemisinin”) and the Chemical Analysis Facility (CAF) at the University of Reading for provision of the 700 MHz NMR spectrometer used in these studies.

1. Duke SO & Paul RN (1993) Development and Fine Structure of the Glandular Trichomes of *Artemisia annua* L. *International Journal of Plant Sciences* 154(1):107-118.
2. Duke MV, Paul RN, Elsohly HN, Sturtz G, & Duke SO (1994) Localization of Artemisinin and Artemisitenone in Foliar Tissues of Glanded and Glandless Biotypes of *Artemisia-Annua* L. *International Journal of Plant Sciences* 155(3):365-372.
3. Olsson ME, et al. (2009) Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of *Artemisia annua* L. *Phytochemistry* 70(9):1123-1128.

4. Yan ZQ, et al. (2015) Mechanism of artemisinin phytotoxicity action: induction of reactive oxygen species and cell death in lettuce seedlings. *Plant physiology and biochemistry : PPB / Societe francaise de physiologie vegetale* 88:53-59.
5. Lange BM & Ahkami A (2013) Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes—current status and future opportunities. *Plant biotechnology journal* 11(2):169-196.
6. Lange BM & Turner GW (2013) Terpenoid biosynthesis in trichomes—current status and

681	future opportunities. <i>Plant biotechnology journal</i> 11(1):2-22.	
682	7. Lapkin AA, Plucinski PK, & Cutler M (2006) Comparative assessment of technologies for extraction of artemisinin. <i>J Nat Prod</i> 69(11):1653-1664.	
683	8. Paddon CJ & Keasling JD (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. <i>Nature reviews. Microbiology</i> 12(5):355-367.	
684	9. Paddon CJ, <i>et al.</i> (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. <i>Nature</i> 496(7446):528-532.	
685	10. Turconi J, <i>et al.</i> (2014) Semisynthetic Artemisinin, the Chemical Path to Industrial Production. <i>Org Process Res Dev</i> 18(3):417-422.	
686	11. Peplow Y (2016) Synthetic malaria drug meets market resistance. <i>Nature</i> 530(7591):389-390.	
687	12. Mercke P, Bengtsson M, Bouwmeester HJ, Posthumus MA, & Brodelius PE (2000) Molecular cloning, expression, and characterization of amorpho-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in <i>Artemisia annua</i> L. <i>Archives of biochemistry and biophysics</i> 381(2):173-180.	
688	13. Teoh KH, Polichuk DR, Reed DW, Nowak G, & Covello PS (2006) <i>Artemisia annua</i> L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. <i>FEBS letters</i> 580(5):1411-1416.	
689	14. Zhang Y, <i>et al.</i> (2008) The molecular cloning of artemisinic aldehyde Delta11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in <i>Artemisia annua</i> . <i>The Journal of biological chemistry</i> 283(31):21501-21508.	
690	15. Teoh KH, Polichuk DR, Reed DW, & Covello PS (2009) Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in <i>Artemisia annua</i> . <i>Botany</i> 87(6):635-642.	
691	16. Soetaert SS, <i>et al.</i> (2013) Differential transcriptome analysis of glandular and filamentous trichomes in <i>Artemisia annua</i> . <i>BMC plant biology</i> 13:220.	
692	17. Brown GD & Sy L-K (2004) In vivo transformations of dihydroartemisinic acid in <i>Artemisia annua</i> plants. <i>Tetrahedron</i> 60(5):1139-1159.	
693	18. Sy LK & Brown GD (2002) The role of the 12-carboxylic acid group in the spontaneous autoxidation of dihydroartemisinic acid. <i>Tetrahedron</i> 58(5):909-923.	
694	19. Graham IA, <i>et al.</i> (2010) The genetic map of <i>Artemisia annua</i> L. identifies loci affecting yield of the antimalarial drug artemisinin. <i>Science</i> 327(5963):328-331.	
695	20. Larson TR, <i>et al.</i> (2013) A survey of artemisinic and dihydroartemisinic acid contents in glasshouse and global field-grown populations of the artemisinin-producing plant <i>Artemisia annua</i> L. <i>Ind Crop Prod</i> 45:1-6.	
696	21. Brown GD (2010) The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of <i>Artemisia annua</i> L. (Qinghao). <i>Molecules</i> 15(11):7603-7698.	
697	22. Ivanescu B, Miron A, & Corciova A (2015) Sesquiterpene Lactones from <i>Artemisia</i> Genus: Biological Activities and Methods of Analysis. <i>J Anal Methods Chem</i> .	
698	23. Wang W, Wang Y, Zhang Q, Qi Y, & Guo D (2009) Global characterization of <i>Artemisia annua</i> glandular trichome transcriptome using 454 pyrosequencing. <i>BMC genomics</i> 10:465.	
699	24. Weng JK, Philippe RN, & Noel JP (2012) The rise of chemodiversity in plants. <i>Science</i> 336(6089):1667-1670.	
700	25. Ma DM, <i>et al.</i> (2015) A Genome-Wide Scenario of Terpene Pathways in Self-pollinated <i>Artemisia annua</i> . <i>Molecular plant</i> 8(11):1580-1598.	
701	26. Ro DK, <i>et al.</i> (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. <i>Nature</i> 440(7086):940-943.	
702	27. Komori A, <i>et al.</i> (2013) Comparative functional analysis of CYP71AV1 natural variants reveals an important residue for the successive oxidation of amorpho-4,11-diene. <i>FEBS letters</i> 587(3):278-284.	
703	28. McCallum CM, Comai L, Greene EA, & Henikoff S (2000) Targeting induced local lesions in genomes (TILLING) for plant functional genomics. <i>Plant physiology</i> 123(2):439-442.	
704	29. Lommen WJ, Schenk E, Bouwmeester HJ, & Verstappen FW (2006) Trichome dynamics and artemisinin accumulation during development and senescence of <i>Artemisia annua</i> leaves. <i>Planta Med</i> 72(4):336-345.	
705	30. Olofsson L, Engstrom A, Lundgren A, & Brodelius PE (2011) Relative expression of genes of terpene metabolism in different tissues of <i>Artemisia annua</i> L. <i>BMC plant biology</i> 11:45.	749
706	31. Sy LK, Zhu NY, & Brown GD (2001) Syntheses of dihydroartemisinic acid and dihydro-epi-deoxyartemisinin B incorporating a stable isotope label at the 15-position for studies into the biosynthesis of artemisinin. <i>Tetrahedron</i> 57(40):8495-8510.	750
707	32. Bryant L, Flatley B, Patole C, Brown GD, & Cramer R (2015) Proteomic analysis of <i>Artemisia annua</i> -towards elucidating the biosynthetic pathways of the antimalarial pro-drug artemisinin. <i>BMC plant biology</i> 15:175.	751
708	33. Knudsmark Jessing K, Duke SO, & Cedergreen N (2014) Potential ecological roles of artemisinin produced by <i>Artemisia annua</i> L. <i>Journal of chemical ecology</i> 40(2):100-117.	752
709	34. Li W, <i>et al.</i> (2005) Yeast model uncovers dual roles of mitochondria in action of artemisinin. <i>PLoS genetics</i> 1(3):e36.	753
710	35. Delabays N, Simonnet X, & Gaudin M (2001) The genetics of artemisinin content in <i>Artemisia annua</i> L. and the breeding of high yielding cultivars. <i>Curr Med Chem</i> 8(15):1795-1801.	754
711	36. Townsend T, <i>et al.</i> (2013) The use of combining ability analysis to identify elite parents for <i>Artemisia annua</i> F1 hybrid production. <i>PLoS one</i> 8(4):e61989.	755
712	37. Larson TR, <i>et al.</i> (2013) A survey of artemisinic and dihydroartemisinic acid contents in glasshouse and global field-grown populations of the artemisinin-producing plant <i>Artemisia annua</i> L. <i>Ind Crop Prod</i> 45:1-6.	756
713	38. Smith CA, Want EJ, O'Maille G, Abagyan R, & Siuzdak G (2006) XCMS: Processing mass spectrometry data for metabolite profiling using Nonlinear peak alignment, matching, and identification. <i>Anal Chem</i> 78(3):779-787.	757
714	39. Tautenhahn R, Bottcher C, & Neumann S (2008) Highly sensitive feature detection for high resolution LC/MS. <i>BMC bioinformatics</i> 9.	758
715	40. Kuhl C, Tautenhahn R, Bottcher C, Larson TR, & Neumann S (2012) CAMERA: An Integrated Strategy for Compound Spectra Extraction and Annotation of Liquid Chromatography/Mass Spectrometry Data Sets. <i>Anal Chem</i> 84(1):283-289.	759
716	41. Guha R (2007) Chemical Informatics functionality in R. <i>J Stat Softw</i> 18(5).	760
717	42. Stacklies W, Redestig H, Scholz M, Walther D, & Selbig J (2007) pcaMethods--a bioconductor package providing PCA methods for incomplete data. <i>Bioinformatics</i> 23(9):1164-1167.	761
718	43. Mccaskill D & Croteau R (1995) Monoterpene and Sesquiterpene Biosynthesis in Glandular Trichomes of Peppermint (<i>Mentha X Piperita</i>) Rely Exclusively on Plastid-Derived Isopentenyl Diphosphate. <i>Planta</i> 197(1):49-56.	762
719	44. Paddon CJ, <i>et al.</i> (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. <i>Nature</i> 496(7446):528-532.	763
720	45. Westfall PJ, <i>et al.</i> (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. <i>Proceedings of the National Academy of Sciences of the United States of America</i> 109(3):E111-118.	764
721	46. Teoh KH, Polichuk DR, Reed DW, Nowak G, & Covello PS (2006) <i>Artemisia annua</i> L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. <i>FEBS letters</i> 580(5):1411-1416.	765
722	47. Zhang Y, <i>et al.</i> (2008) The molecular cloning of artemisinic aldehyde Delta11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in <i>Artemisia annua</i> . <i>The Journal of biological chemistry</i> 283(31):21501-21508.	766
723	48. Teoh KH, Polichuk DR, Reed DW, & Covello PS (2009) Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in <i>Artemisia annua</i> . <i>Botany</i> 87(6):635-642.	767
724	49. Yang J, <i>et al.</i> (2015) The I-TASSER Suite: protein structure and function prediction. <i>Nat Methods</i> 12(1):7-8.	768
725	50. Komori A, <i>et al.</i> (2013) Comparative functional analysis of CYP71AV1 natural variants reveals an important residue for the successive oxidation of amorpho-4,11-diene. <i>FEBS letters</i> 587(3):278-284.	769
726		770
727		771
728		772
729		773
730		774
731		775
732		776
733		777
734		778
735		779
736		780
737		781
738		782
739		783
740		784
741		785
742		786
743		787
744		788
745		789
746		790
747		791
748		792
		793
		794
		795
		796
		797
		798
		799
		800
		801
		802
		803
		804
		805
		806
		807
		808
		809
		810
		811
		812
		813
		814
		815
		816