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

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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 ^{13}C-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 biseriate 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.artemisiaf1seed.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

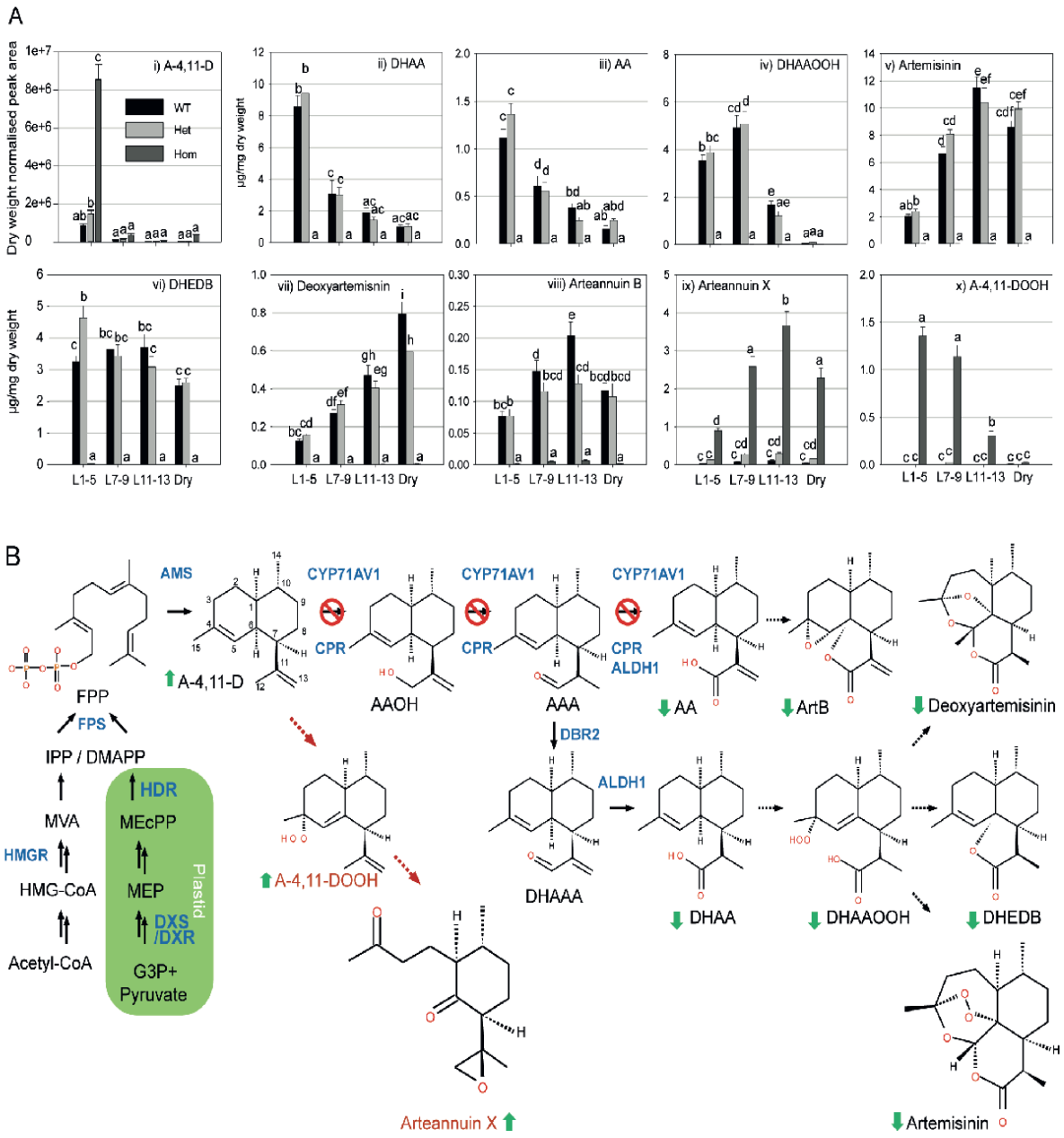

Fig. 1. Effects of *cyp71av1-1* mutation on selected sesquiterpene levels in fresh and dried leaves. A) Level of selected sesquiterpenes were quantified by GC-MS (i) and UPLC-MS (ii)-(x) in fresh leaf (L) 1-5 (juvenile), 7-9 (expanding), 11-13 (mature) as counted from the apical meristem, plus oven-dried 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 – artemsinic alcohol, AAA – artemsinic aldehyde, AA – artemsinic acid, ArtB – arteannuin B, DHAAA - dihydroartemsinic aldehyde, DHAA - dihydroartemsinic acid, DHAAOOH- dihydroartemsinic acid tertiary hydroperoxide, DHEDB – dihydro-*epi*-deoxyarteanniun 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 FPS- Farnesyl diphosphate synthase. 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

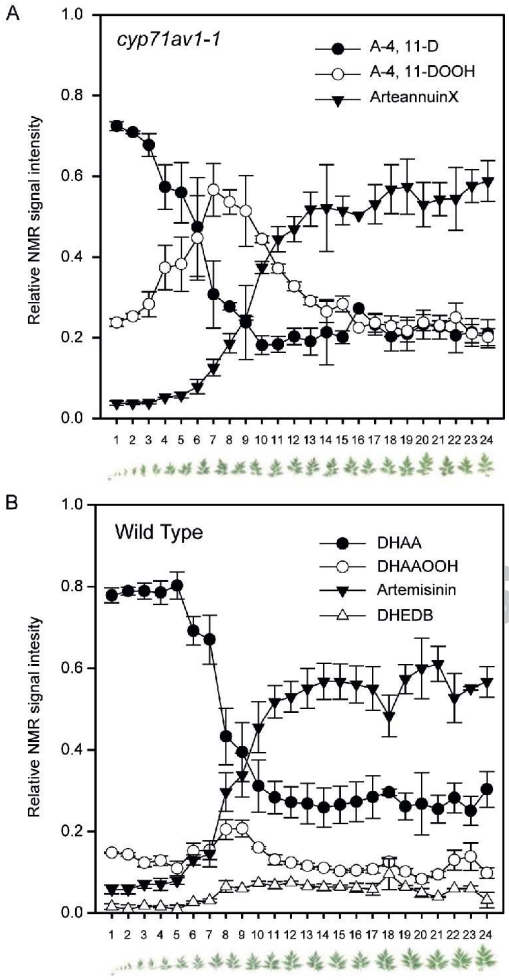

Fig. 2. Developmental patterns of artemisinin and arteannuin X biosynthesis. Leaves 1 to 24 (counting from apical meristem and shown below graphs) detached from the main stem of three *cyp71av1-1* (A) and three wild type plants (B). Chloroform extracts were subjected to NMR analysis (see SI Materials 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 - dihydroartemsinic acid, DHAAOOH- dihydroartemsinic acid tertiary hydroperoxide, DHEDB – dihydroepoxyarteanniun 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

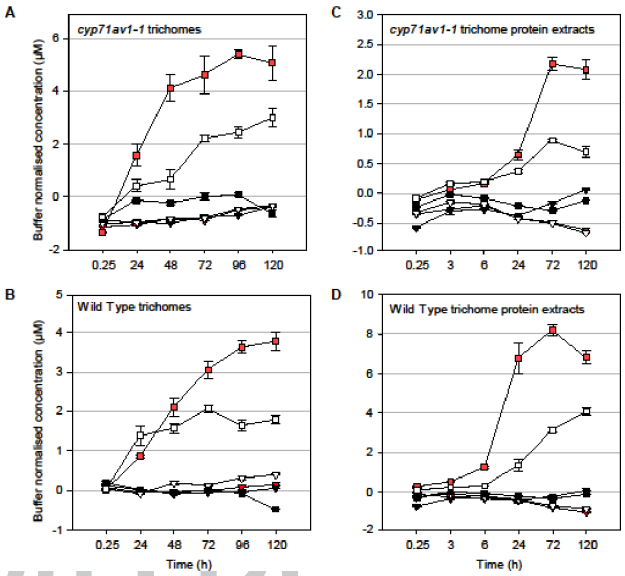

Fig. 3. Feeding intact and protein extracted glandular secretory trichomes (GSTs) with ^{13}C-isotope labelled DHAA. Intact GSTs isolated from young leaves of *cyp71av1-1* (A) or segregating wild type (B) and trichome protein extracts from *cyp71av1-1* (C) or segregating wild type (D) were fed with [U-$^{13}C_{15}$]-DHAA as described in SI materials and methods. The concentration of selected [U-$^{13}C_{15}$]-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 ([U-$^{13}C_{15}$]-DHAA) levels started at 30 - 40uM (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

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 C_{14} 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 ^{1}H 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. ^{1}H 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-$^{13}C_{15}$]-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-$^{13}C_{15}$]-DHAA fed trichomes and no-trichome controls were subjected to UPLC-MS analysis and the mass spectrum of each sesquiterpene ^{12}C monoisotope metabolite was used to predict the mass spectra expected from the corresponding [U-$^{13}C_{15}$]- 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-$^{13}C_{15}$]-artemisinin did not accumulate substantially over the 5-day feeding period in extracts of [U-$^{13}C_{15}$]-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-$^{13}C_{15}$]-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-$^{13}C_{15}$]-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-$^{13}C_{15}$] 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-$^{13}C_{15}$]-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-$^{13}C_{15}$]-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-$^{13}C_{15}$]-artemisinin over the 5 day feeding period (Fig 3C and D red, black and white triangles) while [U-$^{13}C_{15}$]-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 amorpha-4,11-diene prevented us from preparing aqueous solutions of [U-$^{13}C_{15}$]-A-4,11-D for trichrome-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 amorpha-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 amorpha-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 ^{13}C-labelled substrates are presented in the Supplementary Information.

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