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Dissection of the phytohormonal regulation of trichome formation and biosynthesis of the anti-malarial compound artemisinin in *Artemisia annua* plants.

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Artemisinin, glandular trichome, sesquiterpene lactone, jasmonate, cytokinin, gibberellin, fatty acyl-CoA reductase, cDNA-AFLP transcript profiling

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Materials and Methods	905
Results	2212
Discussion	1392
Acknowledgements	47
Number of figures	7
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The sequence data of AA064, AA387, AA400, AA707, and TFAR1 have been deposited in the GenBank with accession numbers FN428575, FN428576, FN428577, FN428578, and GU733320, respectively.

Summary

- Biosynthesis of the sesquiterpene lactone and potent anti-malarial drug artemisinin occurs in glandular trichomes of *Artemisia annua* plants and is subjected to a strict network of developmental and other regulatory cues.
- The effects of three hormones, jasmonate, gibberellin, and cytokinin, were studied at the structural and molecular level in two different *A. annua* chemotypes by microscopic analysis of gland development, and by targeted metabolite and transcript profiling. Furthermore, a genome-wide cDNA-AFLP-based transcriptome profiling was carried out of jasmonate-elicited leaves at different developmental stages.
- Although cytokinin and gibberellin positively affected at least one aspect of gland formation, these two hormones did not stimulate artemisinin biosynthesis. Only jasmonate simultaneously promoted gland formation and coordinated transcriptional activation of biosynthetic gene expression, which ultimately lead to increased sesquiterpene lactone accumulation with chemotype-dependent effects on the distinct pathway branches. Transcriptome profiling revealed a trichome-specific fatty acyl-CoA reductase, TFAR1, the expression of which correlates with trichome development and sesquiterpene biosynthesis.
- TFAR1 is potentially involved in cuticular wax formation during glandular trichome expansion in leaves and flowers of *A. annua* plants. Analysis of phytohormone-modulated transcriptional regulons provides clues to dissect the concerted regulation of metabolism and development of plant trichomes.

Introduction

Artemisinin, a sesquiterpene lactone found in *Artemisia annua* L. (sweet wormwood) plants, has been used in Chinese medicine for centuries. Currently, artemisinin and its semisynthetic derivatives are extensively used in the treatment of malaria, mostly in combination therapies (Haynes, 2006), and have gained additional interest because of their potential in the treatment of several cancers and viral diseases (Efferth, 2007; Efferth *et al.*, 2008). Despite promising advances toward the fermentative production of artemisinin precursors by the expression of biosynthetic genes in microbial hosts (Ro *et al.* 2006; Chang *et al.*, 2007; Arsenault *et al.*, 2008; Zhang *et al.,* 2008), engineering of *A. annua* plants for increased artemisinin production still remains of high interest (Covello, 2008; Graham *et al.,* 2010).

The first committed step in artemisinin biosynthesis (Fig. 1) is the cyclization of farnesyl diphosphate (FDP) to generate amorpha-4,11-diene, catalyzed by amorpha-4,11-diene synthase (ADS) (Mercke *et al.*, 2000; Wallaart *et al.*, 2001). Subsequent oxidation at the C12 position, mediated by the cytochrome P450 enzyme CYP71AV1, leads to artemisinic alcohol (Ro *et al.*, 2006; Teoh *et al.*, 2006). While arteannuin B has been suggested as a late precursor in artemisinin biosynthesis (Sangwan *et al.*, 1993; Zeng *et al.*, 2008), evidence now favours a route from artemisinic alcohol via dihydroartemisinic acid (Bertea *et al.*, 2005; Covello *et al.*, 2007; Covello, 2008). This route is supported by the cloning and characterization of Double Bond Reductase 2 (DBR2) that reduces the Δ11(13) double bond of artemisinic aldehyde, but not of arteannuin B (Zhang *et al.*, 2008), and the cloning of Aldehyde Dehydrogenase 1 (ALDH1) that catalyzes the oxidation of artemisinic and dihydroartemisinic aldehydes (Teoh *et al.*, 2009). The

conversion of dihydroartemisinic acid to artemisinin, and of artemisinic acid to arteannuin B has been suggested to occur via enzyme-independent reactions (Sy & Brown, 2002; Brown & Sy, 2004; Brown & Sy, 2007). Recently, a broad substrate oxidoreductase (RED1) with high affinity for dihydroartemisinic aldehyde and monoterpenes was identified that may have a negative impact on the flux to artemisinin biosynthesis (Rydén *et al*., 2010).

The production of artemisinin occurs in specialized 10-celled biseriate glandular trichomes present on the leaves, stems and inflorescences of *A. annua* plants (Duke *et al.*, 1994; Van Nieuwerburgh *et al.*, 2006). All of the above mentioned biosynthetic enzymes have been shown to be highly expressed in these particular trichomes (Bertea *et al.*, 2005; Teoh *et al.*, 2006, 2009; Zhang *et al.*, 2008), most probably exclusively in the two outer apical cells (Olsson *et al.*, 2009). Large differences in artemisinin content have been reported depending on variety, season, cultivation condition, and plant developmental stage (Ferreira *et al.*, 1995; Wallaart *et al.*, 2000; Delabays *et al.*, 2001; Lommen *et al.*, 2007; Davies *et al.*, 2009; Yang *et al.*, 2009). For instance, artemisinin concentrations are higher in leaves that are formed later in development than those in leaves formed early in the plant's development; this difference has been attributed to a higher trichome density and a higher capacity per trichome in the upper leaves (Lommen *et al.*, 2006). Other leaf traits, such as perimeter, area, and architecture, have recently been proposed as excellent targets for increasing artemisinin production (Graham *et al.*, 2010). However, to date, the regulatory mechanisms that control artemisinin biosynthesis and the formation of the specialized cells in which it takes place are poorly characterized molecularly.

Very recently, a WRKY transcription factor, responsive to the phytohormone jasmonate, has been characterized that regulates the expression of the *ADS* gene (Ma *et*

al., 2009). Another phytohormone, salicylic acid, affects artemisinin biosynthesis as well, presumably by enhancing the conversion of precursor pools and by up-regulating biosynthetic gene expression (Pu *et al.*, 2009). Phytohormones are key molecules that are involved in practically any aspect of a plant's development and its adaptation to the environment. First, the ability of jasmonates, in particular, but also of other phytohormones, to act as elicitors of plant secondary metabolism has been extensively proven. Across the plant kingdom, jasmonates are capable of inducing multiple secondary metabolic pathways synthesizing molecules of an incredible structural variety and of various biochemical origin (Zhao *et al.*, 2005; Pauwels *et al.*, 2009). Second, jasmonates, but also cytokinins, gibberellins and brassinosteroids, are known to modulate epidermal differentiation programs, resulting in increased trichome densities, ectopic trichome formation, or aberrant trichome morphologies, as demonstrated in Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*) (Perazza *et al.*, 1998; Traw & Bergelson, 2003; Li *et al.*, 2004; Boughton *et al.*, 2005; Gan *et al.*, 2007; Maes *et al.*, 2008; Lattarulo Campos *et al.*, 2009; Yoshida *et al.,* 2009; Maes & Goossens, 2010).

The observation that in model plant species, phytohormones can modulate both the onset of secondary metabolism and trichome initiation at the transcriptional level (Maes *et al.*, 2008; Pauwels *et al.,* 2009), provides an excellent rationale to dissect the phenotypic responses of two different *A. annua* cultivars to different phytohormones, in order to unravel the regulatory mechanisms underlying these important traits and eventually identify the gene products involved. The effect of the three phytohormones mostly renowned for their stimulating effect on trichome development, namely cytokinin (6-benzylaminopurine [BAP]), gibberellin (GA_3) and jasmonic acid (JA) was assessed at the developmental, metabolic and transcriptional levels in *A. annua*. In parallel, genome-wide

cDNA-amplified fragment length polymorphism (AFLP)-based transcript profiling of JA-elicited leaves at two different developmental stages revealed transcriptional regulons consisting of genes with expression patterns that correlate with trichome development and metabolism, including a gene encoding a fatty acyl-CoA reductase.

Materials and Methods

Plant material, maintenance, and phytohormonal treatment

Two different *Artemisia annua* L. cultivars were used throughout the experiments. Seeds from the "2/39" (HAP, for high-artemisinin producer) and the "Meise" (LAP, for low-artemisinin producer) cultivars were provided by Pedro Melillo de Magalhães (State University of Campinas, Brazil) and the National Botanic Garden of Belgium (Meise, Belgium), respectively. Seeds of both cultivars were germinated and grown in soil under normal, controlled conditions (21°C; 12 h day/12 h night regime), until they had formed the first four leaves. From then on, seedlings were treated every 2 days with three different phytohormone solutions, containing either 100 µM JA (Sigma-Aldrich, St. Louis, MO, USA), 50 µM BAP (Sigma-Aldrich), or 100 µM GA_3 (Sigma-Aldrich). For each treatment, 2.5 ml of the hormone or mock (water) solutions were applied to the soil and 2.5 ml were sprayed on the leaves of each plant. Treatment was carried out during 5 consecutive weeks and samples were harvested every week.

Measurement of trichome density and glandular trichome cross-sectional area

After 2 weeks of phytohormonal treatment, the 8th leaf (from the bottom) was removed from each plant, mounted on scanning electron microscopy (SEM) stubs with double-sided sticky carbon tape, and examined in a tabletop SEM (TM-1000) under an accelerating voltage of 5 kV. Leaves of at least six different plants per treatment were scanned and digital micrographs recorded. Trichome density was determined for the abaxial leaf epidermis of at least 100 mm^2 per leaf and spanning different areas by using the digital micrographs and the gridlines in Photoshop7. Of the almost elliptical cross-sectional area of the glandular trichomes, the major (A) and minor (B) axes of the ellipse were measured with the program ImageJ (version 1.37; http://rsb.info.nih.gov/ij/). The area was calculated according the formula area = $AB\pi/4$.

Metabolite Profiling

For each treatment and at each time point, leaves of three whole plants were pooled and weighed, and two pools were used for further analysis. Sesquiterpene lactones and precursors were released from the glandular trichomes by a one-minute chloroform extraction and measured by reversed-phase high-pressure liquid chromatography (HPLC) electrospray quadrupole time of flight tandem mass spectrometry (MS) essentially as described (Van Nieuwerburgh *et al.*, 2006).

Quantitative real-time-polymerase chain reaction (qRT-PCR)

RNA was extracted with Plant Reagent (Invitrogen, Carlsbad, CA, USA) and Poly(dT) cDNA was prepared from 1 μg of total RNA with Superscript II reverse transcriptase

(Invitrogen). qRT-PCR was carried out with SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) and gene-specific primers were designed with Primer3 (http://biotools.umassmed.edu/bioapps/primer3_cgi). Expression levels were normalized to those of the *ACTIN* control gene (GenBank Accession Number EU531837). For phytohormone treated samples, three biological repeats were analyzed, each consisting of pooled leaves from three plants, harvested 2 weeks after treatment. For measurement of organ-specific expression, sampling was done as described (Zhang *et al.*, 2008).

cDNA-AFLP-based transcript profiling

For cDNA-AFLP analysis, another elicitation experiment with the Meise cultivar was set up, in which samples were harvested 1 h, 2 h, 4 h, 8 h, 24 h and 48 h after JA or mock treatment. Leaves were harvested from the lower and the upper parts of the plant, and processed separately. Total leaf RNA was extracted as for the qRT-PCR analysis. cDNA-AFLP-based transcript profiling was done essentially as described (Rischer *et al.*, 2006; Vuylsteke *et al.*, 2007).

Full-length cDNA-cloning of *Trichome-specific Fatty Acyl-CoA Reductase 1* (*TFAR1*)

Total RNA was isolated from the glandular trichomes of *A. annua* line 2/39. Single-stranded cDNA was synthesized as described previously (Zhang *et al.*, 2008). The 5′ and 3′-end sequences of *TFAR1* were recovered by a rapid amplification of cDNA ends (RACE)-PCR strategy based on the cDNA-AFLP tag sequence AA387. cDNA encoding the TFAR1 open reading frame was obtained through 33 cycles of PCR with the

glandular trichome cDNA as the template with an annealing temperature of 58°C, Taq DNA polymerase (Invitrogen) and gene-specific oligonucleotide primers 5'-GGATCC**ATG**ATGGAGTTGGGTAGAATTG-3' and 5'-CTCGAG**TCA**TTTAAACTCACGGTGCCTTAG-3', with the *Bam*HI and *Xho*I restriction sites underlined and the start and stop codons in bold. The PCR product was initially cloned into the vector pCR2.1-TOPO (Invitrogen), to give the plasmid pCR2.1-TFAR1, then subcloned into the yeast expression vector pESC-Leu (Stratagene) with the *Bam*HI and *Xho*I restriction sites and yielding the plasmid pESC-Leu-TFAR1.

Functional analysis of TFAR1 in yeast

The plasmid pESC-Leu-TFAR1 and empty vector pESC-Leu were introduced separately into the yeast (*Saccharomyces cerevisiae*) strain WAT11 (Teoh *et al*, 2006). Yeast cultures (10 ml) were grown overnight at 30°C in Leu dropout liquid medium (Clontech, Mountain View, CA, USA) containing 2% (w/v) glucose. After 24 h in an orbital shaker maintained at 250 rpm, cultures were collected by centrifugation, washed three times in sterile deionised water, and resuspended to an A_{600nm} of 0.8 in 10 ml Leu dropout liquid medium containing 2% (w/v) galactose supplemented with a mixture of very-long-chain fatty acids (100 µl of a solution containing 10%(v/v) tergitol and 0.1 g/l of each of the free fatty acids 20:1, 22:1, 24:1, 26:0 and 30:0 (Nu-chek Prep, Elysian, MN, USA)). After incubation at 30°C for 24 h, the cell pellets were collected by centrifugation (8,000*g* for 10 min).

The yeast pellets were prepared and analyzed by gas chromatography (GC)/MS as described (Gagné *et al.*, 2009), except that the final residue was dissolved in 40 µl of

a derivatization solution containing dichloromethane, N,O-bis(trimethylsilyl)acetamide (Sigma-Aldrich) and pyridine (Sigma-Aldrich) (1:1:1) prior to GC/MS analysis. Fatty alcohol reference standards (C24:0-OH and C26:0-OH; Nu-chek Prep) were dissolved in the same derivatization solution prior to GC/MS analysis.

Results

Phytohormones promote the formation of both glandular and filamentous trichomes on leaves of different *A. annua* cultivars

Leaves of *A. annua* possess not only glandular trichomes, in which artemisinin is produced, but also nonglandular, multicellular filamentous trichomes. Measurements based on tabletop SEM revealed a large variation in trichome density within the two *A. annua* cultivars used here, designated HAP and LAP for the 2/39 (from Brazil) and Meise (from Belgium) cultivars, and representing high- and low-artemisinin cultivars, respectively (see below). A higher trichome density with 14.2-fold more filamentous and 1.4-fold more glandular trichomes was observed in control (mock treated) leaves of HAP plants than in those of control LAP plants (Fig. 2a).

To investigate the effect of JA, BAP and GA_3, on the regulation of the two trichome types, an experimental method designed in Arabidopsis to score the effects of these phytohormones (Maes *et al.*, 2008) was adapted to *A. annua* plants. Two general observations could be drawn from this elicitation experiment. First, the two different trichome types were clearly differentially regulated. Whereas all phytohormones increased the density of filamentous trichomes, only BAP and JA, but not GA_3, stimulated

the glandular trichome formation (Fig. 2a and 2c). Second, the phytohormone-triggered trichome promotion trends were identical in the two cultivars, although quantitative differences in induction levels were discernable (Fig. 2a and 2c). For the glandular trichomes, JA and BAP treatments resulted in a 4.3- and 5.5-fold higher trichome density in LAP plants, whereas only a 1.6- and 2.5-fold induction could be observed in HAP plants, respectively. Similarly, filamentous trichome densities increased 23.0-, 22.6- and 7.6-fold in LAP plants and only 1.4-, 2.5-, and 1.5-fold in HAP plants after elicitation with JA, BAP, and GA_3, respectively. Hence, although the "basal" trichome density in LAP leaves was lower than that in HAP leaves, the plasticity with regard to trichome formation seemed higher in the LAP cultivar.

Phytohormones differentially affect glandular trichome size

Besides affecting trichome initiation, previous studies have indicated that phytohormones can also interfere with trichome maturation and size (Maes *et al.*, 2008). To assess whether glandular trichome size was also affected in elicited *A. annua* plants, we measured the cross-sectional area of glandular trichomes, as a representative parameter. Notably, in addition to gland density differences (Fig. 2a), gland size also differed between the two cultivars under control (mock-treated) conditions: trichomes of HAP plants were 26.5% larger than those of LAP plants (Fig. 2b).

In contrast to the effects on trichome density, the phytohormonal effects on gland size were markedly distinct in the two cultivars (Fig. 2b and 2c). JA and GA_3 both increased the size of glandular trichomes in LAP plants, with 65.6% and 45.6%, respectively, but not in HAP plants. Only after BAP treatment a similar pattern was observed in the two

cultivars, namely a clear decrease of 44.0% in trichome size in the two lines. Hence, for both size and density of glandular trichomes, the LAP plants appeared to possess a greater plasticity than the HAP plants. The lack of positive effects on HAP gland size might reflect a certain degree of saturation in this cultivar.

Jasmonate, but not gibberellin or cytokinin, promotes sesquiterpene lactone accumulation in glandular trichome exudates

To analyze how the phytohormonal treatment altered the artemisinin production, we measured the levels of a range of sesquiterpenoids that included artemisinic acid, dihydroartemisinic acid, artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde, dihydroartemisinic aldehyde, artemisinin, and arteannuin B (Fig. 1) in the two cultivars over a time period of 5 weeks (Fig. 3 and Supporting Information (SI) Fig. S1). As for the trichome parameters, both cultivars had different "basal" sesquiterpene lactone levels. The HAP cultivar represents a high-artemisinin chemotype, whereas the LAP cultivar can be considered as a low-artemisinin chemotype. Also dihydroartemisinic acid, artemisinic aldehyde and dihydroartemisinic aldehyde accumulated more in HAP plants, but, conversely, levels of arteannuin B and artemisinic acid were notably lower in HAP than those in LAP plants (Fig. 3 and Fig. S1). Overall, the HAP cultivar was characterized by high dihydroartemisinic acid:artemisinic acid and artemisinin:arteannuin B ratios (approximately 50:1 and 54:1 after 5 weeks of mock treatment, respectively), whereas LAP plants showed an opposite chemotype (ratios of approximately 1:19 and 1:4 after 5 weeks of mock treatment, respectively).

Of the three phytohormones, only JA was capable of eliciting pronounced increases in

sesquiterpene lactone accumulation levels, but with different specificities on the flux through the different pathway branches in the two chemotypes (Fig. 3). The analysis of the individual compounds revealed a clear impact of JA treatment on artemisinic acid, artemisinin, and arteannuin B in LAP plants, on the one hand, and on artemisinic aldehyde, dihydroartemisinic aldehyde, dihydroartemisinic acid, and artemisinin in HAP plants, on the other hand. No significant induction occurred in any of the two *A. annua* chemotypes for artemisinic alcohol or dihydroartemisinic alcohol. In all cases, a maximum elicitation effect was obtained after 3 weeks of treatment, with two notable exceptions, namely the end products artemisinin and arteannuin B in LAP plants. In fact, accumulation levels after 5 weeks of JA treatment for both artemisinin and arteannuin B were higher in LAP plants than those in HAP plants, further underscoring the pronounced plasticity of the LAP trichome machinery and their biosynthetic capacities. Furthermore, even though the absolute amount of arteannuin B remained higher than that of artemisinin in JA-elicited LAP plants, the artemisinin:arteannuin B ratio did increase relative to mock-treated plants (approximately 1:2 versus 1:4, respectively). In HAP plants, JA-elicitation clearly promoted the flux to artemisinin only, with an artemisinin:arteannuin B ratio of approximately 116:1 in JA-elicited plants versus 54:1 in mock-treated plants.

No stimulation of artemisinin or arteannuin B biosynthesis, or of any of their precursors was observed after BAP or GA_3 treatment in either of the two cultivars (Fig. 3 and Fig. S1). On the contrary, the overall tendency pointed rather to a slightly negative effect of both hormones.

Jasmonate induces expression of artemisinin biosynthesis genes in a coordinated manner

To characterize the phytohormonal effects on the regulation of artemisinin biosynthesis, we evaluated the transcriptional regulation of the known biosynthetic genes (Fig. 1) by qRT-PCR. As the maximal metabolite accumulation was observed 3 weeks after elicitation, leaves sampled at the preceding time point, i.e. after 2 weeks of treatment, were chosen for expression analysis.

Expression of all artemisinin biosynthetic genes significantly increased after JA treatment (Fig. 4), which is in clear agreement with the data obtained by metabolite profiling. Expression of *ADS*, *CYP71AV1*, *DBR2*, and the *Cytochrome P450 Reductase* (*CPR*), of which the corresponding proteins catalyze the formation of amorpha-4,11-diene and its ultimate conversion to dihydroartemisinic acid, was induced in both cultivars. The expression of the upstream gene *FDP Synthase* (*FDS*), of which the corresponding enzyme catalyzes the formation of the central sesquiterpene precursor FDP, increased only in the HAP cultivar, and that of *ALDH1*, of which the gene product catalyzes the oxidation of artemisinic and dihydroartemisinic aldehydes, only in the LAP cultivar. Notably, the increase in *DBR2* expression was more pronounced in the HAP cultivar (5- to 7-fold in LAP plants versus 50- to 70-fold in HAP plants), correlating with the specific and marked induction of dihydroartemisinic aldehyde and dihydroartemisinic acid metabolite levels in HAP plants.

Although no pronounced changes in metabolite levels after phytohormonal induction with BAP and GA_3 were observed, some of the artemisinin biosynthetic genes showed altered expression levels in either LAP or HAP plants (Fig. S2). However, in contrast to the concerted upregulation observed after JA elicitation, the patterns were not consistent after either BAP or GA_3 treatments in either of the two cultivars. Most importantly, for both cultivars and both BAP and GA_3 treatments, at least one of the known artemisinin

biosynthesis genes was downregulated, which correlated with the overall metabolite profiles of plants treated with BAP or GA_3. For instance, in HAP plants, *ADS* and *FDS* were downregulated after BAP and GA_3 treatment, respectively, and both BAP and GA_3 downregulated *ADS* and *CYP71AV1* in LAP plants. These observations suggest that BAP and GA_3 could not stimulate sesquiterpene biosynthesis within the glands, in spite of their positive effect on trichome initiation and size, respectively.

Jasmonate-modulated reprogramming of the *A. annua* leaf transcriptome

To characterise in depth the transcriptional response of *A. annua* plants to phytohormones that affect glandular trichome development and metabolism and to reveal novel genes potentially involved in these processes, a genome-wide cDNA-AFLP-based transcriptome analysis was launched. Because LAP plants showed the largest plasticity for all the parameters tested, i.e. sesquiterpene lactone accumulation, gland density, and gland size, this cultivar was chosen as the model system. Similarly, because it was the only phytohormone that positively affected all parameters, JA was selected as the preferred elicitor for a new transcript profiling experiment, in which we focused on early elicitation events, from 0 to 48 h after JA elicitation. Indeed, pilot RT-PCR experiments suggested that the expression of *FDS* and *ADS* in LAP leaves was stimulated within 2 days after JA treatment (data not shown). Furthermore, the transcriptional response in *A. annua* leaves at two different leaf developmental stages was compared. These stages corresponded physically with leaves from the bottom and top part of the same plant that were harvested over 48 h after JA treatment and were designated “lower” and “upper” leaves, respectively. More importantly, these two stages reflected leaves that were either already

(or nearly) fully developed ("lower") versus leaves that were still developing and thus still actively forming trichomes ("upper").

In total, the expression of approximately 12,000 gene tags was visualised. Clustering and sequence analysis of the differentially expressed transcript tags led to the identification of clusters with gene tags corresponding to gene products putatively involved in protein synthesis and fate, transcription, signal transduction, transport, photosynthesis, energy and metabolism, and cell organization and defence (Table S1).

The transcriptional response of the two leaf stages to JA treatment differed remarkably, demonstrating that the context within which the JA signal was perceived was crucial for the shaping of the transcriptional response (Fig. 5). Here, we further concentrated on a cluster of 142 genes, potentially encoding metabolic enzymes and transporters and of which the expression was stimulated by JA (Fig. 5). Among this cluster, tags corresponding to *FDS* and *1-deoxyxylulose 5-phosphate synthase (DXS)* as well as *CYP71AV1* were identified. The former two encode enzymes known to be involved in the biosynthesis of the necessary FDP precursor (Schramek *et al.*, 2009), the latter gene is specifically expressed in trichomes and dedicated to artemisinin biosynthesis (Teoh *et al.*, 2006). Interestingly and particularly relevant to artemisinin biosynthesis and gland formation, all three of these known artemisinin biosynthesis genes were only (*CYP71AV1*) or primarily (*DXS* and *FDP*) upregulated in the upper leaves in response to JA treatment, indicating the importance of the developmental stage for the plants' capacity to synthesize artemisinin (Fig. 5).

In addition, a large number of genes potentially encoding enzymes involved in other secondary metabolic pathways, in particular flavonoid biosynthesis, were identified. Many of these genes had an expression pattern similar to that of artemisinin biosynthesis

genes, i.e. a differential JA-induction in upper versus lower leaves, pointing to a coordinated transcriptional activation of several secondary metabolic pathways within JA-elicited *A. annua* leaves and/or trichomes (Fig. 5; Table S1).

Identification of a novel trichome-specific gene encoding an alcohol-forming very-long-chain fatty acyl CoA reductase

Three gene tags, AA064, AA387, and AA707, were investigated that had been annotated as a putative terpene synthase, an acyl CoA reductase, and an allyl alcohol dehydrogenase, respectively, and that were all induced by JA treatment. AA387 and AA707 were induced in the upper leaves only and clustered with the three known artemisinin biosynthesis genes, whereas AA064 was induced in leaves from both developmental stages and belonged to another cluster (Fig.5).

First, we assessed whether JA induction of these three genes was conserved among different *A. annua* cultivars. Expression analysis in LAP and HAP plants of the first elicitation series confirmed independently that all three genes were inducible by JA in LAP plants and indicated that a similar JA-mediated transcriptional induction also occurs in other *A. annua* cultivars, at least in the HAP line (Fig. S3). Second, because the cDNA-AFLP profiling was done with RNA from whole leaves only, we also analyzed the tissue-specific expression of these three genes in more detail. All of them were expressed in flower buds, leaves, and flower bud trichomes, but negligibly in roots (Fig. 6). Only one gene, corresponding to tag *AA387*, showed a true trichome-specific expression pattern, like that of the known artemisinin biosynthesis genes such as *ADS, DBR2, CYP71AV1,* and *ALDH1* (Fig. 6).

Considering that the expression of *AA387* closely matched that of the established artemisinin biosynthetic genes, we cloned the full-length cDNA corresponding to *AA387* and functionally characterized it. Full-length *AA387*, hereafter referred to as Trichome-specific Fatty Acyl-CoA Reductase 1 (TFAR1), was predicted to be 493 amino acids long, with a molecular weight of 55,914. TFAR1 showed the highest similarity to the family of fatty acyl-CoA reductases (FARs) that includes the jojoba (*Simmondsia chinensis*) FAR involved in seed wax ester biosynthesis and the Arabidopsis CER4 involved in cuticular wax biosynthesis (Metz *et al.*, 2000; Rowland *et al.,* 2006; Doan *et al.*, 2009) (Fig. S4). Expression of *TFAR1* in yeast (*Saccharomyces cerevisiae*), supplemented with very-long-chain fatty acids, resulted in the gene-dependent production of 24:0 and 26:0 primary alcohols (Fig. 7 and Fig. S5), indicating that *TFAR1* encodes a functional fatty acyl-CoA reductase.

Discussion

Despite the pharmaceutical importance of plant-derived artemisinin, relatively little is known about the regulation of its biosynthesis in *A. annua* plants, and the development of the specialized producer organ, the glandular trichome, in which biosynthesis exclusively occurs. Here, we studied the regulation of these fundamental processes by investigating the plants' dynamic response to phytohormonal cues, important modulators of development and metabolism.

Phytohormones differentially affect gland formation and sesquiterpene lactone accumulation

Two different *A. annua* cultivars, characterized by different artemisinin:arteannuin B ratios and trichome parameters, were profiled and three phytohormones, GA_3, BAP and JA, were used that had been shown previously to promote trichome initiation in Arabidopsis (Maes *et al.*, 2008). Arabidopsis has only unicellular filamentous trichomes, whereas *A. annua* has filamentous and glandular trichomes, both of the multicellular type. In Asterids and Rosids, the lineages to which *A. annua* and Arabidopsis belong, respectively, filamentous trichomes have been postulated to be analogous rather than homologous structures and to develop through different transcriptional regulatory networks (Serna & Martin, 2006). Nonetheless, the phytohormonal elicitation patterns for the filamentous trichomes of *A. annua* closely matched those observed for Arabidopsis trichomes. Indeed, all three hormones clearly promoted hairy trichome formation, in the two *A. annua* cultivars tested, suggesting that the formation of at least these filamentous structures obeys similar regulatory cues, although not necessarily by means of similar molecular components.

In contrast, the patterns for promotion of glandular trichome density and size were distinct and showed great variability between the *A. annua* cultivars. First, JA and BAP, but not GA_3, could stimulate gland formation. Second, only after JA treatment, an increase in gland density was accompanied with an increase in gland size, possibly reflecting an advanced maturation and/or increased biosynthetic activity. In contrast, BAP, markedly reduced *A. annua* gland size, as similarly observed in BAP-treated Arabidopsis trichomes. This characteristic had been attributed to the general capacity of cytokinins to keep cells in a division state, thereby stimulating formation of, but preventing full maturation of, for instance, trichome cells (Maes *et al.*, 2008). Third, the

plasticity of trichome capacities differed strikingly between the two cultivars; the cultivar with the lowest "basal" density and size of trichomes (LAP) was far more susceptible to JA elicitation and ultimately displayed more and larger glands than the elicited cultivar with a higher "basal gland score" (HAP). Although it cannot be excluded that this observation would be (partly) due to a different ability to uptake phytohormones, this important trait could be taken into consideration when planning research to increase our fundamental understanding of gland formation or artemisinin biosynthesis or when designing *A. annua* breeding programs for commercial purposes.

Sesquiterpene lactone accumulation was stimulated markedly in JA-elicited plants only and not in plants treated with BAP or GA_3, indicating that an increase in gland density was not necessarily accompanied by an increase in metabolite production. Expression analysis corroborated that this effect could be attributed, at least in part, to the fact that JA is the only phytohormone capable of generating a coordinated transcriptional activation of the biosynthetic genes involved. It is worth noting that our profiling results are consistent with a branched pathway that leads to two related major sesquiterpenes: artemisinin and arteannuin B, with artemisinic aldehyde as the last common intermediate. Based on the different JA-mediated elicitation and accumulation trends in the two chemotypes, the accumulation of dihydroartemisinic acid appears to be associated with the production of artemisinin, whereas that of artemisinic acid with the production of arteannuin B (Fig. 1). The existence of and relative flux through the two branches of the pathway might provide a molecular basis for the high- or low-artemisinin character of particular *A. annua* chemotypes. For instance, in LAP plants, the arteannuin B route seems the most competent, whereas the biosynthetic machinery of HAP plants clearly promotes the flux toward artemisinin (Fig. 3).

A transcriptomics-based screening strategy reveals genes potentially involved in the development and metabolism of glandular trichomes

Because transcriptional coregulation is an important hallmark of genes involved in secondary metabolite pathways in general and, as shown here, also in *A. annua* sesquiterpene lactone biosynthesis, a transcript-profiling-based experimental strategy might well lead to the identification of novel genes encoding enzymes that act, for instance, in arteannuin B biosynthesis, or in metabolic pathways that support or compete with artemisinin biosynthesis in *A. annua* glands. Careful design of such a transcript profiling exercise might also yield novel genes involved in the actual formation of the artemisinin producer organs, the glands. Indeed, although no information is yet available on the identity of the molecular players that steer the formation of trichomes of the glandular type from any plant species, at least some phytohormone-modulated regulatory cues seem to be conserved across the plant kingdom (Maes & Goossens, 2010; this study). Furthermore, from research conducted in Arabidopsis, it became clear that the phytohormone-mediated promotion of unicellular, filamentous trichome formation can be triggered by the transcriptional activation of the components of the established trichome initiation complex (Maes *et al.*, 2008).

Therefore, a cDNA-AFLP-based transcript profiling analysis was launched on JA-elicited plants of the cultivar with the lowest basal metabolite and gland scores, but that showed the highest plasticity and, thus, might inherently display more pronounced transcriptional responses to a particular stimulus. Accordingly, in this analysis known trichome-specific artemisinin biosynthesis genes were identified as well as novel genes

encoding enzymes catalyzing yet unknown metabolic conversions or potential regulatory proteins steering trichome formation and metabolism. Furthermore, by profiling the JA-response in leaves at distinct developmental stages, we demonstrated that the developmental time frame within which the plant or its leaves are elicited, represents a key parameter, which illustrates the importance of the 'context' for the perception of the jasmonate signal and the eventual physiological responses. Notably, the transcriptional upregulation of the artemisinin biosynthetic genes was also observed primarily in developing leaves, indicating that the developmental stage is not only important for the formation of the producer organs themselves but also for their capacity to synthesize artemisinin.

Based on this transcriptome analysis, a new functional FAR, designated TFAR1 and closely related to the Arabidopsis CER4 involved in cuticular wax biosynthesis (Rowland *et al.,* 2006) has been characterized. Like CER4, TFAR1 catalyzes the formation of C24 and C26 fatty alcohols, presumably from fatty acyl-CoAs. Notably, *CER4* expression in Arabidopsis stems and leaves is detected exclusively in epidermal cells and is, in rosette leaves, even confined to trichome cell types (Rowland *et al.*, 2006). Similar trichome-specific expression patterns have also been observed for *CER2* and *YORE-YORE1* (*YRE1*)*/WAX2*, both encoding enzymes catalyzing other steps in cuticular wax production in Arabidopsis (Xia *et al.*, 1997; Kurata *et al.*, 2003). Moreover, several Arabidopsis mutants, defective in loci encoding proteins with either catalytic, regulatory or transport roles in cuticular wax biosynthesis, display either smaller (*yre1*; Kurata *et al.*, 2003), distorted (*desperado*; Panikashvili *et al.*, 2007), fewer (*fiddlehead*; Yephremov *et al.*, 1999) or more (*shine*; Aharoni *et al.*, 2004) trichomes than the wildtype, indicating that cuticular wax production is required for normal trichome development. Hence, taking into

account its activity, its analogy with the Arabidopsis CER4, and its JA-inducible, and trichome- and "developing leaf"-specific expression pattern, we postulate that TFAR1 is involved in cuticular wax formation during glandular trichome expansion in leaves and flowers of *A. annua* plants. Intriguingly, the phytohormone-regulated and the development- and organ-specific expression pattern of *TFAR1* very closely matches that of key artemisinin biosynthetic genes, such as *ADS* and *CYP71AV1*, which might point to the existence of a common regulatory program for both the metabolic content and the structure of the gland. In particular, jasmonates seem to be capable of impinging on this program, as supported by the observation that only JA can stimulate co-ordinately gland initiation, gland size, sesquiterpene lactone biosynthesis and, last but not least, expression of the genes involved.

These results open exciting perspectives for the exhaustive transcriptome mining that is in progress in various laboratories (Wang *et al.*, 2009; Dai *et al.*, 2010; Graham *et al.*, 2010; this work) or that will be undertaken in the near future. Such gene discovery programs will help to fill a major gap in our understanding of plant development: undoubtedly the resulting transcriptome data will reveal genes encoding yet unknown regulatory proteins that determine the formation of glandular trichomes. Ultimately, such genes will be invaluable, not only for successful metabolic engineering of artemisinin biosynthesis in *A. annua*, but also for numerous other high-value compounds produced in the glandular trichomes of other plants.

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References

Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A. 2004. The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in Arabidopsis. *Plant Cell* **16:** 2463–2480.

Arsenault PR, Wobbe KK, Weathers PJ. 2008. Recent advances in artemisinin production through heterologous expression. *Current Medicinal Chemistry* **15:** 2886–2896.

Bertea CM, Freije JR, van der Woude H, Verstappen FWA, Perk L, Marquez V, De Kraker J-W, Posthumus MA, Jansen BJM, de Groot A *et al.* 2005. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Medica* **71:** 40–47.

Boughton AJ, Hoover K, Felton GW. 2005. Methyl jasmonate application induces increased densities of glandular trichomes on tomato, *Lycopersicon esculentum*. *Journal of Chemical Ecology* **31:** 2211–2216.

Brown GD, Sy L-K. 2004. In vivo transformations of dihydroartemisinic acid in *Artemisia annua* plants. *Tetrahedron* **60:** 1139–1159.

Brown GD, Sy L-K. 2007. In vivo transformations of artemisinic acid in *Artemisia annua* plants. *Tetrahedron* **63:** 9548–9566.

Chang MCY, Eachus RA, Trieu W, Ro D-K, Keasling JD. 2007. Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nature Chemical Biology* **3:** 274–277.

Covello PS. 2008. Making artemisinin. *Phytochemistry* **69:** 2881–2885.

Covello PS, Teoh KH, Polichuk DR, Reed DW, Nowak G. 2007. Functional genomics and the biosynthesis of artemisinin. *Phytochemistry* **68:** 1864–1871.

Dai X, Wang G, Yang DS, Tang Y, Broun P, Marks MD, Sumner LW, Dixon RA, Zhao PX. 2010. TrichOME: a comparative omics database for plant trichomes. *Plant Physiology* **152:** 44–54.

Davies MJ, Atkinson CJ, Burns C, Woolley JG, Hipps NA, Arroo RRJ, Dungey N, Robinson T, Brown P, Flockart I *et al.* 2009. Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua*. *Annals of Botany* **104:** 315–323.

Delabays N, Simonnet X, Gaudin M. 2001. The genetics of artemisinin content in *Artemisia annua* L. and the breeding of high yielding cultivars. *Current Medicinal Chemistry* **8:** 1795–1801.

Doan TTP, Carlsson AS, Hamberg M, Bülow L, Stymne S, Olsson P. 2009. Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. *Journal of Plant Physiology* **166:** 787–796.

Duke MV, Paul RN, Elsohly HN, Sturtz G, Duke SO. 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of Artemisia annua L. *International Journal of Plant Science* **155:** 365–372.

Efferth T. 2007 Antiplasmodial and antitumor activity of artemisinin -- from bench to bedside. *Planta Medica* **73:** 299–309.

Efferth T, Romero MR, Wolf DG, Stamminger T, Marin JJG, Marschall M. 2008. The antiviral activities of artemisinin and artesunate. *Clinical Infectious Diseases* **47:** 804–811.

Ferreira JFS, Simon JE, Janick J. 1995. Developmental studies of *Artemisia annua*:

flowering and artemisinin production under greenhouse and field conditions. *Planta Medica* **61:** 167–170.

Gagné SJ, Reed DW, Gray GR, Covello PS. 2009. Structural control of chemoselectivity, stereoselectivity and substrate specificity in membrane-bound fatty acid acetylenases and desaturases. *Biochemistry* **48:** 12298–12304.

Gan Y, Liu C, Yu H, Broun P. 2007. Integration of cytokinin and gibberellin signalling by Arabidopsis transcription factors GIS, ZFP8, and GIS2 in the regulation of epidermal cell fate. *Development* **134:** 2073–2081.

Graham IA, Besser K, Blumer S, Branigan CA, Czechowski T, Elias L, Guterman I, Harvey D, Isaac PG, Khan AM *et al*. 2010. The genetic map of *Artemisia annua* L. identifies loci affecting yield of the antimalarial drug artemisinin. *Science* **327:** 328–331.

Haynes RK. 2006. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction old and new derivatives, stereochemistry and medicinal chemistry requirements. *Current Topics in Medicinal Chemistry* **6:** 509–537.

Kurata T, Kawabata-Awai C, Sakuradani E, Shimizu S, Okada K, Wada T. 2003. The *YORE-YORE* gene regulates multiple aspects of epidermal cell differentiation in *Arabidopsis*. *Plant Journal* **36:** 55–66.

Lattarulo Campos ML, de Almeida M, Lanzoni Rossi M, Pinheiro Martinelli A, Litholdo Junior CG, Figueira A, Rampelotti-Ferreira FT, Vendramim JD, Benedito VA, Pereira Peres LE. 2009. Brassinosteroids interact negatively with jasmonates in the formation of anti-herbivory traits in tomato. *Journal of Experimental Botany* **60:** 4347–4361.

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J, Whalon ME, Pichersky E, Howe

GA. 2004. The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. *Plant Cell* **16:** 126–143.

Lommen WJM, Elzinga S, Verstappen FWA, Bouwmeester HJ. 2007. Artemisinin and sesquiterpene precursors in dead and green leaves of *Artemisia annua* L. crops. *Planta Medica* **73:** 1133–1139.

Lommen WJM, Schenk E, Bouwmeester HJ, Verstappen FWA. 2006. Trichome dynamics and artemisinin accumulation during development and senescence of *Artemisia annua* leaves. *Planta Medica* **72:** 336–345.

Ma D, Pu G, Lei C, Ma L, Wang H, Guo Y, Chen J, Du Z, Wang H, Li G *et al.* 2009. Isolation and characterization of AaWRKY1, an *Artemisia annua* transcription factor that regulates the amorpha-4,11-diene synthase gene, a key gene of artemisinin biosynthesis. *Plant Cell Physiology* **50:** 2146–2161.

Maes L, Goossens A. 2010. Hormone-mediated promotion of trichome initiation in plants is conserved but utilizes species- and trichome-specific regulatory mechanisms. *Plant Signaling & Behavior* **5:** 205–207.

Maes L, Inzé D, Goossens A. 2008. Functional specialization of the TRANSPARENT TESTA GLABRA1 network allows differential hormonal control of laminal and marginal trichome initiation in Arabidopsis rosette leaves. *Plant Physiology* **148:** 1453–1464.

Mercke P, Bengtsson M, Bouwmeester HJ, Posthumus MA, Brodelius PE. 2000. Molecular cloning, expression, and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Archives of Biochemistry and Biophysics* **381:** 173–180.

Metz JG, Pollard MR, Anderson L, Hayes TR, Lassner MW. 2000. Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. *Plant Physiology* **122:** 635–644.

Olsson ME, Olofsson LM, Lindahl A-L, Lundgren A, Brodelius M, Brodelius PE. 2009. Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of *Artemisia annua* L. *Phytochemistry* **70:** 1123–1128.

Panikashvili D, Savaldi-Goldstein S, Mandel T, Yifhar T, Franke RB, Höfer R, Schreiber L, Chory J, Aharoni A. 2007. The Arabidopsis *DESPERADO/AtWBC11* transporter is required for cutin and wax secretion. *Plant Physiology* **145:** 1345–1360.

Pauwels L, Inzé D, Goossens A. 2009. Jasmonate-inducible gene: what does it mean? *Trends in Plant Science* **14:**, 87–91.

Perazza D, Vachon G, Herzog M. 1998. Gibberellins promote trichome formation by up-regulating *GLABROUS1* in Arabidopsis. *Plant Physiology* **117:** 375-383.

Pu G-B, Ma D-M, Chen J-L, Ma L-Q, Wang H, Li G-F, Ye H-C, Liu B-Y. 2009. Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L. *Plant Cell Reports* **28:** 1127–1135.

Rischer H, Orešič M, Seppänen-Laakso T, Katajamaa M, Lammertyn F, Ardiles-Diaz W, Van Montagu MCE, Inzé D, Oksman-Caldentey K-M, Goossens A. 2006. Gene-to-metabolite networks for terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cells. *Proceedings of the National Academy of Sciences of the USA* **103:** 5614-5619.

Ro D-K, Paradise EM, Ouellet M, Fisher KJ, Newman KL, Ndungu JM, Ho KA,

Eachus RA, Ham TS, Kirby J *et al.*, 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* **440:** 940–943.

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L. 2006. *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. *Plant Physiology* **142:** 866–877.

Rydén A-M, Ruyter-Spira C, Quax WJ, Osada H, Muranaka T, Kayser O, Bouwmeester H. 2010. The molecular cloning of dihydroartemisinic aldehyde reductase and its implication in artemisinin biosynthesis in *Artemisia annua*. *Planta Medica*, in press (doi: 10.1055/s-0030-1249930).

Sangwan RS, Agarwal K, Luthra R, Thakur RS, Singh-Sangwan N. 1993. Biotransformation of arteannuic acid into arteannuin-B and artemisinin in *Artemisia annua*. *Phytochemistry* **34:** 1301–1302.

Schramek N, Wang H, Römisch-Margl W, Keil B, Radykewicz T, Winzenhörlein B, Beerhues L, Bacher A, Rohdich F, Gershenzon J *et al*. 2009. Artemisinin biosynthesis in growing plants of *Artemisia annua*. A $^{13}CO_2$ study. *Phytochemistry* **71:** 179–187.

Serna L, Martin C. 2006. Trichomes: different regulatory networks lead to convergent structures. *Trends in Plant Science* **11:** 274-280.

Sy L-K, Brown GD. 2002. The mechanism of the spontaneous autoxidation of dihydroartemisinic acid. *Tetrahedron* **58:** 897–908.

Teoh KH, Polichuk DR, Reed DW, Nowak G, Covello PS. 2006. *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS Letters* **580:** 1411–1416.

Teoh KH, Polichuk DR, Reed DW, Covello PS. 2009. Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua*. *Botany* **87:** 635–642.

Traw MB, Bergelson J. 2003. Interactive effects of jasmonic acid, salicylic acid, and gibberellin on induction of trichomes in Arabidopsis. *Plant Physiology* **133:** 1367–1375.

Van Nieuwerburgh FCW, Vande Casteele SRF, Maes L, Goossens A, Inzé D, Van Bocxlaer J, Deforce DLD. 2006. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry. *Journal of Chromatography A* **1118:** 180-187.

Vuylsteke M, Peleman JD, van Eijk MJT. 2007. AFLP-based transcript profiling (cDNA-AFLP) for genome-wide expression analysis. *Nature Protocols* **2:** 1399-1413.

Wallaart TE, Bouwmeester HJ, Hille J, Poppinga L, Maijers NCA. 2001. Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta* **212:** 460–465.

Wallaart TE, Pras N, Beekman AC, Quax WJ. 2000. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Medica* **66:** 57–62.

Wang W, Wang Y, Zhang Q, Qi Y, Guo D. 2009. Global characterization of *Artemisia annua* glandular trichome transcriptome using 454 pyrosequencing. *BMC*

Genomics **10:** 465.1–465.10.

Xia Y, Nikolau BJ, Schnable PS. 1997. Developmental and hormonal regulation of the Arabidopsis *CER2* gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. *Plant Physiology* **115:** 925–937.

Yang R-Y, Zeng X-M, Lu Y-Y, Lu W-J, Feng L-L, Yang X-Q, Zeng Q-P. 2009. Senescent leaves of *Artemisia annua* are one of the most active organs for overexpression of artemisinin biosynthesis responsible genes upon burst of singlet oxygen. *Planta Medica* **76:** 734–742.

Yephremov A, Wisman E, Huijser P, Huijser C, Wellesen K, Saedler H. 1999. Characterization of the *FIDDLEHEAD* gene of Arabidopsis reveals a link between adhesion response and cell differentiation in the epidermis. *Plant Cell* **11:** 2187–2201.

Yoshida Y, Sano R, Wada T, Takabayashi J, Okada K. 2009. Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in *Arabidopsis*. *Development* **136:** 1039–1048.

Zeng Q, Qiu F, Yuan L. 2008. Production of artemisinin by genetically-modified microbes. *Biotechnology Letters* **30:** 581–592.

Zhang Y, Teoh KH, Reed DW, Maes L, Goossens A, Olson DJH, Ross ARS, Covello PS. 2008. The molecular cloning of artemisinic aldehyde Δ11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. *Journal of Biological Chemistry* **283:** 21501–21508.

Zhao J, Davis LC, Verpoorte R. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnology Advances* **23:** 283-333.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1. Metabolic profiling of artemisinin precursors in elicited *A. annua* plants.

Fig. S2. Regulation of artemisinin biosynthetic gene expression by BAP and GA_3 in LAP and HAP leaves.

Fig. S3. JA elicitation of cDNA-AFLP tags in LAP and HAP leaves.

Fig. S4. Phylogenetic analysis of TFAR1 and related sequences.

Fig. S5. Identification of TFAR1 products in yeast.

Table S1. Overview of JA-modulated cDNA-AFLP tags.

Figure Legends

Fig. 1. Biosynthetic pathway of artemisinin acid and arteannuin B. Dashed arrows indicate steps for which no enzymes have been identified.

Fig. 2. Effect of phytohormonal treatment on leaf trichome development. (a) Trichome density. Numbers in the ordinate indicate the glandular (GT) and filamentous (FT) trichomes per mm^2 in LAP and HAP cultivars. (b) Gland size. Numbers in the ordinate indicate the mean cross-sectional area of the gland (in μm^2) in LAP and HAP cultivars. Error bars are SE (n=6). Statistical significance was determined by Student *t*-test (** $P<0.01$, * $P<0.05$). (c) Representative SEM images of mock-treated and hormone-elicited LAP leaves. Scale bar = 1 mm (top) and 300 μm (bottom).

Fig. 3. Metabolite profiling of elicited *A. annua* plants. Accumulation of artemisinic acid, dihydroartemisinic acid, artemisinin, and arteannuin B in leaves of LAP and HAP cultivars measured 1 to 5 weeks after mock treatment (CON) or treatment with JA, BAP and GA_3. Numbers in the ordinate are μg metabolite per gram fresh weight. Error bars are SE (n=2). Measurements were repeated twice with similar results.

Fig. 4. Regulation of artemisinin biosynthetic gene expression by JA-elicitation in LAP and HAP leaves. Expression of *FDS* (a), *ADS* (b), *CYP71AV1* (c), *DBR2* (d), *ALDH1* (e), and *CPR* (f) verified by qRT-PCR analysis and normalized to that of the *ACTIN* control gene (GenBank Accession Number EU531837). Numbers in the ordinate give the fold induction relative to the expression level in the control sample 1, which was arbitrarily set

at 1. Error bars are SE (n=3).

Fig. 5. cDNA-AFLP transcript profiling of JA-elicited LAP plants. Average linkage hierarchical clustering of LAP cDNA-AFLP tags with high similarity to genes encoding enzymes and transporters. The treatments and time points (in h) are indicated at the top and the gene annotations on the right. Yellow and blue boxes correspond to increased and reduced transcript accumulation, relative to the average accumulation level of all samples respectively. Left, full average linkage hierarchical clustering of all 142 enzyme and transporter tags. Right, expanded view of the pink part of the tree, corresponding to a cluster of 49 genes, the expression of which is induced by JA in the upper leaves primarily, and in which the artemisinin biosynthesis genes *DXS*, *FDP*, and *CYP71AV1* as well as *TFAR1* are present.

Fig. 6. Tissue-specific expression of JA-elicited genes. Tissue-specific expression of genes encoding DBR2 (a) and CYP71AV1 (b), the annotated allyl alcohol dehydrogenase (c), terpene synthase (d), and acyl-CoA reductase (e) verified by qRT-PCR analysis. Numbers in the ordinate are the expression levels relative to that in leaves. Error bars are SE (n=3).

Fig. 7. Heterologous expression of *TFAR1* in yeast. GC/MS total ion current traces of derivatized lipid extracts from induced yeast cultures, supplemented with very-long-chain fatty acids containing the negative control empty vector pESC-Leu (a) and *TFAR1* expression vector pESC-Leu-TFAR1 (b) compared to a standard mixture of fatty alcohols (c).