Phytochemistry 70 (2009) 1123–1128

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Localization of enzymes of artemisinin biosynthesis to the apical cells of glandular secretory trichomes of *Artemisia annua* L.

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ARTICLE INFO

Article history:
Received 26 March 2009
Received in revised form 8 July 2009
Available online 5 August 2009

Keywords:
Amorpha-4,11-diene synthase
Apical cells
Artemisia annua
Artemisinin biosynthesis
Immunogold labeling
Laser microdissection pressure catapulting
Mesophyl cells
Glandular secretory trichomes
Silver enhancement
Sub-apical cells

ABSTRACT

A method based on the laser microdissection pressure catapulting technique has been developed for isolation of whole intact cells. Using a modified tissue preparation method, one outer pair of apical cells and two pairs of sub-apical, chloroplast-containing cells, were isolated from glandular secretory trichomes of *Artemisia annua*. *A. annua* is the source of the widely used antimalarial drug artemisinin. The biosynthesis of artemisinin has been proposed to be located to the glandular trichomes. The first committed steps in the conversion of FPP to artemisinin are conducted by amorpha-4,11-diene synthase, amorpha-4,11-diene hydroxylase, a cytochrome P450 monooxygenase (CYP71AV1) and artemisinic aldehyde Δ11(13) reductase. The expression of the three biosynthetic enzymes in the different cell types has been studied. In addition, the expression of farnesyldiphosphate synthase producing the precursor of artemisinin has been investigated. Our experiments showed expression of farnesyldiphosphate synthase in apical and sub-apical cells as well as in mesophyl cells while the three enzymes involved in artemisinin biosynthesis were expressed only in the apical cells. Elongation factor 1α was used as control and it was expressed in all cell types. We conclude that artemisinin biosynthesis is taking place in the two outer apical cells while the two pairs of chloroplast-containing cells have other functions in the overall metabolism of glandular trichomes.

1. Introduction

Virtually all of the processes we study as biochemists/biologists are distributed non-uniformly among the cells that make up a tissue or an organ. Many processes rely on the distinct features, activities, and interactions of subsets of specialized cells. Therefore, the results of analysis of whole tissue samples usually reflect the metabolism of the major or predominant cell type and may mask biologically relevant and important changes present in a limited number of a particular cell type. Consequently, it is essential to analyze specific cell types to identify and define biologically important processes.

Techniques such as *in situ* hybridization, expression of reporter genes, immunolocalization, and histochemical staining have revealed the cell-specific distributions of individual transcripts, proteins, and metabolites. The molecular analysis of defined cell types from tissues requires the availability of rapid, efficient and accurate methods for obtaining specific groups of cells for further study. The development in the last decade of sophisticated laser-based methods of tissue microdissection has allowed this goal to be achieved by combining microscope-based morphological methods of analysis with a diverse range of very powerful molecular technologies (Day et al., 2005). Normally, this technique is used on either cryosectioned tissue or conventional microtone sectioned paraffin-embedded tissue.

Trichomes are small protrusions of epidermal origin on the surfaces of leaves and other organs of many plants. They are normally divided into two general categories: non-glandular and glandular trichomes. Glandular secretory trichomes range from small structures consisting of a few cells to large and elaborate structures with differentiated basal, stalk and apical secreting cells. One of the most remarkable features of trichomes is their capacity to synthesize, store and sometimes secrete large amounts of specialized metabolites (Schilmiller et al., 2008). These include various classes of terpenes, as well as phenylpropanoid derivatives, acyl sugars, methylketones, and flavonoids. Many trichome borne compounds have significant commercial value as pharmaceuticals, fragrances, food additives, and natural pesticides. For this reason, the prospect

Abbreviations: AAR, artemisinic aldehyde Δ11(13) reductase; ADS, amorpha-4,11-diene synthase; CYP71AV1, amorpha-4,11-diene hydroxylase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; FPP, farnesyl diphosphate; FPPS, farnesyl diphosphate synthase; GST, glandular secretory trichome; LMPC, laser microdissection pressure catapulting.

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doi:10.1016/j.phytochem.2009.07.009

of exploiting glandular secretory trichomes as 'chemical factories' to produce high-value plant products has recently caught the attention of plant biotechnologists.

Studies of the morphology and ultra-structure of glandular secretory trichomes from various plant species have shown significant morphological differences between cells (Hallahan and Gray, 2000). A number of questions regarding the function of the different cells of glandular secretory trichomes may be asked. Do the morphological differences imply a division of biosynthetic functions? Do all or only certain of the gland cells participate directly in the production of secreted metabolites? Does each cell type specialize in synthesis of different compounds? Do those cells with photosynthetically active plastids provide substrate for production of excreted metabolites in adjacent non-photosynthetic cells? Although intact glands can be isolated and studied biochemically, no method has until now been available for separating the different cell types of the gland.

The physiological and metabolic specialization of glandular secretory trichomes and their high expression of biosynthetic enzymes have made them valuable targets for genomic investigations of natural product biosynthesis. Expressed sequence tags data sets derived from trichome-specific cDNA libraries have been used to identify enzymes responsible for the synthesis of terpenoids in mint (*Mentha* × *piperita*) (Lange et al., 2000), phenylpropenes in basil (*Ocimum basilicum*) (Gang et al., 2001), methylketones in tomato (*Solanum lycopersicum*) (Fridman et al., 2005), sesquiterpenes in *Artemisia annua* (Bertea et al., 2005, 2006; Teoh et al., 2006; Zhang et al., 2008), terpenes (Wang et al., 2008) and terpenophenolics (Nagel et al., 2008) in hop (*Humulus lupulus*), and secondary metabolites in pink rockrose (*Cistus creticus*) (Falara et al., 2008). Microarray-based experiments have been used to identify preferentially expressed genes in trichomes of alfalfa (*Medicago sativa*) by comparing transcripts from isolated trichomes to those from stems with the trichomes removed (Aziz et al., 2005). Finally, a proteomics-based approach has been taken to identify enzymes that are selectively express in trichomes from tobacco (Amme et al., 2005) and basil (Xie et al., 2008). The experiments described in these studies rely on 'bulk material' and, consequently, do not reveal the function of different cells of the glandular secretory trichome. Homogenization and analysis of entire glandular secretory trichomes result in an averaged information, which cannot be assigned to particular cell types. Exceptions are the studies on mint (Lange et al., 2000) and basil (Gang et al., 2001; Xie et al., 2008). In these cases, disk-like clusters of 8 and 4 secretory cells, respectively, were obtained from young leaves by mechanical removal using glass beads. However, no report on studies on the metabolic activity of different cell types within multicellular glandular secretory trichomes has, to our knowledge, been published.

During recent years, the sesquiterpenoid artemisinin has gained increasing importance as an antimalarial drug (van Agtmael et al., 1999). Over 60 countries in endemic areas use this drug in combination with another antimalarial drug (artemisinin-based combination therapy) as a first treatment of malaria (White, 2008). Artemisinin is produced by the plant *A. annua* L. (sweet wormwood or annual wormwood). The biosynthesis of artemisinin has been proposed to be located to multicellular glandular secretory trichomes (Duke et al., 1993). Using glanded and glandless biotypes of *A. annua*, Duke et al. (1994) showed that all extractable artemisinin is localized to the subculticular space of trichomes. No artemisinin could be extracted from the glandless biotype indicating that the sesquiterpenoid is produced and stored in glandular trichomes.

2. Results and discussion

The glandular secretory trichome of *A. annua* is a 10-celled biseriate structure of two stalk cells, two basal cells, and three pairs of secretory cells (Duke et al., 1993). The cuticle of the six secretory cells is separated from the cell walls to form a bilobed sac as shown in Fig. 1A and B. At maturity, the apical cells contain proplastids or leucoplasts but no thylakoids. The two cell pairs below the apical cell pair contain chloroplasts as shown by autofluorescense in Fig. 1C. Artemisinin and other sesquiterpenoids (Duke et al., 1994; van Nieuwerburgh et al., 2006) as well as monoterpenoids (Tellez et al., 1999) are excreted into and stored in the subcuticular space.

The first committed steps in the conversion of farnesyl diphosphate (FPP) to artemisinin are conducted by amorpha-4,11-diene synthase (ADS) (Mercke et al., 2000) and amorpha-4,11-diene hydroxylase, a cytochrome P450 monooxygenase (CYP71AV1) (Teoh et al., 2006) as outlined in Fig. 2. Recently a third enzyme of artemisinin biosynthesis, artemisinic aldehyde Δ11(13) reductase (AAR), was cloned (Zhang et al., 2008). In plants, two pathways for the biosynthesis of isopentenyldiphosphate/dimethylallyldiphosphate (IPP/DMAPP), the building blocks of terpenoids, are operative; the mevalonate pathway in the cytoplasm and the methyl-erythritol phosphate (MEP) pathway located to plastids, i.e. chloroplasts. Recently, some strong evidence that part of the mevalonate pathway is localized to peroxisomes was reported (Sapir-Mir et al., 2008). Mono- and diterpenes are synthesized in plastids from geranyl- and geranylgeranyl diphosphate, respectively, while sesqui and triterpenoids, as well as sterols, are synthesized in the cytoplasm from FPP.

In this study, we have used immunogold labeling with silver enhancement to localize the key enzyme of artemisinin biosynthesis, i.e. ADS. Paraffin-embedded sections of young leaves of

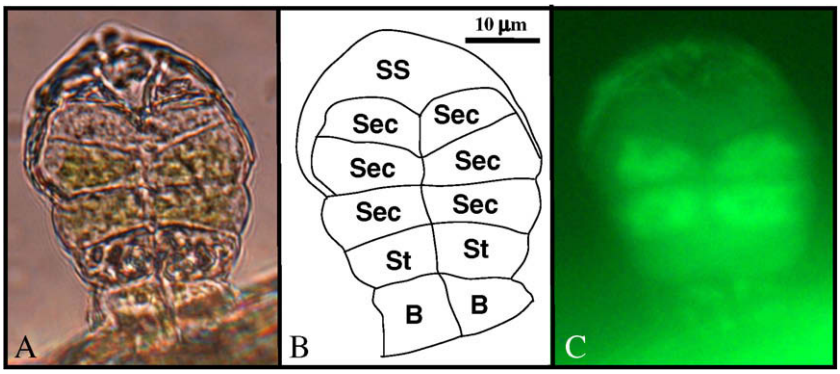

Fig. 1. (A) Glandular secretory trichome of *A. annua*. (B) Schematic drawing showing the different cells; B: basal cells; St: stalk cells; Sec: secretory cells; SS: subcuticular space. (C) Autofluorescence of chloroplasts in sub-apical secretory cells with the FITC filter (λ_{ex} = 480 nm; λ_{em} = 535 nm).

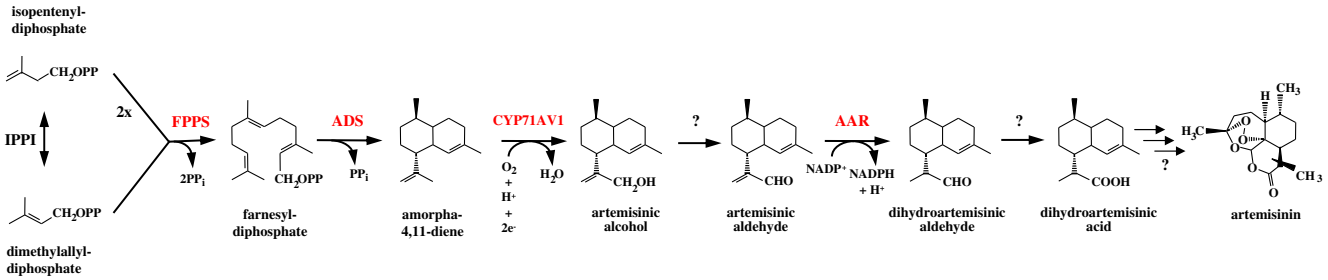

Fig. 2. Putative biosynthetic pathway of artemisinin in *Artemisia annua*. FPPS: farnesyldiphosphate synthase; ADS: amorpha-4,11-diene synthase; CYP71AV1: amorphadiene-12-hydroxylase; AAR: artemisinic aldehyde Δ11(13) reductase.

A. annua were treated with rabbit polyclonal ADS antibodies and goat anti-rabbit antibodies with gold conjugates followed by silver enhancement (Hayat, 1989). Light-microscope was used to examine the samples and the positive labeling was seen as a dark brown precipitate due to silver deposition on the gold particle of the secondary antibodies. Control with pre-immune serum showed no staining (Fig. 3A). A strong positive staining was detected in the glandular trichomes (Fig. 3B). No staining is observed in other cells of the leaf. From these results we may conclude that ADS is expressed only in glandular trichomes of *A. annua*. This is in good agreement with previous studies on the accumulation of artemisinin in glandular trichomes of the plant (Duke et al., 1994).

In order to investigate the function of different cells of multicellular glandular secretory trichomes and to answer at least some of the above questions, a method based on the LMPC technique has been developed for isolation of different whole cells from *A. annua* glandular secretory trichomes. To our knowledge, neither laser capture microdissection nor LMPC has been performed on non-sectioned plant tissue (Nelson et al., 2006) but the harvest by pressure catapulting of single cells from suspension cultures has been reported (Stich et al., 2003). Using a modified tissue preparation method (fixation with 4% para-formaldehyde) we have dissected whole cells from glandular secretory trichomes of *A. annua* as shown in Fig. 4 by performing LMPC on fixed plant tissue that was neither dehydrated, embedded nor sectioned. We have isolated the outer pair of apical cells (Fig. 4D) and two pairs (i.e. a four cell aggregate) of sub-apical, chloroplast-containing cells (Fig. 4E). With this method whole cells are isolated which can be used for studies on the transcriptome, the proteome as well as the metabolome. With conventional LMPC using embedded sectioned cells, 10–20 cuts are required to obtain the content of a whole cell. However, the method presented here is only applicable to protruding tissues like trichomes and possibly root hairs. Whole glandular secretory trichomes were isolated by cutting through the basal cells of the trichome. Finally, mesophyl cells were also isolated by LMPC.

In order to confirm that artemisinin is biosynthesized in glandular secretory trichomes of *A. annua* and to investigate if artemisinin is biosynthesized in a particular cell type, first strand cDNA synthesized from RNA extracted from apical, sub-apical, whole trichomes or mesophyl cells was used as template in the PCR amplifications of transcripts encoding FPPS, ADS, CYP71AV1, and

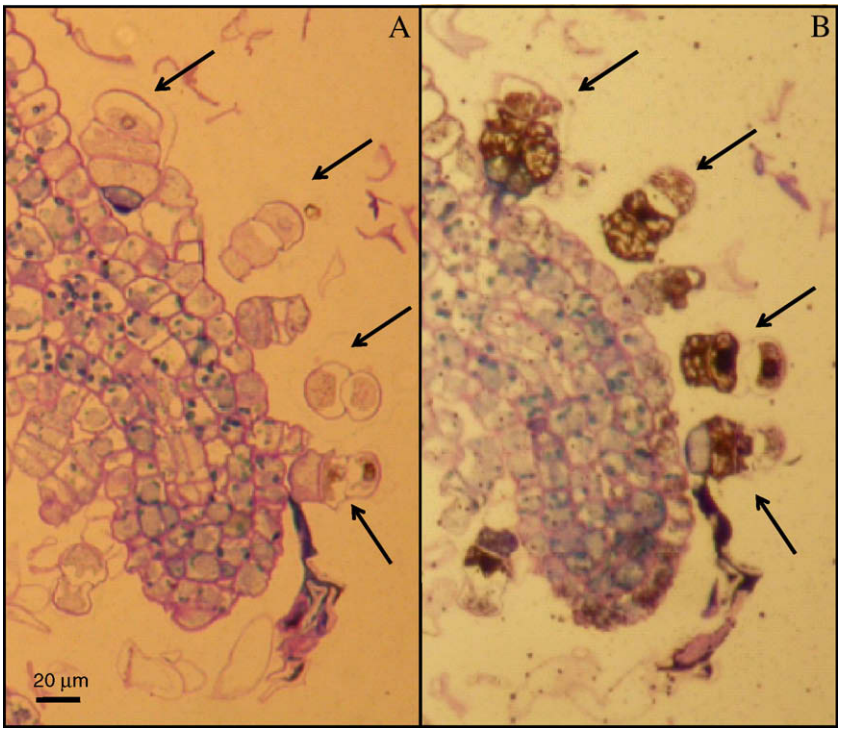

Fig. 3. Immunogold labeling of amorpha-4,11-diene synthase. (A) Transverse cut of a young leaf incubated with rabbit pre-immune serum showing unlabeled trichomes (paragon stained). (B) Transverse cut of a young leaf incubated with polyclonal rabbit ADS antibodies and detected by using secondary goat anti-rabbit antibodies conjugated with gold particles with subsequent silver enhancement.

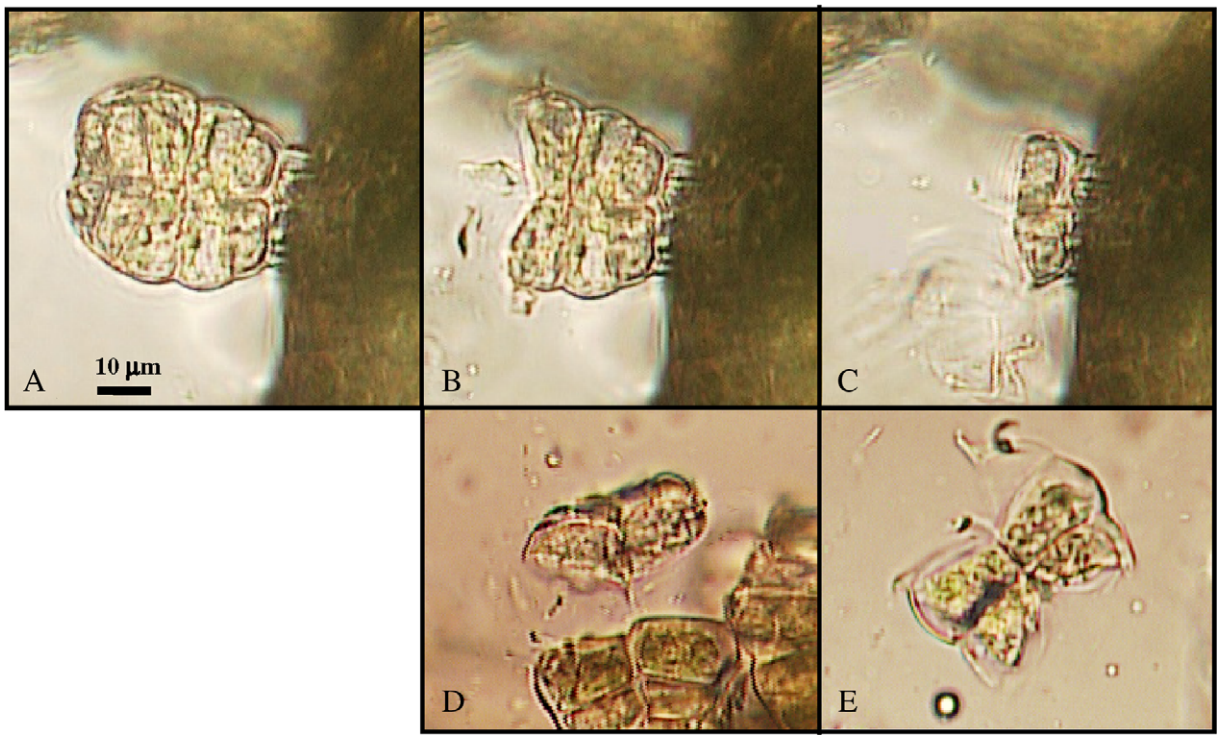

Fig. 4. Microdissection of glandular trichomes of *A. annua*. (A) Intact fixed glandular secretory trichome. (B) Trichome after dissection and collection of the apical cell pair. (C) The remaining basal and stalk cells of the trichome after dissection and collection of the two sub-apical cell pairs. (D) Apical cell pair after microdissection and before collection. (E) Two sub-apical cell pairs after microdissection and before collection.

AAR (Fig. 2). Elongation factor 1α was used as positive control and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) as a marker for plastids.

Three independent experiments all showed expression of FPPS in both apical and sub-apical cells (Fig. 5). This is expected since FPP is required for sterol biosynthesis. Sterols are generally components of cellular membranes. However, a very interesting finding is that the three enzymes of artemisinin biosynthesis were only amplified from the apical cells. No detectable amplification was observed in the sub-apical cells (Fig. 5). Accordingly, it may be concluded that at least the initial steps of artemisinin biosynthesis is taking place exclusively in the apical cells. It is, however, highly likely that the entire biosynthesis of artemisinin from FPP takes place in these cells. Artemisinin is highly phytotoxic to *A. annua* itself (Duke et al., 1987), making secretion from the producing tissues into a storage compartment a necessity. Synthesis in the apical cells with a big surface area towards the subcuticular space would simplify an efficient sequestration. The enzyme DXR involved in the plastidial MEP-pathway was amplified from the chloroplast-containing sub-apical cells but not from apical cells. Furthermore, FPPS was expressed in mesophyl cells while no amplification of ADS, CYP71AV1 and AAR was observed in these cells (data not shown). This is in agreement with the suggestion that artemisinin is only produced in trichomes of *A. annua* (Duke et al., 1994).

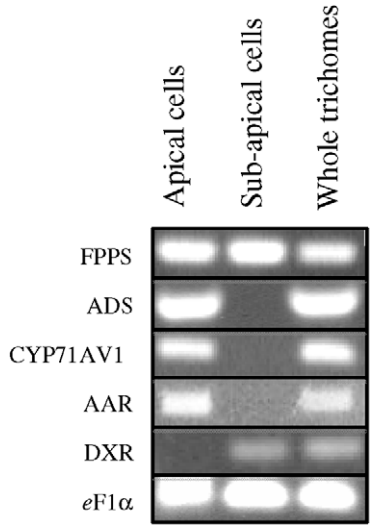

Fig. 5. PCR amplification of various transcripts using cDNA from apical, sub-apical, whole trichomes and mesophyl cells as indicated. FPPS: farnesyldiphosphate synthase; ADS: amorpha-4,11-diene synthase; CYP71AV1: amorphadiene-12-hydroxylase; AAR: artemisinic aldehyde Δ11(13) reductase; DXR: 1-deoxy-D-xylulose-5-phosphate reductoisomerase; *e*F1α: elongation factor 1α.

3. Concluding remarks

From our studies it is evident that different cells of multicellular glandular secretory trichomes have different tasks in the overall metabolism of the trichomes. Apical cells produce the sesquiterpenoid artemisinin and possibly other sesquiterpenoids while sub-apical cells are most likely involved in the production of monoterpenoids. The morphological differences imply a division of biosynthetic function. We may conclude that artemisinin is biosynthesized in the two apical cells of the GSTs and it is highly likely that other enzymes of artemisinin are also exclusively expressed in these cells. The progress in cloning enzymes of artemisinin biosynthesis has been slow even though a number of cDNA libraries from glandular trichomes have been used for this purpose. cDNA obtained from apical cells is enriched in transcripts encoding enzymes of artemisinin biosynthesis and may therefore be an excellent source for the cloning of these enzymes and to study the biosynthesis of this important antimalarial drug in an unmasked

environment. Furthermore, it is highly likely that the cells with photosynthetically active plastids provide energy and carbon source for terpenoid production in adjacent (apical) cells. Finally, the tissue preparation method developed in combination with the LMCP technique to isolate individual and intact cells from glandular secretory trichomes may be a valuable tool for gathering further insight into the (secondary) metabolism of different cells of multicellular plant trichomes.

4. Experimentals

4.1. Tissue preparation for immunolocalization

Young leaf primordia and the first developed leaves of *A. annua* were fixed, dehydrated and embedded gradually in LR White resin (Sigma Aldrich). Samples were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h in room temperature. Vacuum was applied during the early stage of fixation. The samples were dehydrated in a series of aqueous ethanol (30%, 50%, 70%, 90%, 95%: 30 min each step) and further dehydrated with two washes in 100% ethanol for 30 min followed by overnight incubation in 100% ethanol. They were then progressively infiltrated with LR White resin (LRW) by incubation in mixtures of pure LRW: 100% ethanol of 1:3 for 4 h, 1:1 for 4 h, 3:1 over night and 100% LRW for 24 h. The infiltrated samples were embedded in LRW and polymerized for 24 h at 58 °C. Semi-thin sections (1.25 μm) were cut from the LRW embedded samples with a Leica RM 2135 manual microtome and mounted on glass microscope slides.

4.2. Immunogold labeling and silver enhancement for light microscopy

The fixed and embedded sections were rinsed in PBS containing 1% BSA (PBS/BSA) followed by incubation in blocking solution of 5% normal goat serum in PBS/BSA in a wet chamber for 30 min. After washing with PBS/BSA the sections were treated with primary antibody (rabbit anti-serum raised against ADS; diluted 1:100) at 4 °C overnight. Controls were made using rabbit pre-immune serum. After washing with PBS containing 0.002% Tween-20 (PBST) (4 × 5 min), the sections were treated with secondary antibody solution, i.e. goat anti-rabbit IgG conjugated to 1 nm gold (BB International, Cardiff, UK), diluted 1:200 in PBST for 2 h at room temperature. The slides were washed with PBST (4 × 5 min) and with water (3 × 2 min) and dried at room temperature. Subsequently, the samples were treated with silver enhancer according to the manufacturer's instructions (BB International, Cardiff, UK). The sections were studied under a light microscope (Nikon Eclips E400) and the positive labeling was seen as a dark brown precipitate due to silver deposition one the gold particle of the secondary antibodies. Finally the sections were counterstained with paragon and mounted with Mountex (HistoLab Products AB, Gothenburg, Sweden) and documented.

4.3. Tissue preparation for laser microdissection

A. annua L. were grown 12 h days and 12 h nights at 22 °C to a height of ~1 m followed by flower bud induction at 8 h days and 16 h nights at 22 °C. Slices of leaves (around 5 mm in width) and flower buds were fixed in 4% phosphate buffered formaldehyde (PFA) (HistoLab Products AB, Gothenburg, Sweden) and subjected to two rounds of 15 min vacuum to assist sinking and infiltration for 12 h at 4 °C. The tissue was finely cut with a razor blade to 0.5–1 mm in thickness and spread on a SuperFrostPlus microscope slide (Menzel-Gläser, Germany) in a drop of PFA. Prior to microdissection, a radom search for trichomes suitable for microdissection of apical and sub-apical cells were localized, i.e. trichomes perpendicular to the laser beam.

4.4. Fluorescence microscopy

The tissue was prepared as above and all micrographs were captured using a Nikon e400 C-SHG1 fluorescence microscope equipped with a digital camera, using filter settings for FITC (l_{ex} = 480 nm; l_{em} = 535 nm), Texas Red (l_{ex} = 570 nm; l_{em} = 625 nm) and UV. Autofluorescence was separated in the red and green channels and brightness adjusted using NIS-elements imaging software v. 2.20 (Nikon, Badhoevedorp, The Netherlands).

4.5. Laser microdissection pressure catapulting (LMPC)

The P.A.L.M® MicroLaser System fitted with a P.A.L.M® RoboMover (PALM, Wolfratshausen, Germany) was used to microdissect the fixed tissue directly from the SuperFrostPlus microscope slide. The laser consisted of a 337-nm nitrogen laser coupled with the light path of an inverted light-microscope (Axiovert 135; Carl Zeiss, Oberkochen, Germany) and focused through a 10× or 40× dry objective lens. The RoboMover is a computer-controlled microscope stage and micromanipulator. The laser was laterally fixed and object positioning was achieved by horizontal movement of the microscope stage. For microdissection the laser energy was adjusted for each individual cut by observing the laser's interaction with the tissue. The area around each cell(s) was cut until the target cells were completely separated from the neighboring cells. The neighboring cells were examined for disruption or other damage before catapulting of the target cells. For catapulting the energy level of the laser was set to about twice of that used when cutting and focused slightly below the target cells. One single laser pulse ejected the cells into the cap of a 0.65 ml microcentrifuge

Table 1
Primers used in PCR of transcripts from apical or sub-apical cell or whole trichomes of *A. annua* GSTs. FPPS: farnesyldiphosphate synthase; ADS: amorpha-4,11-diene synthase; CYP71AV1: amorphadiene-12-hydroxylase; AAR: artemisinic aldehyde Δ11(13) reductase; eF1α: elongation factor 1α.

Primer	Primer sequence (5′–3′)	Annealing temperature (°C)	Size of fragment (bp)
FPPS-F	CTTTAAGTGCTCCTGGTTAGTTGTC	54	
FPPS-R	AGGTGATCAGCTTCTTGTAACTTGT	54	191
ADS-F	AATGGAGCGTTCAAGCAA	50	
ADS-R	CTCTTCCTTGTGCAATGA	50	339
CYP71AV1-F	AACGGAGATTGTGAAAGAGATACTG	50	
CYP71AV1-R	TCTCGTTAGTTTTACTGGAGGTGGT	50	194
AAR-F	TACTAGAGGTGGTTGAAGACTATCG	50	
AAR-R	TCTAATACCAACTCGGTCTGTACC	50	232
DXR-F	CAGATTGTGTTACTGTTGTCACAGG	55	
DXR-R	GGTCAAGATAATACGCCTTAGAGC	55	247
*e*F1α-F	CTGTAACAAGATGGATGCCACTAC	55	
*e*F1α-R	CAGTCAAGGTTTGTGGAACCT	55	176

tube containing 30 μl lysis buffer mounted 1–3 mm over the microscope slide. Apical cells, sub-apical cells and whole GSTs were dissected, isolated and catapulted. 1–4 cells were collected in each cap, centrifuged down into the tube and stored at −80 °C. Mesophyl cells were isolated by LMPC from young leaves. Great care was taken not to obtain any cells from glandular trichomes in the mesophyl cell preparation.

4.6. RNA extraction

RNA was extracted from aliquots of 15–30 cells of each cell type (apical, sub-apical, whole GSTs and mesophyl) using the RNA Nanoprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Each preparation was eluted in 20 μl elution buffer. The RNA was DNase treated using DNase I (Fermentas, St. Leon-Rot, Germany) to remove remaining genomic DNA.

4.7. First Strand cDNA Synthesis

RNA (15 μl) was reverse transcribed using RevertAid™ H Minus-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) primed with 0.5 μg oligo$(dT)_{18}$ primer. (2 μl RNA was used as template in a negative control for DNA contamination). RNA was removed from the first strand cDNA by RNase treatment using RNase H (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The cDNA preparation was cleaned using the PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

4.8. PCR

PCR was performed using specific primers according to Table 1. First strand cDNA (1 μl) was used as template and PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare) were used for the PCR reactions according to the manufacturer's instructions. For FPPS, CYP71V1 and AAR, the first PCR product (1–2 μl) was used for a second amplification using the same primers. *e*F1α was selected as a housekeeping control gene and the primers were based on the sequence from *Glycine max* (Bilyeu et al., 2003). Control PCR reactions were also performed with RNA as template to check for genomic DNA contamination. PCR cycling was performed at 94 °C (3 min), 40 cycles at 94 °C (30 s), various annealing temperatures (Table 1) (30 s), 72 °C (30 s or 2 min and 30 s when using primer 6) and final elongation at 72 °C (10 min). Reaction products (14 μl) were separated on standard 1.5% agarose gels. Gel and running buffer was 0.5× TBE (45 mM Tris–borate, pH 8.0, 1 mM EDTA) containing 5 μg/ml ethidium bromide. To verify the specificity of the PCR reactions all PCR products were excised from the gel, purified using the QIAquick Gel Extraction Kit (Qiagen), cloned (pGEM-T Easy Vector) and sequenced (MWG-Biotech AG, Ebersberg, Germany).

Acknowledgements

Professors Jonathan Gershenzon and Joe Chappell are acknowledged for valuable suggestions on this publication. The financial support from the Crafoord Foundation, the National Graduate School in Pharmaceutical Science and University of Kalmar is acknowledged.

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