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

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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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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

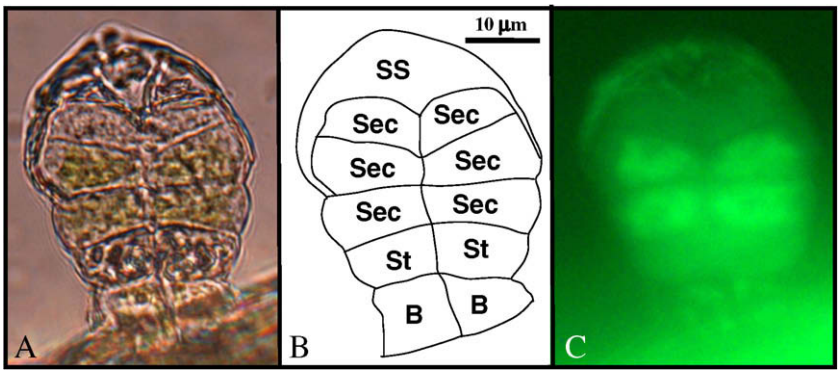

Fig. 1. (A) Glandular secretory trichome of *A. annua*. (B) Schematic drawing showing the different cells; B: basal cells; St: stalk cells; Sec: secretory cells; SS: subcuticular space. (C) Autofluorescence of chloroplasts in sub-apical secretory cells with the FITC filter (λ_{ex} = 480 nm; λ_{em} =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