

Capitate glandular trichomes of *Helianthus annuus* (Asteraceae): ultrastructure and cytological development

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Abstract Previous studies have shown that capitate glandular trichomes (CGT) of the common sunflower, *Helianthus annuus*, produce sesquiterpene lactones (STL) and flavonoids, which are sequestered and accumulated between the apical cuticle and the wall of the tip cells. To explore the cellular structures required and putatively involved in the STL biosynthesis and secretion, the present study was focused on the development of CGT and the comparison of the ultrastructure of its different cell types. Gradual maturation of flowers in the capitulum of the sunflower provided the possibility to study the simultaneous differentiation from the primordial to the secretory stage of CGT located by light microscopy (bright field, differential interference contrast and fluorescence) as well as transmission electron microscopy. It was shown that the CGT of sunflower anthers had a biseriate structure with up to 14 cell pairs. In mature trichomes, the apical cells called secretory cells were covered entirely by a large cuticle globe, which enclosed the resinous terpenoids and was specialised in thickness and structure. The secretory cells lacked chloroplasts and contained mainly smooth endoplasmic reticulum (sER). Conspicuous cell wall protuberances and an accumulation of mitochondria nearby occurred in the horizontally oriented cell walls. The cytological differences between stalk cells and secretory cells indicate a different function. The dominance of sER suggests its involvement in STL biosynthesis and cell wall protuberances enlarge the surface of the plasmamembrane of secretory cells and may be involved in the secretion processes of STL into the subcuticular space.

Keywords Sunflower · Light microscopy · Transmission electron microscopy

Introduction

Trichomes are widespread epidermal structures on vegetative and reproductive organs of plants providing a multitude of functions for ecological interactions and the protection against physical or biological stress (Wagner 1991; Spring 2000; Werker 2000). Within the Asteraceae, a characteristic type of capitate glandular trichome (CGT) is frequently occurring and is known to sequester predominantly terpenoids, flavonoids and other resinous plant metabolites into a subcuticular space formed around the trichome tip. In the past, numerous studies had focused on the chemical identification of the metabolites in CGT and particularly on the bioactive and bitter tasting sesquiterpene lactones (STL) of which more than 5,000 different structures were characterised to date (Seaman 1982; and reviews by Fraga 1987, 2011). In contrast, relatively few investigations were performed dealing with the ultrastructure and development of CGT in Asteraceae. Stahl (1956) was among the first authors who characterised the CGT on the surface of *Achillea millefolium* L. flowers and described their biseriate nature, their secretory activity and their senescence after the cuticular globe has been completed. A more recent study on *A. millefolium* provided insights in the early stages of trichome development and advanced microscopical techniques such as scanning (SEM) and transmission electron microscopy (TEM) revealed cytological details which allowed to suggest functional differentiation in stalk and secretory parts of CGT (Figueiredo and Pais 1994). Although further studies on *Vernonia galamensis* Gilbert (Favi et al. 2008), *Stevia rebaudiana* (Bert.) Bert (Monteiro et al. 2001) and *Artemisia annua* L. (Duke and Paul 1993) reported similar features for CGT of other Asteraceae, some characteristic differences in the mode of cell division, the presence of plasmodesmata and crystals or the morphology of plastids became visible. The investigation of *Sigesbeckia jorullensis* Kunth (Heinrich et al. 2010) showed that the structure of CGT might differ even within the same plant. Similarly, the

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common sunflower *Helianthus annuus* L. also harbours two different types of glandular trichomes: the uniseriate linear glandular trichomes (LGT), which produce bisabolene type sesquiterpenes (Spring et al. 1992; Göpfert et al. 2010) and the typical biseriate CGT (Spring and Bienert 1987). The latter follows the abovementioned concept of secreting bioactive STL (Spring et al. 1989; Hausen and Spring 1989) into a cuticular globe at the trichome tip. However, they consist of more than the typically reported 10 cells and produce flavonoids in addition to the terpenoids (Göpfert et al. 2005)

The paucity of studies in Asteraceae focusing on the early development of CGT and their cellular ultrastructure is owed to the fact that the formation of the trichomes generally starts extremely early in the ontogeny of the plant organ on which they are located. Primordial of trichomes in *S. rebaudiana* were found on immature leaves in the apical bud (Monteiro et al. 2001). In sunflower, early stages of CGT were also observed in the plant apex (Spring and Bienert 1987), but a much better site to study their development was found in the premature capitulum, where CGT on anther appendages of disc flowers gradually developed from the central florets to the margin. Chemical analysis of the CGT metabolites combined with expression studies of enzymes involved in the STL biosynthesis allowed the differentiation of CGT in a presecretory, secretory and post-secretory stage (Göpfert et al. 2005). Hence, the young capitulum of sunflower appeared to be a suitable model for a detailed cytological investigation of CGT. For the current study, we dissected disc florets harbouring trichomes in a presecretory and secretory stage in order to unravel the CGT development from the unicellular primordium to the full differentiation where metabolites are sequestered into the subcuticular globe surrounding the trichome tip. Special emphasis was put on the cellular ultrastructure where TEM investigation was employed to compare the organisation between the basal cells, the stalk cells and the secretory cells of sunflower CGT.

Materials and methods

Plants of *H. annuus* genotype HA300 were grown for 8 weeks under greenhouse conditions with a photoperiod of 18-h light and a constant temperature of 21 °C. For microscopy studies, young sunflower capitula, about 2.5 cm in diameter (ray flowers not yet visible) were divided into halves and disc flowers of different maturity were excavated to gain the trichome-harboring anthers. The capitula at that stage had an average of 12 rows of disc flowers from the centre to the margin. For developmental investigations, florets of rows 2, 5, 7 and 12 were used.

Fresh trichomes were removed from the anthers with a lancet and investigated using bright-field and differential interference contrast (DIC). For the accurate determination of the

number of cells in one trichome, a nuclear staining with the fluorochrome Hoechst 33258 (1 µg/ml in 5 % Tween 20 over night at 4 °C; Filter 2, 365 nm/420 nm, Zeiss, Göttingen, Germany) was applied. For investigation of chlorophyll distribution, untreated CGTs were observed with Filter 5, 395 nm/440 nm (Zeiss, Göttingen, Germany).

Single anthers were fixed in 2.5 % (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate buffer (pH 7.2) for 1 h. After three washing steps in buffer, a second fixation in 1 % osmiumtetroxide, buffered with 0.1 sodium phosphate buffer (pH 7.2), followed. For dehydration, the progressive lowering of temperature method was applied (1.5 h in 30 % ethanol at 0 °C; 1.5 h in 50 % ethanol at -20 °C; over night in 70 % ethanol at -35 °C; 1.5 h in 100 % ethanol at -35 °C). After warming to RT, the samples were embedded in LR-White Resin (Science Service, Munich, Germany). The infiltrated samples were transferred to gelatine capsules and polymerized at 55 °C for 24 h.

For light microscopy, semithin cross sections (1,500 nm) of anthers were produced with an ultratom (Ultracut UCT, Leica, Wetzlar, Germany) using glass knives. Sections were stained for 45 min in 0.05 % aqueous toluidine blue (Merck, Darmstadt, Germany). For transmission electron microscopy, ultrathin sections were prepared using a diamond knife (Drukker International; Cuijk, Netherlands). Sections were collected on Pioloform-carbon-coated copper grids, stained with uranyl acetate for 15 min, followed by lead citrate for 60 s, and investigated in a transmission electron microscope (EM 10, Zeiss, Oberkochen, Germany) at 60 kV. Fresh trichomes and semithin sections were investigated with the light and fluorescence microscopes Axioplan (Zeiss, Göttingen, Germany) coupled to a digital camera (Canon, Powershot 650). EM negatives were scanned (Epson Perfection 2450 Photo). Brightness and contrast of all images were adjusted using Photoshop CS2 (Adobe).

Results

Trichome development

The earliest developmental stage of CGT from sunflower was found on anthers of the youngest disc flowers in the middle of capitulum (row 2). Together with CGT of flowers from row 5, they were in the presecretory phase. Older flowers of rows 7 and 12 generally contained CGT in the phase of STL secretion as was visible by the formation of a cuticular globe at the trichome tip. Within florets of row 5 and older, the CGT of an anther were all nearly in the same developmental stage. Only in the youngest flowers, a gradient of maturation was observed between older CGT at the tip of anther appendages to younger ones at the base.

The CGT development started with the expansion of single epidermal cells with a large central nucleus, and prominent nucleoli were observed (Fig. 1a). The cells of such trichome initials had dense cytoplasm with smooth endoplasmic reticulum (sER), some short cisternae of rough endoplasmic reticulum (rER), Golgi stacks, small vacuoles, numerous mitochondria, and proplastids, which sometimes contained starch grains (not shown). After the first anticlinal division (Fig. 1b, white arrow), a series of periclinal divisions from both of the resulting cells followed. A displacement observed between the horizontal cell walls of the cell pairs indicated that the divisions of both basal cells were independent of each other (Fig. 1c). At this stage, the vacuoles increased in size and some contained small crystals (Fig. 1d). The thickness of the cuticle around the apical cells also increased (Fig. 1d, black to white arrow) and plastids contained some membrane elements.

At the beginning of the secretory phase of CGT, one or two separated cuticle globes were formed on top of each of the two apical cells (Fig. 2a). Trichomes at this stage of development had numerous crystals and a differentiation between stalk and secretory cells became obvious. Two (Fig. 2a) to four (Fig. 2b) of the apical cell pairs appeared transparent in LM investigations when compared to the subjacent cells, which showed green colouration due to the maturation of chloroplasts. This

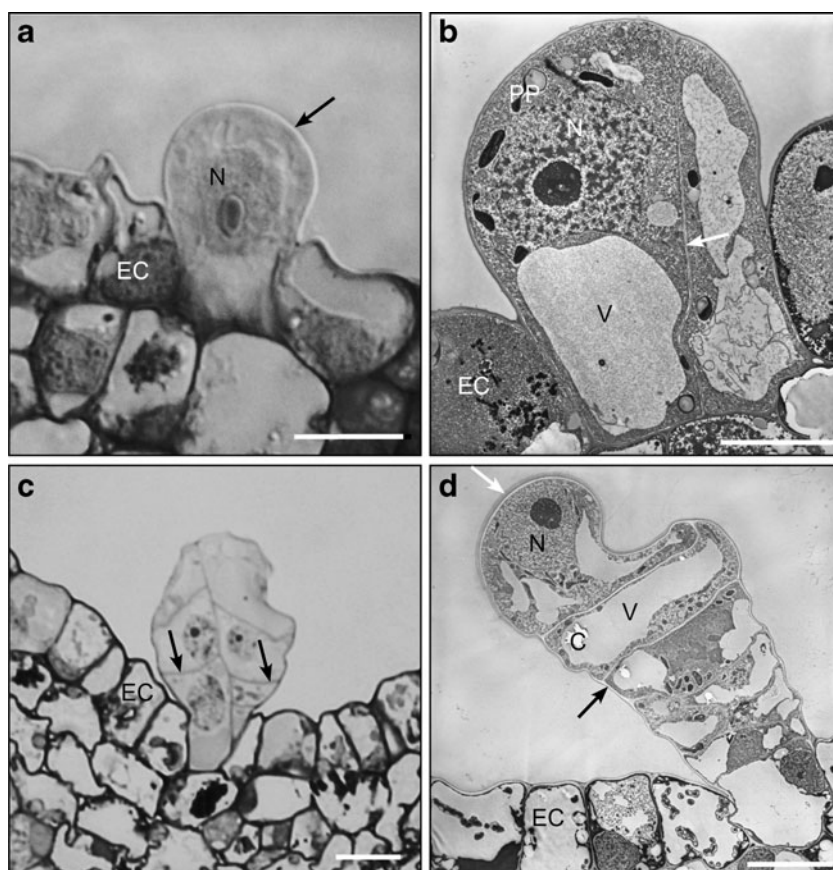
marked the border between the stalk cells and the secretory cells. The expansion of the glands progressed when the two single globes fused to one large cuticle globe, which finally covered the whole surface of the secretory cells (Fig. 2b).

Ultrastructure of the mature trichome

The mature gland had a biseriate structure and consisted of two to four pairs of secretory cells, five to seven pairs of stalk cells at maximum and one pair of basal cells (Fig. 2c). All cell types had large central nuclei (Fig. 2d) and vacuoles with large crystals. The basal cells differed from surrounding epidermal cells by the presence of a few chloroplasts (data not shown). The thickness of the cuticle remained constant at about 130 nm from the trichome basal cells to the regular epidermal cells (Fig. 1b, c).

The stalk cells showed the same cellular structures as the basal cells, but had more chloroplasts (Fig. 2e) and there seemed to be an increasing ratio of sER towards the secretory cells (Fig. 3). All cells showed clear vacuoles and some of them contained crystals. The horizontal cell walls of the uppermost stalk cells and the secretory cells showed conspicuous protuberances. This typical feature of secretory cells was most characteristic for the two uppermost cell layers (Fig. 4), which later on got covered by the cuticular globe. The thickness

Fig. 1 Light- and transmission electron micrographs showing the early stages of development of capitate glandular trichomes of *Helianthus annuus*: **a** Light microscopy (DIC) of a Toluidine Blue stained section; **c** Light microscopy (bright field) of Toluidine Blue stained section; **b**, **d** Transmission electron microscopy; **a** expansion of a single epidermal cell (arrow) with a central nucleus (N), EC=epidermal cell, scale bar=10 μ m; **b** two cell stage after the first anticlinal division (arrow), N=nucleus, V=vacuole, PP=proplastid with starch grain, EC=epidermal cell, scale bar=5 μ m; **c** six cell stage after periclinal divisions, the cells of one pair divided probably independent of each other (arrows), EC=epidermal cell, scale bar=10 μ m; **d** early stage of cell specialisation: increase in cuticle thickness (black arrow to white arrow) and small crystal (C) in the vacuole (V), N=nucleus, EC=epidermal cell, scale bar=10 μ m



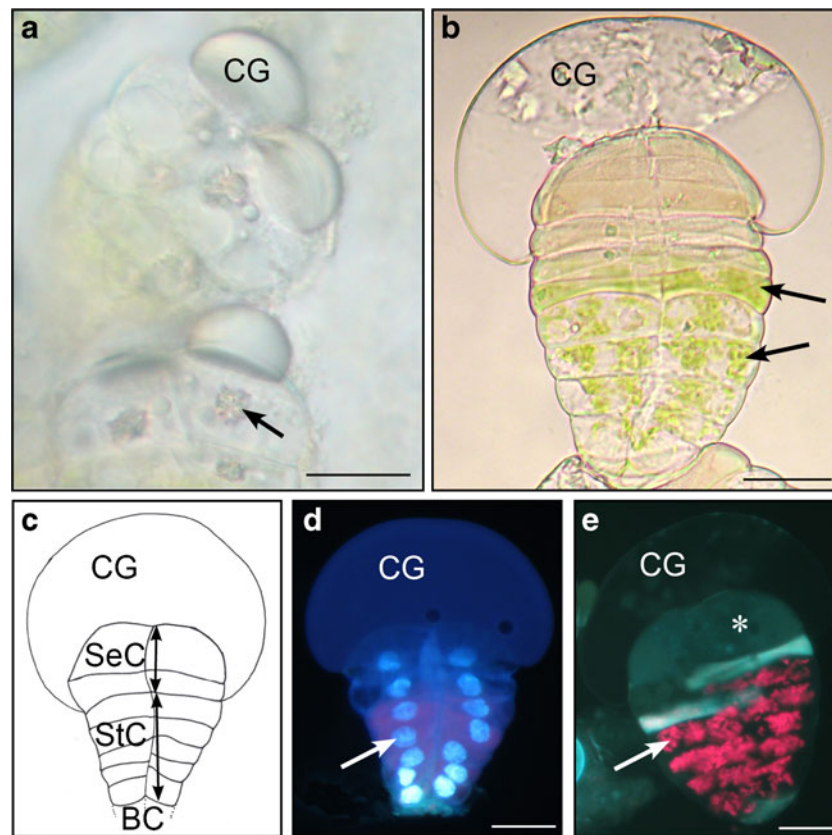


Fig. 2 Light- and fluorescence micrographs showing later stages of trichome development from capitate glandular trichomes (CGT) of *H. annuus*; **a** light microscopy (DIC) of untreated CGT; **b** light microscopy (bright field) of untreated CGT; **c** fluorescence microscopy of CGT stained with Hoechst 33258; **d** fluorescence microscopy of untreated CGT; **e** CGT at the beginning of STL secretion into a single cuticle globe (CG) at the top of each apical cell, *arrow* shows crystal, *scale bar*=20 μ m;

b mature CGT with completely developed cuticle globe (CG), chloroplasts are only abundant in the stalk cells (*arrows*), *scale bar*=20 μ m; **c** cytological scheme of a CGT consisting of a cuticle globe (CG), secretory cells (SeC), stalk cells (StC) and basal cells (BC); **d** mature CGT with large central nuclei (*arrow*) demonstrate the biseriate structure, *scale bar*=20 μ m; **e** mature CGT showing red chlorophyll fluorescence (*arrow*) only present in the stalk cells, secretory cell (*asterisk*), *scale bar*=20 μ m

of the cuticle increased from the basal cells along the stalk cells and reached up to 340 nm (Fig. 1d, 4). Starting from the distal end of the top cell layer, the cuticle detachment from the cell wall progressed lateral when secretion of STL continued and

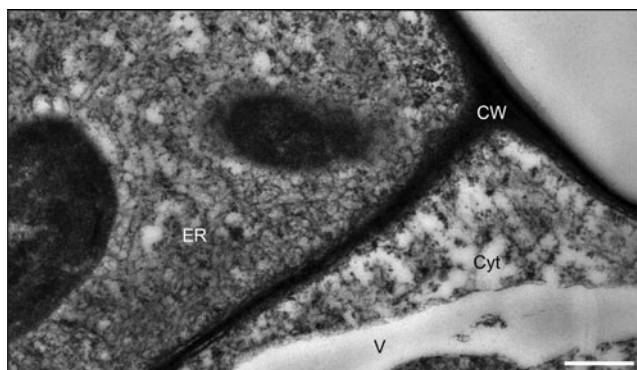


Fig. 3 Transmission electron micrograph showing a transition from a stalk cell with abundant ER (ER) to a stalk cell with mainly cytoplasm (Cyt), V=vacuole, CW=cell wall, *scale bar*=1 μ m

required space for their accumulation (Figs. 4 and 2b). At that stage, the cuticle above the secretory cells reached a relatively constant thickness of up to 1 μ m and no pores or micro channels were observed. The inner side of the cuticle was more electron-dense than the outer part (Fig. 4). In younger trichomes, a thin electron dense layer between the cell wall and the cuticle was observed as well (Fig. 5). In the region of the first detachment of the cuticle, a thickening of the cell wall from about 130 nm to 500 nm was observed (Fig. 4). There were no plasmodesmata detected in the whole CGT. The secretory cells had numerous mitochondria, often near the protuberances, and the cytoplasm consisted mainly of a network of tubular sER (Fig. 6a), but also contained some regions with rER (Fig. 6b). Occasionally, they also had lipid bodies of variable sizes (Fig. 4). The plastids of the two apical cells were leucoplasts, containing globuli with electron dense material (Figs. 4 and 5). In addition, leucoplasts with internal membrane systems (Fig. 4) but lacking chlorophyll (Fig. 2e) were observed in secretory cells.

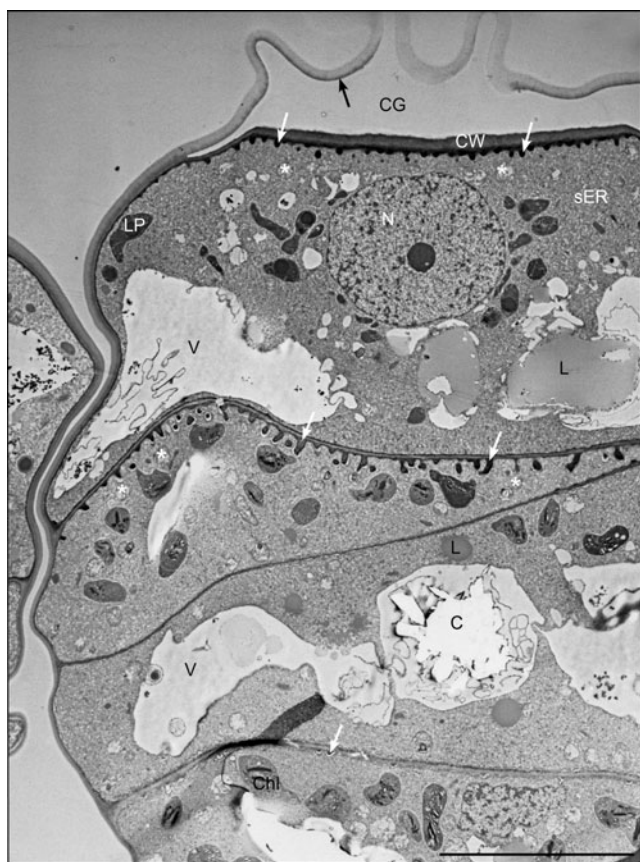


Fig. 4 Transmission electron micrograph showing the apical part of a CGT in the secretory stage. The apical cell has leucoplasts with electron-dense globuli (LP) and numerous mitochondria (white asterisks) near the protuberances (white arrows). The cuticle formed a subcuticular space (CG) and shows an electron-dense layer at the inner side (black arrow). Protuberances (white arrows) are most pronounced in the two apical cells. The subjacent cells show only few small ones. Smooth endoplasmic reticulum (sER) is well developed in every cell, CW=cell wall, N=nucleus, L=lipid body, V=vacuoles, some contain crystals (C), Chl=chloroplast, scale bar=10 μ m

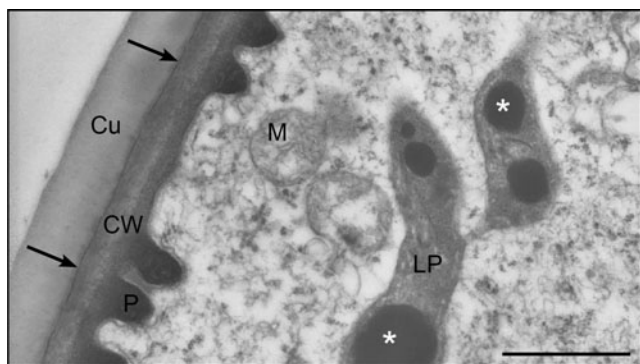


Fig. 5 Higher magnification of a TEM micrograph showing the cell wall (CW) of a secretory cell with protuberances (P) and the electron-dense layer (black arrow) between cell wall and cuticle (Cu), LP=leucoplasts with electron-dense globuli (asterisks), M=mitochondrion, scale bar=1 μ m

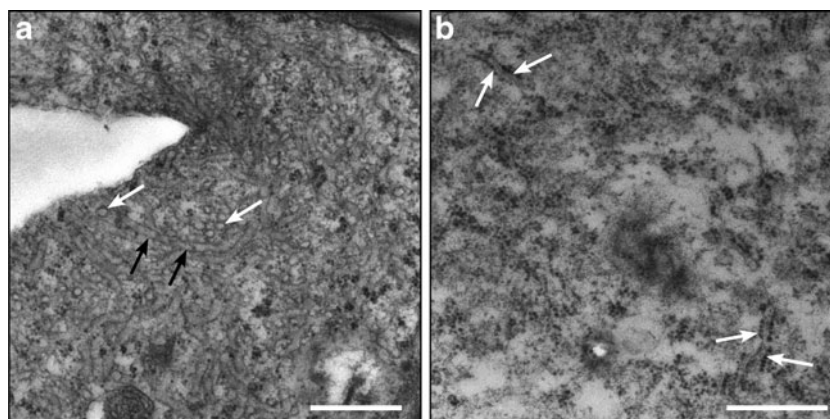
Discussion

The investigation of CGT of sunflower showed many characteristics typical for the biserial type of glandular hairs in certain other genera of the Asteraceae (Hellwig 1992). Unlike in *Achillea* (Figueiredo and Pais 1994), *Stevia* (Bert.) Bert (Monteiro et al. 2001) and *Artemisia* (Duke and Paul 1993), sunflower CGT developed very early during the formation of the plant organs. Unlike in *Vernonia* (Favi et al. 2008), we could never observe mature and immature trichomes simultaneously on the same floret or leaf. Only on the youngest flowers, a slight gradient of maturation was found with primordial stages more prevalent at the basis of the anthers and further developed, but still presecretory stages located towards the tip.

The development of the CGT in *H. annuus* was quite similar to those of *A. annua* (Duke and Paul 1993). After the first anticlinal division of a single, prolonged epidermal cell (trichome primordium) only periclinal divisions followed, thus forming the biserial type of CGT typical for Asteraceae. But contrary to the trichomes of *A. annua*, *S. rebaudiana* and *V. galamensis*, the differentiation of CGT in sunflower deviated from a common concept in terms of cell divisions and functional aspects of the cells. In the aforementioned taxa, the number of periclinal divisions was strictly limited to four, thus providing structurally homogenous CGT consisting of 10 cells (five cell pairs), two of which functioning as basal cells, two as stalk cells and six as secretory cells. In sunflower CGT, we found up to 11 divisions and a maximum of 12 cell pairs. As a consequence, the number of stalk and secretory cells increased.

The differentiation of the CGT into these three cell types was suggested by Duke and Paul (1993) who defined the three apical cell pairs of glands of *A. annua* as secreting cells because they bordered the subcuticular space. Whether secretion really occurs on the cell wall of all three cell layers was not shown yet and may be questioned, because the cell wall protuberances, typical for secretory cells, predominantly occurred on the distal cell wall of CGT in *A. annua*. In sunflower, such protuberances were found in at least the upper two cell layers and to a lesser degree also in the horizontal walls of subjacent cell rows. A clear distinction in the cell layers of sunflower CGT was the presence or absence of chloroplasts. While the cells bordering the cuticular globe contained only leucoplasts, the subjacent cell layers contained fully developed chloroplasts. According to this observation, we suggest to define the chloroplast-containing cells as stalk cells. This is in contrast to *A. annua*, where chloroplasts occurred up to the subapical cell layer (Duke and Paul 1993). It had been suggested that the chloroplasts are involved in the artemisinin biosynthesis (Duke et al. 1994). But experiments involving laser capture microdissection showed that the first step from FPP to dihydroartemisinin aldehyde could be carried out in the apical cell pair without

Fig. 6 Transmission electron micrographs showing ER formation in cells of a CGT in the secretory stage; **a** network of tubular sER of a secretory cell, single cisterna with cross sections (*white arrows*) and longitudinal sections (*black arrows*), *scale bar*=1 μ m; **b** mixed ER of a secretory cell with the typical ribosome formation of rER (*white arrows*), *scale bar*=1 μ m



any chloroplasts present (Olsson et al. 2009). Although it was previously shown that light can influence the biosynthetic activity of sunflower CGT (Spring et al. 1986), a direct involvement of chloroplasts in the biosynthesis of STL is rather unlikely. Although the incorporation of isopentenyl pyrophosphate (IPP), derived from the plastidal methylerythritol phosphate pathway could be shown in chamomile sesquiterpenes (Adam et al. 1999), there is no general doubt in the cytoplasmic localisation of the sesquiterpene biosynthesis depending on mevalonate-derived IPP (Lichtenthaler et al. 1997).

The dominant compartment of all CGT cells of sunflower was the tubular smooth ER. This supports the assumption that the MVA pathway is involved in the synthesis of STL and that the recently identified key enzymes of the STL biosynthesis could be associated with the sER (Göpfert et al. 2009; Nguyen et al. 2010; Ikezawa et al. 2011). This corroborates with results of Gleizes et al. (1980) who detected labelled sesquiterpene hydrocarbons in membrane fractions of isolated ER. An extensive network of sER was also described in glands of *A. millefolium* (Figueiredo and Pais 1994), *Helichrysum aureonitens* (Afolayan and Meyer 1995), *A. annua* (Duke and Paul 1993) and *S. jorullensis* (Heinrich et al. 2010). However, independent of the plant family or the metabolites produced, most glands were shown to possess abundant sER (Schnepf 1972; Ascensão and Pais 1998; Sacchetti et al. 1999; Turner et al. 2000). Hence, a final proof for the involvement of the sER in STL biosyntheses will require immunocytological techniques using antibodies for the characterised enzymes.

The transport of metabolites from the secretory cells into the cuticle globe could not be resolved completely. The cell wall protuberances observed in the apical cell layers of sunflower CGT are typical for transfer cells, which have secretory or absorbing functions. Transmission electron microscopy showed also that the cell wall protuberances were more electron-dense than other cell wall areas. Similar wall protuberances were observed in cells of trichomes from *L. clandestina* (Schnepf 1964) and *A. annua* (Duke and Paul 1993) and often correlated with a high number of mitochondria in their neighbourhood supporting the idea of energy-consuming

transport processes along the plasmamembrane of cell wall protuberances. Alike in CGT of the other Asteraceae, the secretory process in sunflower CGT started with the detachment of the cuticle from the cell wall of the uppermost cell layer and subsequently proceeded along the other secretory cells. In *A. millefolium*, plasmodesmata were found between the cell layers, which could account for a cell-to-cell transport of metabolites to the site of secretion (Figueiredo and Pais 1994), but no such structures were detected in CGT of sunflower. For that reason, it remains undisclosed, whether the subapical cell layers secrete laterally at a later stage of trichome maturation or transfer their synthesised metabolites via exo-/endocytosis into the tip cells.

The detached cuticle of CGT has the function of accommodating the predominantly hydrophobic metabolites of sunflower. The release of STL into the environment normally results through mechanical rupture of the cuticle. No structures like pores or micro channels were observed. To withstand the mechanical stress from an increasing accumulation of compounds, the cuticle of sunflower CGT showed a considerable thickening in those parts, which were involved in the formation of the globe. A constant setup of the cuticle was similarly detected in glands of *Cannabis sativa* (Mahlberg and Kim 1991) and seems to be a prerequisite to prevent premature disruption. In contrast to *S. jorullensis* (Heinrich et al. 2010) and peppermint (Turner et al. 2000), where a reduction of thickness of the cuticle was observed during globe formation, the thickness of the cuticle in sunflower CGT remained constant. This suggests a putative additional setup of cuticle layers during the expansion of the globe. This assumption was supported by the detection of an electron-dense layer on the inner side of the cuticle. A similar structure was described in studies on trichomes of *Lathraea clandestina* (Schnepf 1964) and *C. sativa* (Hammond and Mahlberg 1978; Mahlberg and Kim 1991) and it was supposed that this layer consists of pectin, which is lysed by enzymes for detachment of the cuticle. We assume that the hydrophilic pectin layer at the inner side of the cuticle globe of sunflower CGT might have an important function in preventing the lipophilic STL from diffusing through the cuticle.

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Conflict of interest The authors declare that they have no conflict of interest.

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