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Sub-cellular trafficking of phytochemicals explored using auto-fluorescent compounds in maize cells

Yakang Lin, Niloufer G Irani and Erich Grotewold*

Address: Department of Plant Biology and Plant Biotechnology Center, The Ohio State University, Columbus, OH 43210, USA

Email: Yakang Lin - ykl8@yahoo.com; Niloufer G Irani - irani.8@osu.edu; Erich Grotewold* - grotewold.1@osu.edu

* Corresponding author

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Abstract

Background: Little is known regarding the trafficking mechanisms of small molecules within plant cells. It remains to be established whether phytochemicals are transported by pathways similar to those used by proteins, or whether the expansion of metabolic pathways in plants was associated with the evolution of novel trafficking pathways. In this paper, we exploited the induction of green and yellow auto-fluorescent compounds in maize cultured cells by the PI transcription factor to investigate their targeting to the cell wall and vacuole, respectively.

Results: We investigated the accumulation and sub-cellular localization of the green and yellow auto-fluorescent compounds in maize BMS cells expressing the PI transcription factor from an estradiol inducible promoter. We established that the yellow fluorescent compounds accumulate inside the vacuole in YFBs that resemble AVIs. The green fluorescent compounds accumulate initially in the cytoplasm in large spherical GFBs. Cells accumulating GFBs also contain electron-dense structures that accumulate initially in the ER and which later appear to fuse with the plasma membrane. Structures resembling the GFBs were also observed in the periplasmic space of plasmolyzed cells. Ultimately, the green fluorescence accumulates in the cell wall, in a process that is insensitive to the Golgi-disturbing agents BFA and monensin.

Conclusions: Our results suggest the presence of at least two distinct trafficking pathways, one to the cell wall and the other to the vacuole, for different auto-fluorescent compounds induced by the same transcription factor in maize BMS cells. These compartments represent two of the major sites of accumulation of phenolic compounds characteristic of maize cells. The secretion of the green auto-fluorescent compounds occurs by a pathway that does not involve the TGN, suggesting that it is different from the secretion of most proteins, polysaccharides or epicuticular waxes. The yellow auto-fluorescent compounds accumulate in a vacuolar compartment, in structures that resemble the AVIs present in many cells accumulating anthocyanins. Together, our studies suggest that the accumulation of auto-fluorescent compounds can provide a powerful tool to dissect the trafficking of phytochemicals, knowledge necessary for the efficient engineering of plant metabolism.

Background

Largely as a consequence of their sessile life styles, plants

accumulate hundreds of thousands of specialized compounds (phytochemicals), primarily derived from

secondary metabolism [1]. These phytochemicals are often synthesized in cellular compartments different from where they accumulate [2]. Many phytochemicals are secreted, either in a constitutive fashion, or in response to specific biotic or abiotic stress conditions. The secretion of phytochemicals has been best described for gland cells [3] and for roots [4]. In some cases, the secretion involves specific proteins, such as ABC transporters [5], responsible for example in the secretion of antifungal terpenoids in *Nicotiana plumbaginifolia* [6]. In other instances, vesicles participate in the transport of phytochemicals from the site of synthesis to the cell surface, as shown for the secretion of capsaicinoid vanillyl amides in placenta from red pepper [7], or for the secretion of epicuticular waxes by cork cells in sorghum [8]. In most cases, however, no mechanisms have been proposed to explain the transport of small molecules, often highly reactive or toxic, within cells, or even between cells, as observed in alkaloid biosynthesis in *C. roseus* [9].

In contrast to the ease by which the transit of fluorescence-tagged proteins is followed, the study of the pathways by which small molecules move from one sub-cellular compartment to another has been complicated by the difficulties associated with tracking these movements. Phytochemicals are difficult to observe within the cell using immunological techniques, and pigmented compounds such as the flavonoid-derived anthocyanins become colored only after they reach their final destination, in this case the vacuole or vacuole-like compartment [10]. The anthocyanin pigments accumulate in the central vacuole. As is the case for other phenylpropanoids and flavonoids, anthocyanins are likely to be synthesized on the cytoplasmic face of the endoplasmic reticulum (ER) [11,12]. The transport of anthocyanins to the vacuolar compartment may involve specialized glutathione *S*-transferases that serve as flavonoid carrier proteins [13,14]. More recently, the *tt12* gene from Arabidopsis was shown to encode a member of the MATE (multidrug and toxic compound extrusion) family of transporters involved in the sequestration of flavonoids to the vacuole of seed coat endothelium [15].

We have exploited the accumulation of two auto-fluorescent compounds induced by the *P1* transcription factor in Black Mexican Sweet (BMS) maize cells in culture [16] to start to dissect possible mechanisms by which small molecules traffic within plant cells. *P1* encodes a R2R3 MYB transcription factor that regulates the accumulation of 3-deoxy flavonoids in maize floral organs [17]. The expression of *P1* in BMS cells resulted in the accumulation of several flavonoids and phenylpropanoids [16]. *P1*-expressing BMS cells display a number of morphological changes, including a dilatation of the ER and the accumulation of electron-dense structures that appear to accumu-

late originally in the ER to then migrate to the cell surface to fuse with the plasma membrane [16]. *P1*-expressing BMS cells also accumulated an increased quantity of yellow- and green auto-fluorescent compounds targeted to the vacuole and cell wall, respectively [16].

Here, we provide a detailed characterization of the BMS cells accumulating green and yellow auto-fluorescent compounds after the expression of *P1*. These compounds accumulate in green and yellow fluorescent bodies (GFB and YFB respectively), which are targeted to the cell wall and vacuole, respectively. The YFBs accumulate in the large central vacuole in structures that resemble anthocyanic vacuolar inclusions (AVIs) [18]. We show that the secretion of the green auto-fluorescent compounds to the cell wall is not inhibited by agents that disrupt the *trans*-Golgi network (TGN). Together, our studies suggest that these auto-fluorescent compounds represent a powerful tool in dissecting the trafficking of phytochemicals, and indicate a possible novel secretion mechanism that does not involve the TGN network.

Results

Accumulation of green and yellow auto-fluorescent bodies in maize BMS cells

The large size of Black Mexican Sweet (BMS) maize cells provides a good opportunity to investigate the sub-cellular trafficking of phytochemicals. BMS cells expressing the *P1* R2R3 MYB transcription factor from the *CaMV* 35S promoter (35S::*P1*) accumulate yellow- and green auto-fluorescent compounds targeted to the vacuole and cell walls, respectively [16]. Because 35S::*P1* cells in culture tend to suppress the expression of the *P1* transgene after extended propagation, we investigated the trafficking and sub-cellular localization of the yellow- and green auto-fluorescent compounds in BMS cells expressing *P1* from an estradiol inducible promoter (ERE::*P1*, [19]). The addition of 10 μ M estradiol to ERE::*P1* BMS cells induces the accumulation of green- and yellow auto-fluorescent compounds, often co-existing in the same cell (Fig. 1).

The accumulation of the green auto-fluorescent compounds is first observed in the cytoplasm of ERE::*P1* cells 2–3 days after the addition of estradiol (Fig. 2A). Six to seven days after induction, a fraction of the cells starts showing green fluorescence on the cell surface. Interestingly, even after 9–10 days following the induction with estradiol, only 2% of the total cells in a culture contain green fluorescent compounds in either the cytoplasm or the cell surface (Fig. 2A). In contrast to the green auto-fluorescence, the yellow auto-fluorescent compounds are frequently found in cells not treated with estradiol (Fig. 2B), but the induction of *P1* expression increases the number of cells accumulating these compounds (Fig. 2B), as well as the amount of the yellow fluorescence in the cells. The

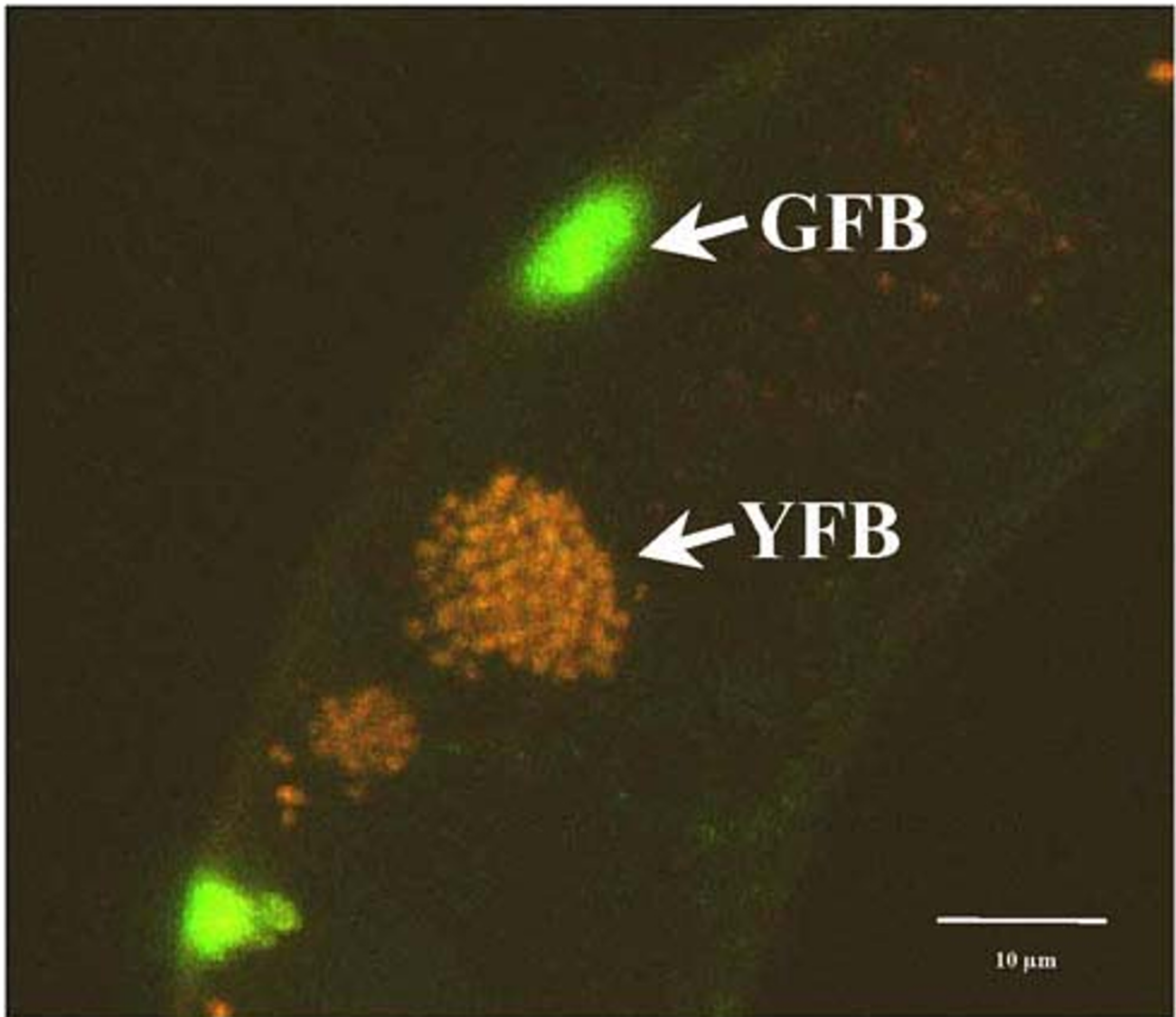


Figure 1

Accumulation of green and yellow auto-fluorescent compounds in maize cells expressing the *P1* gene. Confocal microscopy image of a maize BMS cell expressing the *P1* gene from the estradiol-inducible promoter (false colors in image). The green and yellow fluorescent bodies are indicated as GFB and YFB respectively.

fluorescent spectral properties of methanol extracts obtained from ERE::P1 BMS cells induced for 7 days were compared to those of control BMS cells not expressing P1. Maximum fluorescence was observed at 568 nm (in a rather broad peak, excitation 440 – 460 nm), consistent with the microscopic visualization of the green and yellow fluorescence with an FITC filter [16].

Accumulation of the yellow fluorescent compounds to the central vacuole

The yellow auto-fluorescent compounds accumulate in the central vacuole in the form of discrete structures (YFBs, yellow fluorescent bodies) (Fig. 3A,3B). The number of YFBs per vacuole is variable, with some vacuoles having just a few (Fig. 3A), while others can have 50 or more YFBs (Fig. 1). The yellow fluorescence can also be found in the cytoplasm in the form of small bodies that

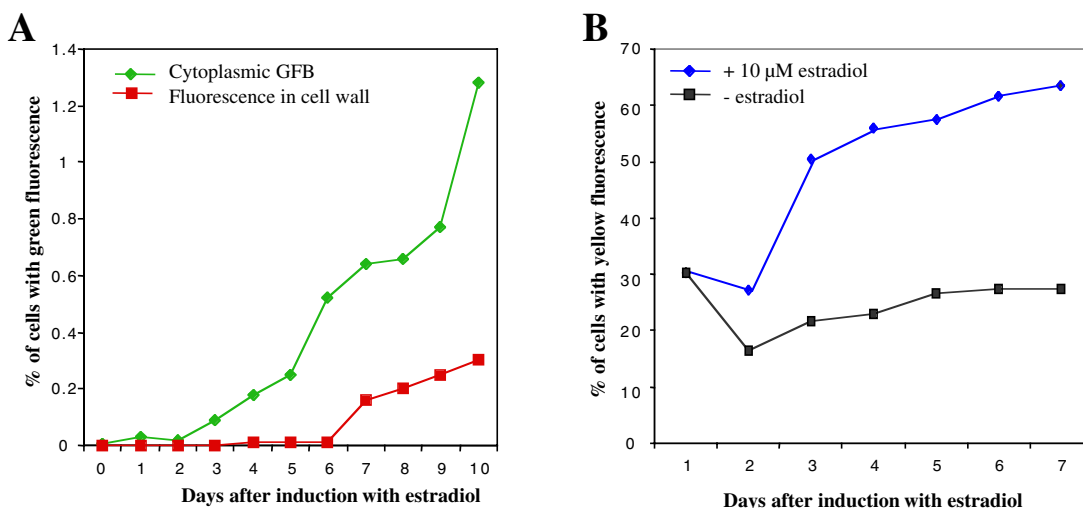


Figure 2

Accumulation of green and yellow auto-fluorescence in ERE::PI cells. (A) Accumulation of green fluorescence in ERE::PI cells after induction with estradiol. The y-axis indicates the percentage of cells with cytoplasmic GFBs (green line) or cell wall green fluorescence (orange line). (B) Accumulation of yellow auto-fluorescence in ERE::PI cells treated (blue line) or not treated (black line) with estradiol. The y-axis indicates the percentage of cells displaying yellow fluorescence.

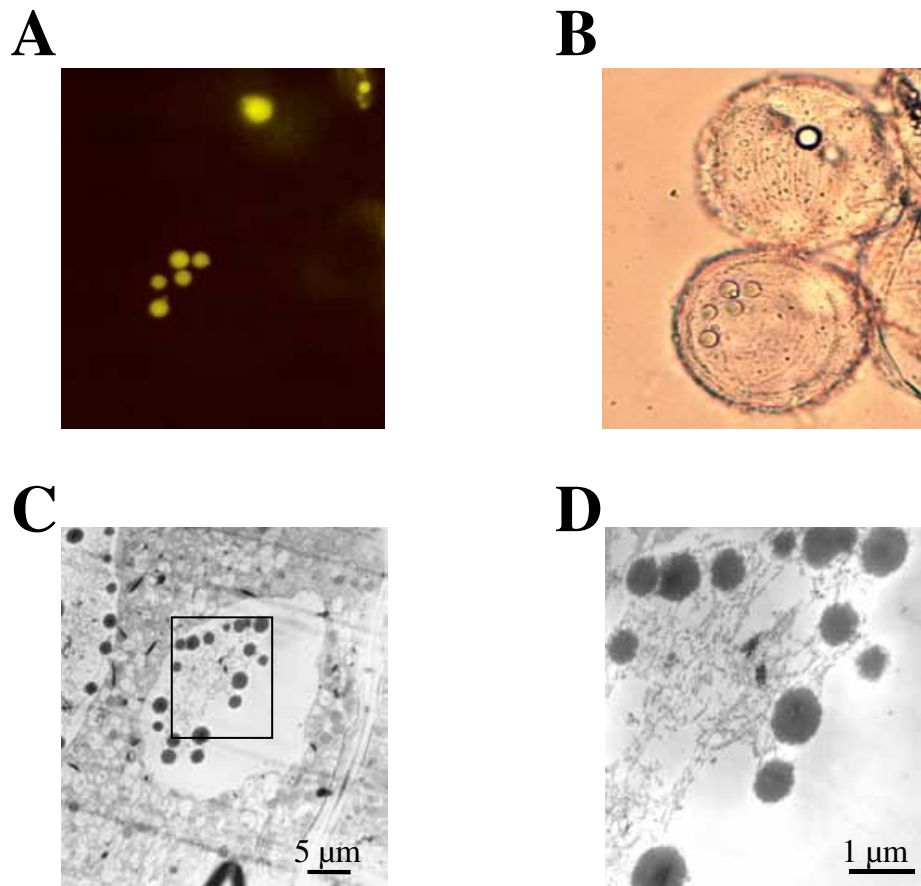
resemble vesicles (Fig. 1). Thus, it is tempting to speculate that the cytoplasm is the initial site of synthesis of the yellow auto-fluorescent compounds, which traffic to the vacuole through vesicle-like structures.

The accumulation of the YFBs in the vacuole is intriguing and resembles the anthocyanic vacuolar inclusions (AVIs) often found in the petals of many red and blue flowers [18]. Under the epifluorescence microscope, the YFBs have a regular boundary (Fig. 3A,3B). However, transmission electron microscope (TEM) micrographs show vacuolar electron-dense bodies of a size similar to the YFBs, yet with an irregular boundary (Fig. 3C,3D). While we sus-

pect that the fixing conditions might be responsible for the irregular boundary in the TEM micrographs, we cannot conclusively conclude from these findings whether a membrane encircles the vacuolar YFBs or not.

Transport of the green fluorescent compounds to the cell wall

Similar to the yellow fluorescent compounds, the green auto-fluorescent compound initially accumulates in the cytoplasm in the form of small bodies that resemble vesicles, which coalesce into very large GFBs (Fig. 4A,4B). At longer times, after the induction with estradiol, an increased number of cells accumulate GFBs close to the

**Figure 3**

Localization of the YFB to the vacuole. (A) Epifluorescence microscopy and (B) bright field image of a cell accumulating YFBs in the large central vacuole. (C) TEM micrograph showing YFBs in the central vacuole, and (D) corresponds to a higher magnification of the region in a box in (C).

cell wall (Fig. 4C), suggesting a migration of these structures towards the periphery of the cells. The green fluorescence observed in the cell walls (Fig. 4D) indicates that this is the ultimate destiny of the green auto-fluorescent compounds. Consistent with these observations, protoplasts of ERE::P1 cells induced for 7–10 days with estradiol accumulate GFBs in the cytoplasm, but lack green fluorescence in the cell surface (not shown). To further examine the accumulation of the green auto-fluorescent compounds in the cell surface, we took advantage of the rare examples of plasmolized cells normally present in BMS cultures. When we examined a large number of estradiol-induced ERE::P1 BMS cells, we frequently found

structures resembling the GFBs in the periplasmic space of plasmolized cells (Fig. 5A,5B).

The presence of GFBs coincides with the presence of ER-derived electron-dense bodies

To better determine the origin and the anatomy of the GFBs, cells accumulating these bodies were separated with a micromanipulator from a ERE::P1 BMS suspension culture induced for 4 days with estradiol. Cells were fixed and examined by transmission electron microscopy (TEM). Green fluorescing cells contained electron-dense spherical bodies (indicated with arrows in Fig. 6). These electron-dense bodies, absent in cells not accumulating GFBs, are found closely associated with structures that resemble the

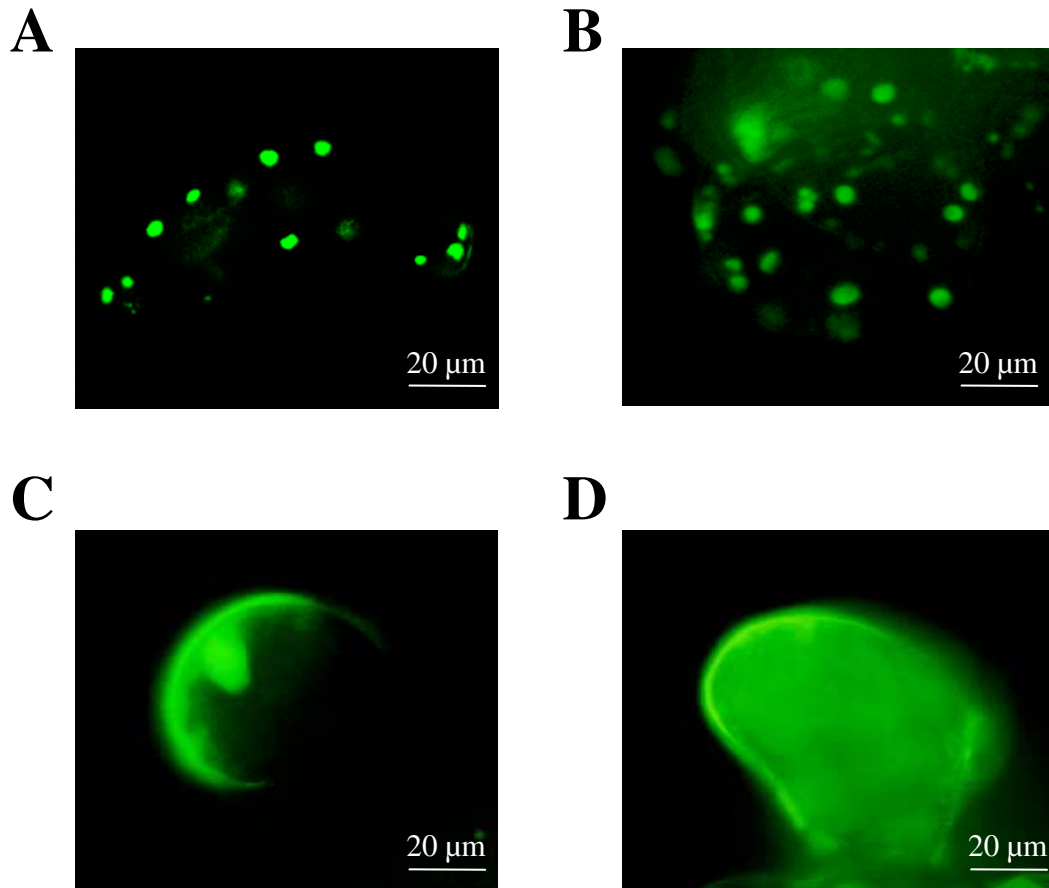


Figure 4

Localization of GFPs in ERE::P1 induced BMS cells. (A-D) Epifluorescence microscopy of cells present in ERE::P1 cells induced for 7–10 days with 10 μ M estradiol representing the accumulation of GFPs in the cytoplasm (A, B), their migration to the cell surface (C) and release of green fluorescence to the cell wall (D).

expanded ER (Fig. 6C) characteristic of BMS over-expressing P1, as well as close to the plasma membrane (Fig. 6E,6F). The size of these electron dense bodies rarely exceeds 300 nm (Fig. 6). Similar electron-dense structures were previously observed in 35S::P1 cells [16]. Contrary to the GFPs, which were sometimes observed in the periplasmic space, the electron-dense structures were found to fuse with the plasmalemma [16].

Effect of Golgi-disturbing agents on the secretion of the green fluorescent compounds

To determine whether the secretion of the green auto-fluorescent compounds involves the Golgi apparatus and the *trans* Golgi network (TGN), we investigated the effect of

Golgi-disturbing agents with respect to the accumulation and distribution of the green auto-fluorescent compounds present in induced ERE::P1 cells. Brefeldin A (BFA) blocks protein secretion by promoting the disassembly of the Golgi apparatus [20,21]. The treatment of plant cells with BFA results in the complete loss of vesicle formation from the TGN and the generation of ER-Golgi hybrid compartments [22]. We rationalized that, if the TGN were involved in the secretion of the green auto-fluorescent compound to the cell wall, as is the case for the secretion of cell wall polysaccharides [23], epicuticular waxes [8] and proteins, then the treatment with BFA should abolish the cell wall fluorescence.

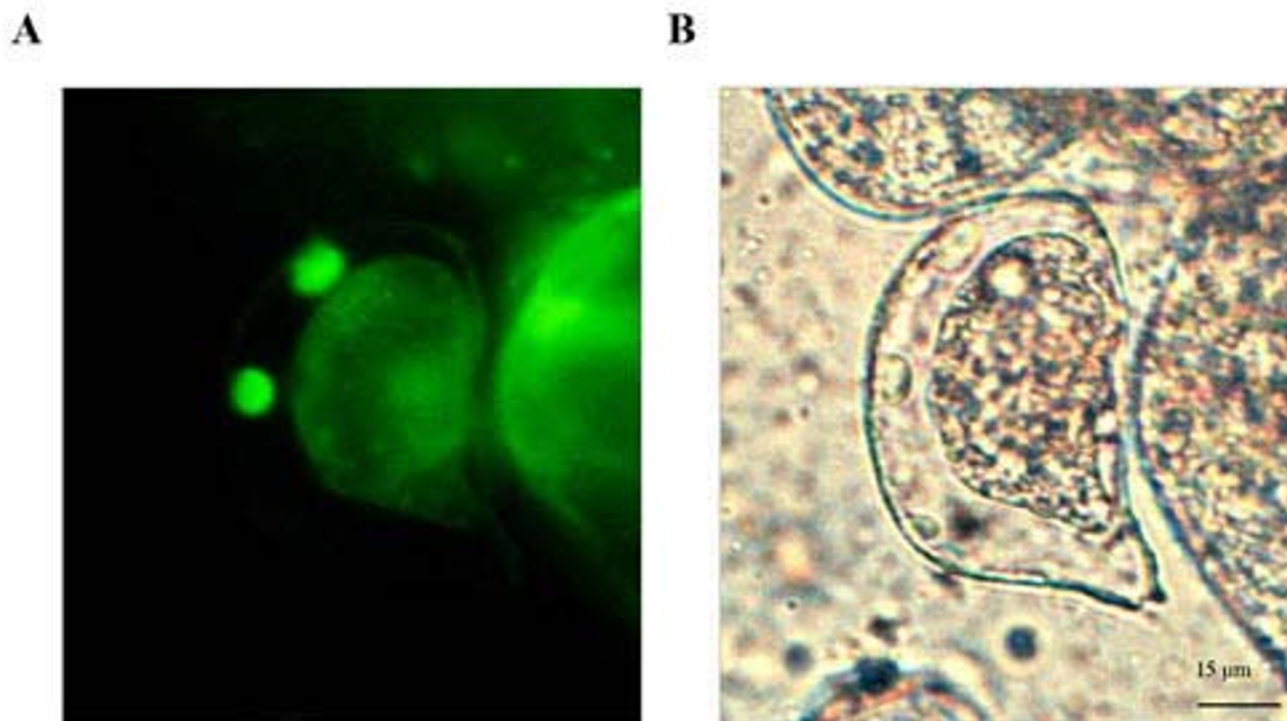


Figure 5
Accumulation of green fluorescence in the periplasmic space in plasmolyzed ERE::P1 BMS cells. (A) Epifluorescence microscopy and (B) bright field image of a plasmolyzed BMS cell accumulating two green fluorescent bodies in the periplasmic space.

When ERE::P1 cells were treated simultaneously with 10 μM estradiol and 10 $\mu\text{g/ml}$ of BFA, we observed a dramatic increase in the number of cells accumulating cytoplasmic GFBs (Fig. 7A). At eight days, for example, more than 16% of the estradiol- and BFA-treated cells accumulated green fluorescence (Fig. 7A), compared with less than 1% of the cells treated only with estradiol (Fig. 2A). The increase in green fluorescence-accumulating cells was already evident at 2–4 days after induction and continued until day ten, at which time cells started accumulating brown pigmentation, suggestive of cell death (not shown). Strikingly, however, the number of cells with green fluorescence in the cell wall increased proportionally, yet in a delayed fashion, to the number of cells with GFBs (Fig. 7A). The treatment of ERE::P cells with just 10 $\mu\text{g/ml}$ of BFA (no estradiol) resulted in a very low induction of green fluorescence-accumulating cells (Fig. 7A), presumably due to the low level of P1 expression in the absence of the hormone. In addition to the increased presence of cytoplasmic and cell wall fluorescence, estradiol-induced cells treated with BFA often accumulated abnormal membranous structures filled with green fluorescence (Fig. 7B). These structures were only present in cells

treated with BFA and were never observed in ERE::P1 cells treated only with estradiol. These structures may correspond to ER compartments filled with green fluorescent compounds, which would further suggest the ER as the possible site of initial accumulation for this compounds. Alternatively, the tubular organizations (Fig. 7B) may correspond to the sponge-like structures also observed in tobacco BY-2 cells after prolonged exposure (5 hours) to BFA [22].

To conclusively establish that the integrity of the TGN is not necessary for the secretion of the green auto-fluorescent compound, we treated ERE::P1 cells with estradiol and monensin, another TGN disturbing agent that has a different mechanism of action than BFA [21]. Treatment of ERE::P1 cells with 10 μM estradiol and 10 μM monensin resulted in more than 17% of the ERE::P cells accumulating green fluorescence in the cell wall after just 2 days, up to 30% after 3 days, while in the control with just 10 μM monensin (no estradiol) only 0.5% ERE::P1 cells contained green fluorescence compound(s) in the cell walls (not shown). However, the treatment with monensin resulted in accelerated cell death, making it

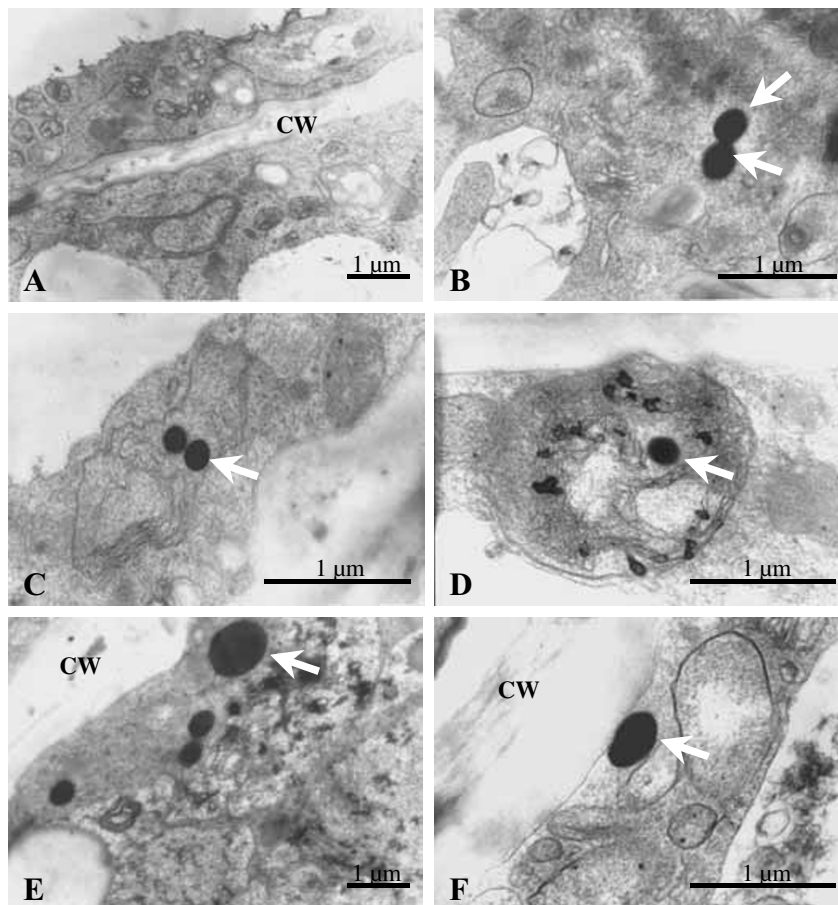


Figure 6

Ultrastructure alterations of BMS cells expressing PI from the inducible ERE promoter. (A) BMS cell prior to induction. (B-F) Cells 4 days after the addition of estradiol. Cells containing green fluorescent bodies (GFB) were selected using a micro-manipulator under the fluorescence microscope. The white arrows indicate electron-dense structures uniquely associated with the presence of the GFBs. CW indicates the cell wall.

impossible to investigate the effect of this agent at longer times. Different from BFA, the number of cells with cytoplasmic GFBs did not increase significantly over 2% during the five days of treatment. This observation suggests that both BFA and monensin increase cell wall fluorescence; the distinct effects on the cytoplasmic fluorescence may reflect the different mechanisms by which BFA and monensin function. The accumulation and vacuolar localization of the yellow fluorescent compounds was not affected by the treatment with BFA or monensin (not shown).

Discussion

Here, we investigated the accumulation of green and yellow auto-fluorescent compounds in maize BMS cultured cells as potential markers to follow the sub-cellular trafficking of plant small molecules. Gaining a better understanding of the process by which phytochemicals move within and between plant cells is central for the manipulation of plant metabolism.

We show that the green and yellow auto-fluorescent compounds are targeted to distinct sub-cellular compartments: the green fluorescent compound to the cell wall and the yellow fluorescent compound to the

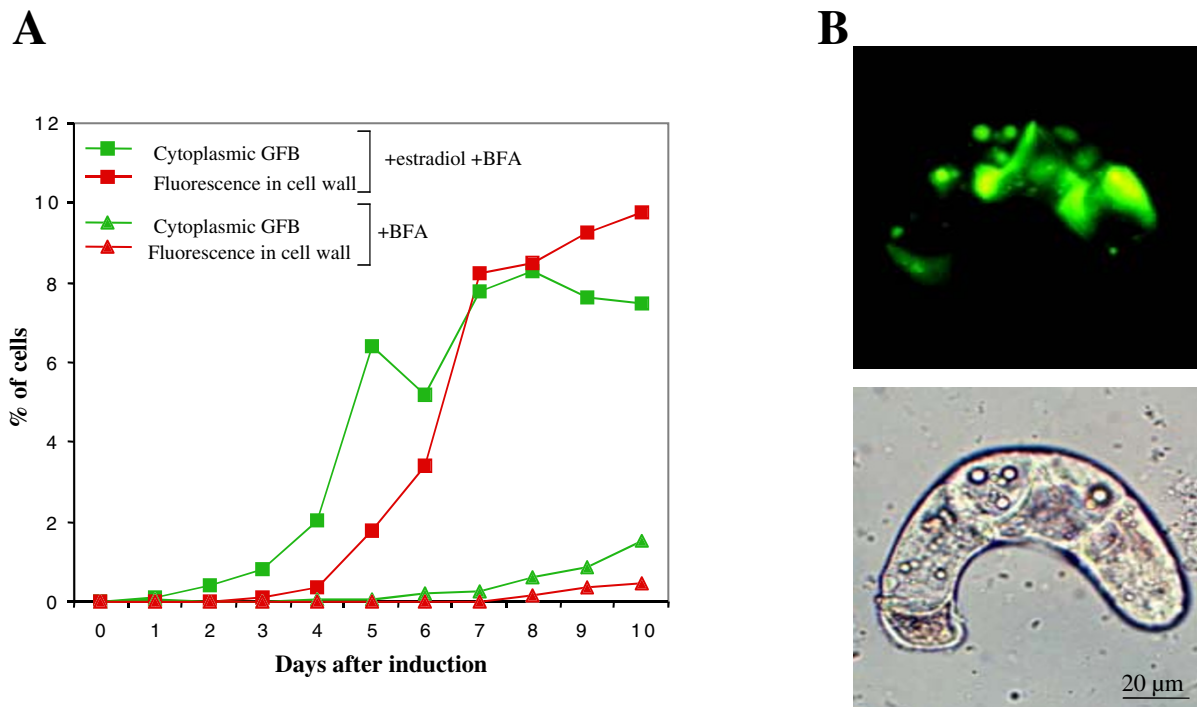


Figure 7

Effect of BFA on the accumulation of green auto-fluorescent compounds. (A) Accumulation of green fluorescence compounds in ERE::PI cells after treatment with BFA, induced (squares) or not induced (triangles) with estradiol. The y-axis indicates the percentage of cells with cytoplasmic (green line) GFBs or cell wall (red line) green fluorescence. (B) Epifluorescence and bright field microscopic image of ERE::P BMS cell 6 days after treatment with estradiol and 10 µg/ml of BFA indicating the tubular structures sometimes filled with green fluorescence that appear after the treatment with BFA.

vacuole. The cell wall and the vacuole are two main sites for the accumulation of phenolic compounds [2]. Thus, establishing the pathways used by these fluorescent compounds to traffic to these two structures can significantly advance our understanding of how important phytochemicals derived from phenolic metabolism are transported to the cell wall (e.g. lignins, phlobaphene pigments) or the vacuole (e.g. anthocyanins, sinapoyl esters, condensed tannins).

The yellow auto-fluorescent compounds appear to initially accumulate in small vesicles in the cytoplasm. In the

vacuole, the yellow fluorescent compounds are present in YFBs. These YFBs have striking similarities to the AVIs and anthocyanoplasts present in many cells accumulating high levels of anthocyanins [18], and in BMS cells expressing the C1 and R regulators [16]. We have not yet established whether a membrane encloses these compounds, an issue that has been controversial with the AVIs [18].

The green fluorescent compounds also accumulate initially in the cytoplasm. Small vesicle-like structures containing these compounds coalesce to form the large GFBs. The GFBs appear to migrate to the cell surface where

the green fluorescent compounds are released to the cell wall. The presence of the GFBs strongly correlates with the appearance of electron-dense spherical bodies evident under the TEM. Similar to the GFBs, these spherical structures can be found at the cell surface, even fusing to the plasma membrane. They are also present inside the expanded ER, suggesting that the ER is probably their initial accumulation site [16]. Interestingly, a recent study on *Arabidopsis* roots determined that at least two flavonoid biosynthetic enzymes, chalcone synthase and chalcone flavanone isomerase, are localized in electron-dense bodies that resemble in size and localization the electron-dense structures observed here [24]. Similar vesicle-like structures have been also previously observed for the localization of a flavonol *O*-glucosyltransferase in *Chrysosplenium americanum* cells [25], and for *Primula kewensis*' chalcone synthase [26]. These findings open the possibility that cytoplasmic structures resembling the electron-dense bodies described here function not only as the sites of storage or transport of specific phytochemicals, but also participate in specific steps in their synthesis, similar to what has been found for alkaloid vesicles [27].

While it is tempting to speculate that electron-dense structures as observed in the TEMs and the GFBs, so far observed only in P1-expressing cells, are one and the same, their sizes are strikingly different. The GFBs are often up to 3 μm in diameter, while the spherical electron-dense structures are rarely larger than 0.3 μm in size. We cannot rule out the possibility that the size difference is a consequence of sample fixation for the TEM studies. Alternatively, the GFBs and the electron-dense bodies may correspond to different structures induced by the expression of P1 or by the accumulation of P1-regulated compounds.

The treatment of ERE::P1 cells with estradiol and the TGN disturbing agents BFA or monensin does not result in an inhibition of the accumulation of the green fluorescence in the cell wall, as it would be expected if the secretion of these compounds follows a pathway similar to proteins, i.e. through the TGN. Rather, the accumulation of the green fluorescence increases dramatically in the cell wall. An enzymatic polymerization or degradation of the green fluorescent compounds in the cell wall could explain the increase in cell wall fluorescence in the presence of BFA or monensin. The secretion of the enzyme responsible for this would be blocked by BFA and monensin, resulting in the observed increase in cell wall green fluorescence. In contrast, the accumulation of the yellow fluorescent compounds in the vacuole is not increased by BFA or monensin, suggesting that the transport of the green and yellow fluorescent compounds to the cell wall and the vacuole respectively, occurs by different mechanisms.

The results presented here suggest the existence of a secretion pathway in BMS cells for the green fluorescent compounds that does not require the TGN. Structural analyses have previously suggested the existence of secretion pathways that involve the direct fusion of the ER to the plasma membrane, or of vesicles derived from the ER that do not appear to involve the TGN [3]. What is the nature of the GFBs present in the BMS cells expressing P1? Based on our current knowledge, we cannot rule out the formal possibility that the GFBs are proteinaceous or crystalline in nature. Indeed, we have been so far unable to isolate these bodies by differential centrifugation from gently lysed protoplasts, in conditions under which the YFBs retain their identity and fluorescent properties (not shown). However, the observation of GFBs in the periplasmic space of induced ERE::P1 BMS cells resembles the secretion of exosomes by many animal cells in culture [28]. Exosomes are believed to derive from late multi-vesicular compartments such as endosomes or the secretory lysosomes characteristic of dendritic and some hemopoietic cells [29]. A possible origin of the GFBs from an endosomal or vacuolar compartment would be consistent with the observed insensitivity to BFA and monensin, as a secretion pathway originating from these compartments would be after the Golgi block caused by the drugs. Alternatively, the GFBs could correspond to some specialized domain of the ER that has the ability to fuse with the plasma membrane. Determining the identity of the GFBs and the presence of similar structures in cells secreting other metabolites should provide fundamental information regarding the mechanisms used by plants for the trafficking of phytochemicals to the cell surface.

Conclusions

The green and yellow auto-fluorescent compounds are potentially powerful tools to investigate the mechanisms by which specialized compounds (phytochemicals) traffic within plant cells, from their site of synthesis to the compartment where they ultimately accumulate. While the chemical identity and the site of synthesis of these compounds remain to be established, it is evident that the possibility to follow these compounds within the cell by their fluorescing properties will allow the isolation and biochemical characterization of the structures in which they accumulate. The secretion of the green fluorescent compounds evidently occurs by a mechanism distinct from the secretion of proteins. Whether this involves a specialized vesicular system is currently under investigation. Establishing the pathways involved in the sub-cellular trafficking of phytochemicals is essential for the successful engineering of plant metabolism.

Methods

Treatment of BMS cells with estradiol and Golgi disturbing agents

BMS cells were grown and maintained in the conditions previously described [30]. Newly sub-cultured cells were used for treatments of estradiol and Golgi disturbing agents. For the induction with estradiol, 25 μ l of a stock solution of 20 mM β -estradiol 17-propionate (Sigma) in ethanol was added to 50 ml of BMS cell culture suspension. For the treatments with the Golgi disturbing agents, stock solutions of 20 mg/ml of BFA (Sigma) and 20 mM of monensin (Sigma) were prepared in ethanol. The treatment with BFA was done at a final concentration of 10 μ g/ml and monensin at 10 μ M. For microscopy examinations, 0.5–1 ml of cell culture was taken out every 24 hours after treatment.

Microscopy methods

The BMS cells were diluted to 100,000 cells/ml with cell culture medium. For epifluorescence experiments, 50 μ l of diluted cells were loaded on slides with a 20 \times 40 mm cover slip. The cells containing GFBs or YFBs were counted and photographed under a Zeiss Axiovert-100 fluorescent microscope coupled to a Bio-Rad MRC-1024 imaging system, using a blue filter adequate for FITC detection. Pictures images were taken using Kodak 35 mm slide film (160 T). At least 10,000 living cells were observed in each sample.

Electron Microscopy

The cells containing YFBs and GFBs were picked up using a micro-manipulator (MMO-203, Narishige) under the fluorescence microscope. After double fixations in 2.5% glutaraldehyde and 1% OsO₄, the cells were embedded in Spurr's Low Viscosity resin. Ultrathin sections were prepared with an ultramicrotome (Reichert Ultracut E). The sections were mounted on 100 mesh nickel grids coated with Formvar film and stained with 1% uranyl acetate for 10 min and 0.3% lead citrate for 5 min. Observation and photography were conducted using a Philips 301 transmission electron microscope.

List of abbreviations

AVI, anthocyanin vacuolar inclusions; BFA, Brefeldin A; BMS, Black Mexican Sweet; ER, endoplasmic reticulum; GFB, green fluorescent body; TEM, transmission electron microscope; TGN, *trans* Golgi Network; YFB, yellow fluorescent body.

Authors' contributions

Y.L. carried out all the microscopy experiments of ERE::P1 cells. N.I. investigated the effect of BFA on the TGN network in BMS cells. E.G. was involved in the design and supervision of this project and drafted the manuscript.

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References

1. Verpoorte R: **Plant secondary metabolism**. In *Metabolic engineering of plant secondary metabolism* Edited by: Verpoorte R, Alfermann AW. Kluwer Academic Publishers, Dordrecht; 2000:1-29.
2. Grotewold E: **Subcellular trafficking of phytochemicals**. *Rec Res Devel Plant Physiol* 2001, **2**:31-48.
3. Kronstedt-Robards E, Kronstedt AW: *Exocytosis in gland cells* Cambridge Univ. Press, U.K; 1991.
4. Walker TS, Bais HP, Grotewold E, Vivanco JM: **Root exudation and rhizosphere biology**. *Plant Physiol* 2003, **132**:44-51.
5. Theodoulou FL: **Plant ABC transporters**. *Biochim Biophys Acta* 2000, **1465**:79-103.
6. Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J, Boutry M: **A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion**. *Plant Cell* 2001, **13**:1095-1107.
7. Zamski E, Shoham O, Palevitch D, Levy A: **Ultrastructure of capsinoid-secreting cells in pungent and nonpungent red pepper (*Capsicum annuum* L.) cultivars**. *Bot Gaz* 1987, **148**:1-6.
8. Jenks MA, Rich PJ, Ashworth EN: **Involvement of cork cells in the secretion of epicuticular wax filaments on *Sorghum bicolor* (L.) moench**. *Int J Plant Sci* 1994, **155**:506-518.
9. St.-Pierre B, Vazquez-Flota F, De Luca V: **Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate**. *Plant Cell* 1999, **11**:887-900.
10. Stafford HA: *Flavonoid metabolism* CRC Press, Inc, Boca Raton, USA; 1990.
11. Hrazdina G, Wagner GJ: **Metabolic pathways as enzyme complexes: evidence for the synthesis of phenylpropanoids and flavonoids on membrane associated enzyme complexes**. *Arch Biochem Biophys* 1985, **237**:88-100.
12. Winkel-Shirley B: **Evidence of enzyme complexes in the phenylpropanoid and flavonoid pathways**. *Physiol Plant* 1999, **107**:142-149.
13. Marrs KA, Alfenito MR, Lloyd AM, Walbot V: **A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *bronze 2***. *Nature* 1995, **375**:397-400.
14. Mueller LA, Goodman CD, Silady RA, Walbot W: **AN9, a petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein**. *Plant Physiol* 2000, **123**:1561-1570.
15. Debeaujon I, Peeters AJM, Leon-Kloosterziel KM, Koornneef M: **The TRANSPARENT TESTA 12 gene of *Arabidopsis* encodes a multi-drug secondary transporter-like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium**. *Plant Cell* 2001, **13**:853-871.
16. Grotewold E, Chamberlain M, St Claire G, Swenson J, Siame BA, Butler LG, Snook M, Bowen B: **Engineering secondary metabolism in maize cells by ectopic expression of transcription factors**. *Plant Cell* 1998, **10**:721-740.
17. Grotewold E, Drummond B, Bowen B, Peterson T: **The Myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset**. *Cell* 1994, **76**:543-553.
18. Markham KR, Gould KS, Winefield CS, Mitchell KA, Bloor SJ, Boase MR: **Anthocyanic vacuolar inclusions – their nature and significance in flower colouration**. *Phytochemistry* 2000, **55**:327-336.
19. Bruce W, Folkerts O, Garnaat C, Crasta O, Roth B, Bowen B: **Expression profiling of the maize flavonoid pathway genes controlled by estradiol-inducible transcription factors CRC and P**. *Plant Cell* 2000, **12**:65-80.
20. Klausner RD, Donaldson JG, Lippincott-Schwartz J: **Brefeldin A: Insights into the control of membrane traffic and organelle structure**. *J Cell Biol* 1992, **116**:1071-1080.

21. Dinter A, Berger EG: **Golgi-disturbing agents.** *Histochem Cell Biol* 1998, **109(5-6)**:571-590.
22. Ritzenthaler C, Nebenfuhr A, Movafeghi A, Stussi-Garaud C, Behnia L, Pimpl P, Staehelin LA, Robinson DG: **Re-evaluation of the effects of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera.** *Plant Cell* 2002, **14**:237-261.
23. Driouich A, Faye L, Staehelin LA: **The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins.** *Trends Biochem Sci* 1993, **18**:210-214.
24. Saslowsky D, Winkel-Shirley B: **Localization of flavonoid enzymes in Arabidopsis roots.** *Plant J* 2001, **27**:37-48.
25. Latchinian-Sadek L, Ibrahim RK: **Flavonol ring B-specific O-glucosyltransferases: purification, production of polyclonal antibodies, and immunolocalization.** *Arch Biochem Biophys* 1991, **289**:230-236.
26. Schopker H, Kneisel M, Beerhues L, Robenek H, Wiermann R: **Phenylalanine ammonia-lyase and chalcone synthase in glands of *Primula kewensis* (Wats, W)-Immunofluorescence and immunogold localization.** *Planta* 1995, **196**:712-719.
27. Facchini PJ: **Alkaloid biosynthesis in plants: biochemistry, cell biology, molecular regulation, and metabolic engineering applications.** *Annu Rev Plant Physiol Plant Mol Biol* 2001, **52**:29-66.
28. Thery C, Zitvogel L, Amigorena S: **Exosomes: composition, biogenesis and function.** *Nat Rev Immunol* 2002, **2(8)**:569-579.
29. Andrews NW: **Regulated secretion of conventional lysosomes.** *Trends Cell Biol* 2000, **10**:316-321.
30. Dias A, Brown J, Bonello P, Grotewold E: **Metabolite profiling as a functional genomics tool.** In *Plant Functional Genomics: Methods and Protocols Volume 236*. Edited by: Grotewold E. Humana Press, NJ; 2003:415-426.

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