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Subcellular compartmentalization in protoplasts from *Artemisia annua* cell cultures: Engineering attempts using a modified SNARE protein

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ABSTRACT

Plants are ideal bioreactors for the production of macromolecules but transport mechanisms are not fully understood and cannot be easily manipulated. Several attempts to overproduce recombinant proteins or secondary metabolites failed. Because of an independent regulation of the storage compartment, the product may be rapidly degraded or cause self-intoxication. The case of the anti-malarial compound artemisinin produced by *Artemisia annua* plants is emblematic. The accumulation of artemisinin naturally occurs in the apoplast of glandular trichomes probably involving autophagy and unconventional secretion thus its production by undifferentiated tissues such as cell suspension cultures can be challenging.

Here we characterize the subcellular compartmentalization of several known fluorescent markers in protoplasts derived from Artemisia suspension cultures and explore the possibility to modify compartmentalization using a modified SNARE protein as molecular tool to be used in future biotechnological applications. We focused on the observation of the vacuolar organization in vivo and the truncated form of AtSYP51, 51H3, was used to induce a compartment generated by the contribution of membrane from endocytosis and from endoplasmic reticulum to vacuole trafficking.

The artificial compartment crossing exocytosis and endocytosis may trap artemisinin stabilizing it until extraction; indeed, it is able to increase total enzymatic activity of a vacuolar marker (RGUSChi), probably increasing its stability. Exploring the 51H3-induced compartment we gained new insights on the function of the SNARE SYP51, recently shown to be an interfering-SNARE, and new hints to engineer eukaryote endomembranes for future biotechnological applications.

1. Introduction

Compartmentalization mechanisms in plants are not fully understood and cannot be easily manipulated. This is a main reason for the failure of attempts to overproduce several recombinant proteins and secondary metabolites. In the case of secondary metabolism, the overexpression of biosynthetic enzymes may often be insufficient to overproduce the molecule of interest. It is for example the case of the anti-malarial compound artemisinin produced by *Artemisia annua* plants. Artemisinin is a sesquiterpene lactone endoperoxide, which is synthesized in the cytosol from the general isoprenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). The biosynthesis pathway has been elucidated and the genes involved are known (Ting et al., 2013).

Since malaria is one of the most serious health problems in many tropical countries, causing more than 660,000 deaths every year (World Malaria Report 2012, World Health Organization, WHO), artemisinin production has been the object of intense research using several strategies: overexpressing key enzyme genes in *A. annua* plants (Nafis et al., 2011; Tang et al., 2014), other plants (Ting et al., 2013) or yeast (Paddon et al., 2013); enhancing biosynthesis by blocking competitive pathways (Chen et al., 2011); altering regulation by transcription factors (Lu et al., 2013; Tang et al., 2014); by stress indirect regulation (Caretto et al., 2011) and by increasing biomass (Banyai et al., 2011) or the density of the specific cell type where the metabolite is accumulated, the glandular trichomes (Maes and Goossens, 2010).

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None of these strategies appeared fully satisfying because the biosynthesis of the metabolite and its accumulation in the glandular trichome are under independent regulatory mechanisms that need to be coordinated. Until now the overexpression of biosynthetic enzymes in heterologous systems, such as yeast, appears the most attractive strategy but the strategy to improve artemisinin compartmentalization and accumulation in *A. annua* plants was not yet fully explored.

Exploitation of plant cell natural compartmentalization is not new and is often proposed for the accumulation of recombinant proteins in seeds (Khan et al., 2012).

A. annua plants accumulate artemisinin in the extremely specialized endomembrane system of few cells in the glandular trichomes. To attempt to induce cells of other tissue to trap artemisinin, as glandular trichomes do or just with similar results, it is essential to make the point on *A. annua* cells compartmentalization. It is known that the initial enzymes involved in the artemisinin biosynthesis are located in the cytosol and in the plastid but at least AaCYP71AV1 is located in the endoplasmic reticulum (ER; Ting et al., 2013). The accumulation of the metabolite occurs in the apoplast of glandular trichomes and it seems to arrive there by autophagy-related exosome secretion (Krause et al., 2013). Several examples of this mechanism were recently reviewed (Kulich and Zarsky, 2014) and are well represented in maize cells where autofluorescent membrane-bound bodies derived from the ER were induced and secreted upon the induction of the P1 transcription factor (Lin et al., 2003). Similarly to those autofluorescent compounds, phenylpropanoids are found in the cell walls esterified to the polysaccharides, but they reach the apoplast travelling from the ER to plasma membrane (PM) in small membrane vesicles aggregating into bigger structures before fusing with the PM. These compartments might be generated by autophagy-related process similar to that observed in epidermal cells of leaves invaded by pathogens, where the mechanism is based on multivesicular bodies (MVB)-derived exosomes (Meyer et al., 2009; Kulich and Zarsky, 2014).

Hypothesizing the involvement of some autophagocitotic process, we investigated the natural cellular compartmentalization of *A. annua* cells in suspension cultures; we used a modified SNARE to alter cell endomembrane organization. In particular we found that a truncated form of AtSYP51, deleted of its N-terminus, was able to induce a stable compartment generated by the contribution of membranes from endocytosis and ER-to-vacuole traffic. We explored the biological characteristic of this artificially generated compartment gaining new insight on the function of the SNARE SYP51, recently shown to be an interfering-SNARE (De Benedictis et al., 2013), and new hints for the development of compartmentalization engineering for future biotechnological applications.

2. Materials and methods

2.1. Plant material

Artemisia annua L. cell suspension cultures were established and maintained as described by Caretto et al. (2011) in G6 medium. Briefly, suspension cultures were maintained in MS medium (Murashige and Skoog, 1962) supplemented with 2 mg L^{-1} of 2,4-dichlorophenoxyacetic acid and 0.15 mg L^{-1} 6-benzylaminopurine. Cultures were incubated on a rotary shaker (120 rpm) at 25 °C under continuous fluorescent white light (125 μmol photons m^{-2} s^{-1}) and were subcultivated every 35 days in 500 mL Erlenmeyer flasks by transferring 15 mL of the 35-day-old suspensions into 85 mL fresh G6 medium. Growth of these cell suspension cultures (henceforth referred to as G6 cells) was monitored by measuring dry weight during the culture cycle. Cell viability was assayed using the fluorescein diacetate staining method (Wildholm, 1972).

2.2. Plasmid constructions for G6 cells transformation

51H3 and GFP:At51H3 were used here for the first time. The Qc domain including the trans-membrane domain of AtSYP51 (named H3) was amplified with the primer #159 (GGGG ACA AGT TTG TAC AAA AAA GCA GGC TGG ATG CGACAAGTTATGAGAG) and #160 (GGGG AC CAC TTT GTA CAA GAA AGC TGG GTA TTA-CATATACTTAACCAACA) using Phusion polymerase (Finnzymes). The resulting fragment, containing also the TM domain, was cloned in pDONOR221 P1–P2 (Invitrogen) to yield a pENTRY clone. This plasmid was recombined by a LR Clonase (Invitrogen) in pK7WGF2 (Plant System Biology, Gent University, Belgium) to get GFP:51H3 and in pB7WG2 (Plant System Biology, Gent University, Belgium) to get 51H3.

The other constructs used in this study, 51F and 51T, Cherry:BP80 (De Benedictis et al., 2013); RFP:At122F (named RFP122F in Faraco et al., 2011); GFPgl133Chi (Paris et al., 2010); AleuGFPgl133, RFP-KDEL, ST52-mRFP (De Caroli et al., 2011a); cytosolic GFP (Rehman et al., 2008), RGUSChi and secRGUS (Di Sansebastiano et al., 2007); were described previously in different publications.

2.3. Protoplasts preparation and transformation

Protoplasts were prepared from G6 cells harvested from 14 or 21 day-old suspension culture. The manipulation was performed as previously described for other plant material, under normal white light (De Benedictis et al., 2013; Di Sansebastiano et al., 2014). Protoplasts were examined with a confocal laser-microscope (LSM 710 Zeiss, ZEN software, GmbH, Jena, Germany). GFP was detected within the short 505–530 nm wavelength range, assigning the green colour, RFP within 560–615 nm assigning the red colour. Excitation wavelengths of 488 and 543 nm were used. The laser power was set to a minimum and appropriate controls were made to ensure there was no bleed-through from one channel to the other. Images were processed using Adobe Photoshop 7.0 software (Mountain View, CA, USA).

2.4. Protein extraction from protoplasts and enzymatic GUS tests

To perform RGUS activity evaluation protoplasts were lysed by three consecutive cycles of freezing (in liquid nitrogen) and thawing. The soluble proteins and part of the microsomal fraction were separated from large insoluble residues by centrifugation for 5 min at 10,000 × *g*. This lysate extract was directly used to measure enzymatic activity of rat-glucuronidase RGUSChi (Di Sansebastiano et al., 2007) by using a TECAN Infinite-200 fluorimeter (TECAN, Switzerland) as previously described (De Benedictis et al., 2013).

3. Results

3.1. Protoplasts preparation and transformation from Artemisia annua cultivated cells (G6)

We have established and maintained an in vitro culture of *A. annua* cells, named G6, able to produce a set of important bioactive secondary metabolites. In previous papers we demonstrated that G6 cells were able to produce the antimalarial compound artemisinin (Caretto et al., 2011) and that the addition to the medium of chemical elicitors, such as methyl jasmonate and β-cyclodextrins, induced the increase of both intracellular and extracellular levels of artemisinin (Durante et al., 2011).

G6 cells were harvested from 14 or 21 day-old suspension culture and treated as described in Section 2. All experiments produced vital protoplasts efficiently transformed. No evident differences could be reported for cells of the two selected ages in terms of

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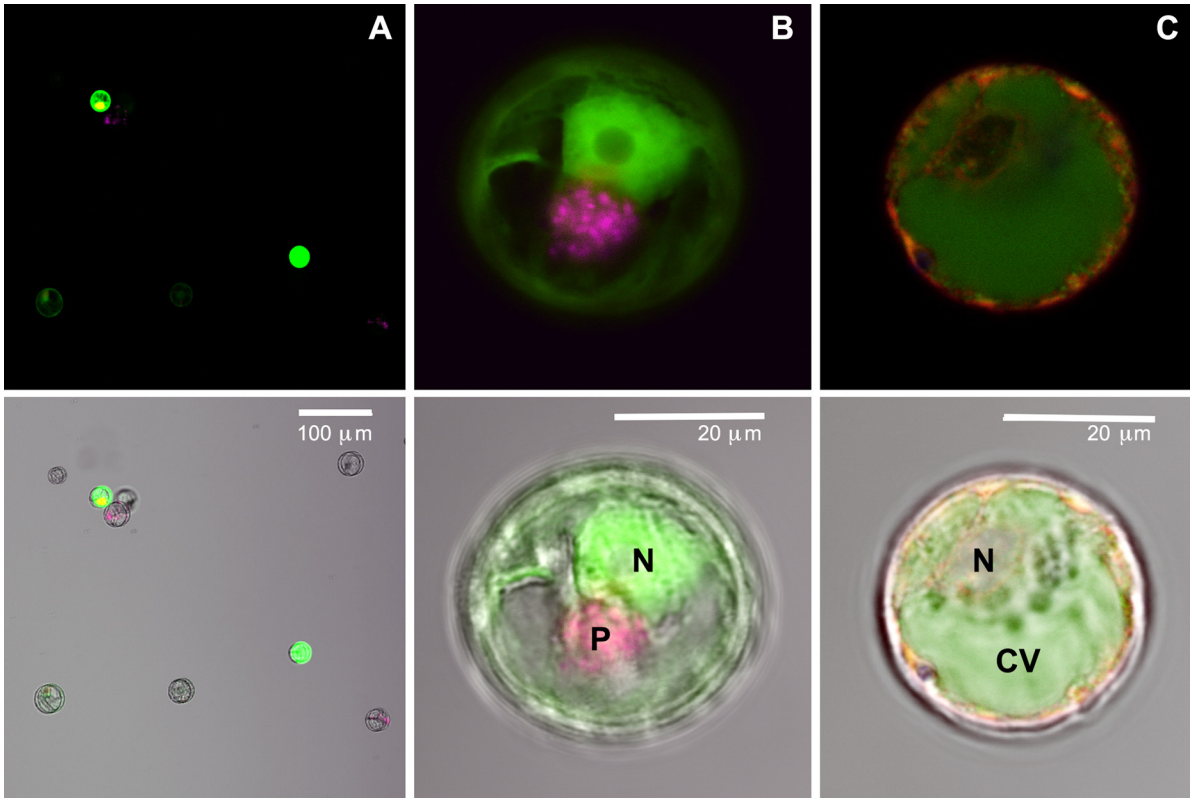

Fig. 1. Examples of protoplasts from *A. annua* cell suspension cultures. (A) Low magnification confocal image of cells, in UV light (upper frame) and transmitted light (lower frame), density of population and abundance of transformants expressing GFP are visible (4 cells out of 8 in focus); (B) cell expressing cytosolic GFP (in green), particularly visible within the nucleus (N), with pro-plastids (P) autofluorescence (in magenta); and (C) cell expressing GFPgl133Chi (in green) labelling the central vacuole (CV), and RFP-KDEL (in red), labelling the endoplasmic reticulum forming an evident ring around the nucleus (N). (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of the article.)

viability and transformation efficiency. Transformation efficiency was around 50% (Fig. 1A) or above, depending on supercoiled DNA quality and on freshly prepared PEG solution. About 20 μg plasmid DNA was used each 700.000 cells.

Cytosolic GFP fluorescence can be easily visualized. Autofluorescence is limited to small clusters of pro-plastids emitting red fluorescence (Fig. 1B). Red Fluorescent Protein (RFP, excited by 543 nm laser and emitting 560–615 nm) can be easily distinguished from pro-plastid and plastid autofluorescence (>650 nm). GFP and RFP can be co-expressed and label distinct endomembranous compartments such as ER and vacuole (as shown by RFP-KDEL and GFPgl133Chi in Fig. 1C).

The pilot experiments presented here, showed that protoplasts from *A. annua* suspension cultures are a perfect tool to study endomembrane compartment biogenesis and metabolite accumulation.

3.2. G6 vacuolar compartmentalization and effect of AtSYP51 variants

G6 protoplasts have a normal ER-export machinery to the Golgi apparatus, as demonstrated by the limited co-localization of the cis-Golgi marker ERD2:YFP with the ER marker RFP-KDEL (Fig. 2A). G6 protoplasts tend to present a single central vacuole (CV). It can be evidenced with two independent vacuolar markers that follow alternative routes, AleuGFPgl133 and GFPgl133Chi (Stigliano et al., 2013). The presence of both markers in the CV after more than 24 h of transient expression indicates a simple vacuolar compartmentalization (Fig. 2B and C).

Nonetheless, during transient expression of GFPgl133Chi it was possible to observe that vacuolar sorting was indirect. The vacuolar

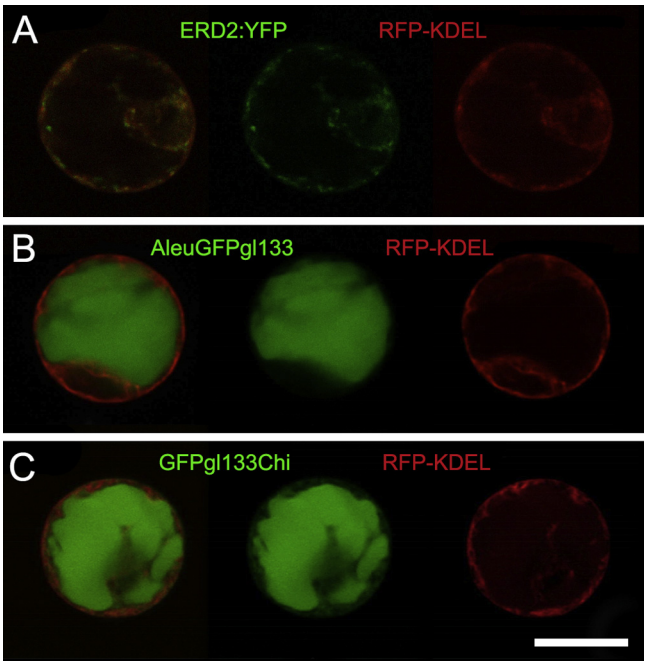

Fig. 2. Merged and separated fluorescent emissions (GFP in green and RFP in red) of G6 protoplasts co-transformed with the red marker RFP-KDEL and the green marker ERD2:YFP (A), AleuGFPgl133 (B) and GFPgl133Chi (C). Scale bar 20 μm.

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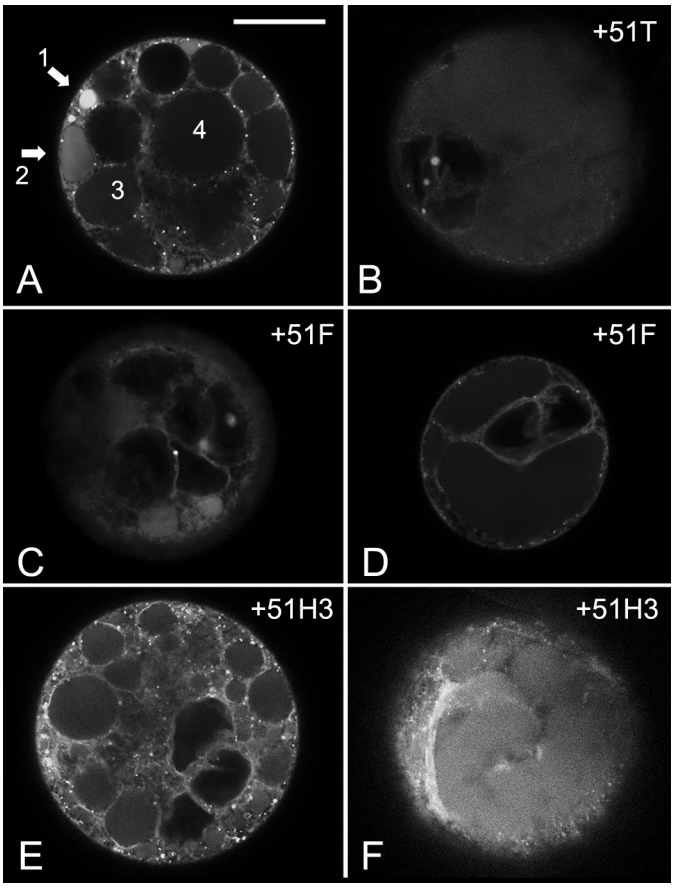

Fig. 3. GFPgl133Chi fluorescence after 16 h of transient expression in G6 protoplasts. (A) In control conditions the markers accumulate more evidently in smaller vacuoles (evidenced with arrows, 1, 2) probably generated de novo while larger vacuoles (3, 4) do not appear to accumulate GFP directly, suggesting that they are pre-existing compartments; co-transformation with AtSYP51 variants 51T (B), 51F (C and D), 51H3 (E and F) caused alterations of the distribution pattern. Scale bar 20 μm.

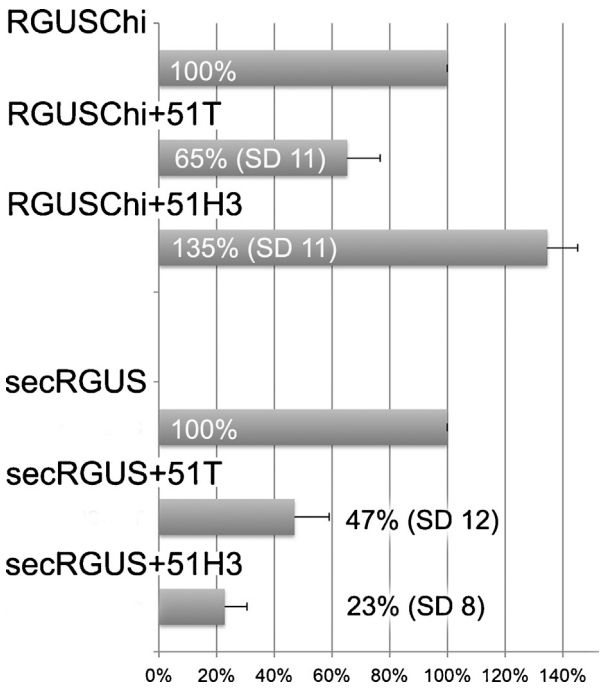

Fig. 4. Effect of AtSYP51 variants on the vacuolar marker RGUSChi and on secreted marker secRGUS. RGUSChi total activity has an average 35% increase while all other co-expressions cause total activity reduction. Number of independent experiments: $n = 3$.

marker was first accumulated in smaller vacuoles (Fig. 3A1) whose content was then diluted in vacuoles of increasing size (Fig. 3A2–4), in agreement with recent finding about vacuolar transport (Viotti, 2014). The clear difference in GFP intensity among vacuoles clearly indicated that transport was not direct from ER to the pre-existing central vacuoles.

It was recently shown (De Benedictis et al., 2013) in Arabidopsis that a Qc-SNARE protein named SYP51, localized on tonoplast and on post-Golgi compartments up-to-now indicated as TGN (De Benedictis et al., 2013), was important for the specific transport of GFPChi (equivalent to GFPgl133Chi) also when compared to the very similar SYP52 (De Benedictis et al., 2013; Faraco et al., 2013). SYP51 interferes with traffic to tonoplast thus we co-expressed GFPgl133Chi with three variants of SYP (Suppl. Fig. 1): a C-terminal truncated form (51T), a native form (51F) and a N-terminal truncated form (51H3). Variant 51T accelerated the labelling of CV by GFPgl133Chi (Fig. 3B) that is normally exported more slowly from the ER. Variant 51F interfered with vacuolar transport as expected and GFP stayed longer in small vacuoles (Fig. 3C) or never arrived to the CV (Fig. 3D). Variant 51H3 had the interesting effect to visibly increase fluorescence in general, in intermediate small vacuole (Fig. 3E) as well as in the CV (Fig. 3F).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.11.016.

To test the hypothesis that variant 51H3 could increase GFP fluorescence by improving the stability of the protein altering the environment of the vacuole lumen, we measured the effect on an enzymatic marker based on the rat-beta-glucuronidase with the same vacuolar sorting determinant, RGUSChi (Di Sansebastiano et al., 2007). Results confirmed indeed a positive effect of 51H3 on vacuolar proteins stability, revealed in this case by the increase in RGUSChi total activity (Fig. 4). The 51T variant did not induce an increase of enzymatic activity for the vacuolar marker and, in any case the effect on the secreted marker was negative.

3.3. AtSYP51 and 51H3 variant localization in Artemisia annua protoplasts

Transiently expressing GFP:At51F in *A. annua* protoplasts, it was possible to label the tonoplast (Fig. 5A). The deletion of the AtSYP51 N-terminus was tested as possible cause of vacuolar fusion perturbation. In fact, the expression of the construct derived from the fusion of GFP with the C-terminus of AtSYP51 (GFP:At51H3) caused the formation of several abnormal compartments of large (Fig. 5B) and small size (Fig. 5C) which were typical for this construct. These compartments were independent from Golgi apparatus as evidenced by lack of co-localization during co-expression with the Golgi marker ST52-mRFP (Fig. 5B; De Caroli et al., 2011a). The relation with pre-vacuolar compartments (PVCs), labelled by Cherry:BP80, was more complex. In fact in some cells the two markers were clearly distinct (Fig. 5C) but in others (visibly characterized by higher fluorescence) the markers co-localized in small compartments or clusters of small compartments and labelled together the GFP:51H3 typical compartments (Fig. 5D). GFP:51H3 labelled the outer membrane while Cherry:BP80 appeared solubilized in the compartment lumen.

Low efficient transient transformation of *A. annua* plantlets was sufficient to confirm that the GFP:51H3 typical compartments formed also in differentiated tissue cells (Suppl. Fig. 2).

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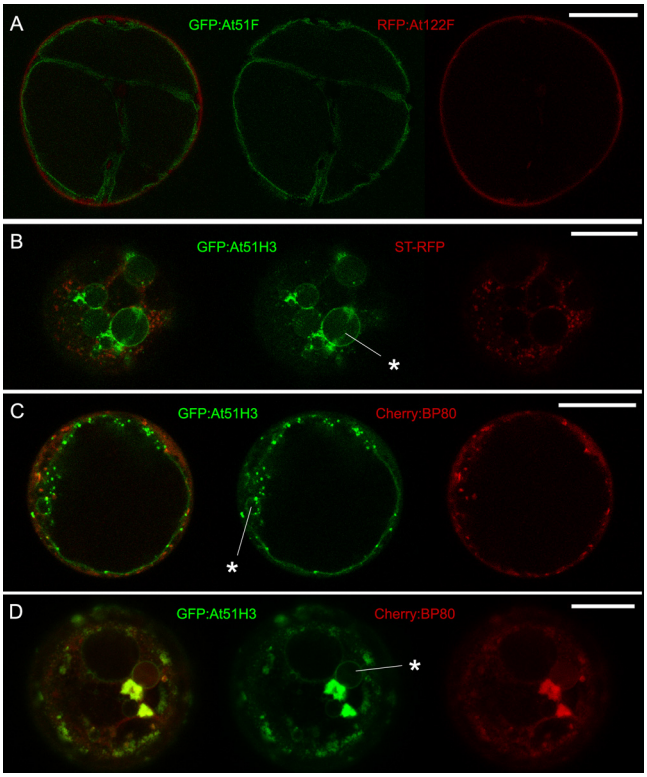

Fig. 5. Merged and separated fluorescent emissions (GFP in green and RFP in red) of G6 protoplasts co-transformed with (A) GFP:At51F and the plasma membrane marker RFP:At122F; (B) GFP:At51H3 and the Golgi marker ST52-mRFP; (C and D) GFP:At51H3 and the pre-vacuolar marker (PVC) Cherry:BP80. The asterisk evidences the abnormal compartments characteristically induced by GFP:At51F. Scale bar 20 μm. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of the article.)

4. Discussion

In previous papers we have described the establishment and maintenance of *A. annua* in vitro cultures and demonstrated that they were able to produce the antimalarial compound artemisinin (Caretto et al., 2011). Interestingly the results showed that suspension cultures were also able to secrete artemisinin into the growth medium. Furthermore the addition to the medium of chemical elicitors such as methyl jasmonate and β-cyclodextrins, resulted a useful tool to induce the increase of both intracellular and extracellular levels of artemisinin (Durante et al., 2011). In addition, we have recently evaluated the ability of the β-CD-treated *A. annua* cells suspension cultures to produce other important bioactive compounds such as carotenoids, quinones and chlorophylls (Rizzello et al., 2014). The accumulation of these compounds is totally dependent on their proper compartmentalization. The compartmentalization of secondary metabolite synthesis within plant cells is only partly known. Many biosynthetic machineries are distributed between cytoplasm and endomembranous compartments. It is particularly difficult to distinguish between cytoplasm and ER surface-localized components. Analysis by iPSORT (http://ipsort.hgc.jp/) suggests that some artemisinin biosynthetic enzymes should be cytosolic for the lack of signals (ADS – amorpha-4,11-diene synthase), other enzymes (Dbr2 – artemisinic aldehyde delta(11(13)) reductase; CPR – cytochrome P450 reductase) have plastid targeting signals and some other have translocation peptides for the ER (CYP71AV1, AldH1). The way artemisinin gets accumulated in the glandular trichomes remains unclear but it probably includes autophagy because precursor derived from cytosolic and plastidial reactions seems to be finally transformed by enzymes most probably translocated in the secretory pathway. The sorting and accumulation mechanism was not sufficiently investigated but it would not be a surprising mechanism. Anthocyanin autophagy-related vesicular import pathway from ER into the vacuole is an example of compartmentation with the help of autophagocitosis (Kulich and Zarsky, 2014).

Here we explored the compartmentalization of *A. annua* cells in suspension cultures by producing protoplasts and transiently expressing several fluorescent proteins using methods mediated from other model systems (Di Sansebastiano et al., 2001; De Benedictis et al., 2013; Di Sansebastiano et al., 2004). Protoplasts are widely used as standard system for transient expression studies to define proteins sub-cellular localization, including cell wall deposition (De Caroli et al., 2011b, 2014), to assay promoter activities and other cellular processes. Protoplasts loose in part their tissue specificity and can be used to assay genes and proteins normally expressed in any plant organ. At the same time they maintain their tissue specificity and endomembrane organization within the time required for transformation and analyses of transient expression. Protoplasts partially maintain the characteristics of the originary cell and give different results for the localization of tagged proteins and for specific promoter activity (Faraco et al., 2011). For these reasons protoplasts should also maintain the behaviour of cells in suspension cultures for the short lapse of time required for transient expression of fluorescent markers.

In *A. annua* protoplasts several compartments were labelled with different fluorescent probes, cytosol (GFP), ER (RFP-KDEL), central vacuole (GFPgl133Chi and AleuGFP), Golgi (ST52-mRFP), PVC (Cherry:BP80) and tonoplast (GFP:At51F). These recombinant proteins showed to be valid markers for the study of *A. annua* cells subcellular compartmentalization.

It was observed that, despite G6 cells have a single central vacuole, the vacuolar protein GFPgl133Chi, sorted in a Sar1 independent way, reaches the vacuole through a series of small vacuoles fusion (Fig. 3A). These multiple fusion events imply the formation of separated vacuolar compartments generated in a COPII independent way. Independence from COPII vesicle can be deduced by the presence of GFPgl133Chi in the lumen. In fact this marker secreted by a Sar1 independent mechanism seems to be representative of the ER-to-vacuole pathway (Stigliano et al., 2014) and to support new models revolutionizing the classic view of vacuolar sorting (Viotti, 2014; Robinson, 2014).

The use of GFP:At51F to further investigate the dynamic of the tonoplast was due to the recent findings showing the sorting of GFPChi (equivalent to GFPgl133Chi) specifically dependent on SYP51 activity in Arabidopsis (De Benedictis et al., 2013). The functional specificity is characteristic of several SNAREs belonging to multigenic families (Silva et al., 2010) and is generally related to regulated processes. The interesting distribution pattern of the deletion mutant GFP:At51H3, labelling small vacuolar compartments well separated from the central vacuole, indicates the possibility to delay the fusion process.

In other SNAREs, mainly Qa-SNAREs, the N-terminus is a regulatory domain and also in this case its removal enhances the protein reactivity. SYP51 seems to be trapped on a membrane where it should just transit. Other H3 mutants have been prepared in the past for other SNARE proteins and induced aberrant structures (Di Sansebastiano et al., 2006), anyhow in this case this structure represents an interruption of traffic and, apparently, not a diversion to a wrong final destination. If the small vacuoles induced by GFP:At51H3 have not the same characteristics of the central vacuole, generally lytic, they may maintain their content more stable, so providing an advantage in the accumulation and stability of interesting products. We tested this hypothesis by measuring the enzymatic activity of RGUSChi, a vacuolar marker sorted by the same COPII independent machinery as GFPgl133Chi. This marker is,

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indeed, stabilized by the co-expression of the non-tagged deletion mutant of SYP51, 51H3.

We ignore the mechanisms through which GFP:At51H3 and apparently 51H3 (since it is not directly visualized) induce and stabilize the small vacuoles but we can hypothesize that the deletion of the N-terminus open the active Qc domain of the SYP51 to incorrect interactions that are still being investigated in Arabidopsis. The alteration of endomembranes organization via a SNARE mutant is anyhow an exciting opportunity to manipulate plant cell compartmentalization, due to its exceptional versatility (Di Sansebastiano et al., 2009).

Exploitation of plant cell natural compartmentalization was the strategy adopted in several successful attempts but we introduce here the idea of compartmentalization engineering. In nature, the modification of the plant cell endomembranes seems to be a common feature among most single-stranded positive ss(+)RNA and even some DNA viruses that use viral-induced organelles to support viral replication and spread (Patarroyo et al., 2013). For example *comoviridae* such as Grapevine fanleaf nepovirus (GFLV) replicates on ER-derived membranes (Ritzenthaler et al., 2002).

It is now necessary to study the localization of the artemisinin biosynthetic enzymes to better understand how to alter their compartmentalization, also using the 51H3 variant of SYP51. Indeed engineering *A. annua* plants remains an attractive option to produce more artemisinin. Recently the group of Keasling reported to have successfully produced 25 g/L of artemisininic acid from 1 L of yeast culture (Paddon et al., 2013) but, even if this opens the possibility of industrial bioproduction, the scale-up cost remains a problem and will only be overcome by an overproducing Artemisia plantation or Artemisia tissue culture in which artemisinin could be accumulated in every single vegetative cell. Up to now the genetic engineering strategies on *A. annua* plants have included the modification of the biosynthetic enzymes and the differentiation of trichomes (Tang et al., 2014), but not the compartmentalization of the metabolite.

The alteration of the membrane transport by the formation of a stable compartment between the exocytotic and the endocytotic pathway could intercept the processes just mentioned and trap cargo molecules in an intracellular compartment keeping them more stable and abundant until extraction.

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