LIVE IMAGING AND SUB-CELLULAR LOCALIZATION OF THE A9-GUS-GFP CHIMERIC PROTEIN EXPRESSED IN NICOTIANA BENTHAMIANA TRANSGENIC PLANTS

K. Stefanova1, R. Wightman2, A. Atanassov1, I. Atanassov1
AgroBioInstitute, Sofia, Bulgaria1
University of Manchester, Manchester, UK2
Correspondence to: Katerina Stefanova
E-mail: katia_karserdarova@yahoo.com

ABSTRACT
The fusion of the signal peptide region from the Arabidopsis A9 anther-specific gene to a GUS-GFP reporter protein results in direction of the chimeric protein to ER and its further secretion out of the cell, following expression in Nicotiana benthamiana cell suspension. The detailed observations of the sub-cellular translocation and exocytosis of the chimeric A9-GUS-GFP protein have been largely restricted by the strong fluorescence originating from to the accumulation of high levels of the fluorescent protein in the endo-membrane compartments and plasma membrane. We report using of cold-pretreatment and photo bleaching for more detail fluorescent microscopy observation of the sub-cellular compartmentalization and secretion pattern of the A9-GUS-GFP protein in leaf cells of the transgenic N. benthamiana plants.

Keywords: GFP, signal peptide, secretion, confocal microscopy, FRAP technology

Introduction
Live cell-imaging of chimeric proteins composed of the green fluorescent protein (GFP) and a protein of interest is an important tool for studying sub-cellular localization, translocation and the function of signal peptide sequences (3, 5, 10). These methods have been applied to the investigation and dissection of the plant secretory pathway, which is composed of several functionally distinct membrane-bound compartments including endoplasmic reticulum (ER) and the Golgi apparatus (GA), (3, 6, 7). Observations of chimeric GFP proteins has revealed determinants that are important in the function of individual compartments and has revealed how these compartments are formed and how they interact with each other (2, 4, 8, 9). In a previous study we report that the fusion of the signal peptide region from the Arabidopsis A9 anther specific gene to a GUS-GFP reporter protein results in its secretion out of the cell via the ER in a cell suspension of Nicotiana benthamiana (14). Microscopy of Brefeldin A treated samples provides evidence that the reporter protein is transported through the classical secretory pathway involving ER and GA. Detailed observations of the sub-cellular translocation and exocytosis of the chimeric A9-GUS-GFP protein have been largely restricted by the strong fluorescent signal originating from the endo-membrane compartments and plasma membrane due to the accumulation of high levels of the chimeric protein.

Here we present data, using fluorescence microscopy techniques, of cold-pretreated and photobleached samples from transgenic N. benthamiana plants expressing the A9-GUS-GFP protein, allowing for more detailed observations of its sub-cellular compartmentalization and secretion pattern.

Materials and Methods

Plant Material
Seeds from transgenic N. benthamiana plants constitutively expressing the A9-GUS-GFP chimeric protein, as previously was described (14), were sterilized with 25% bleach for 15 min and plated on half strength MS media with 1.5% agar. The Petri dishes with the plated seeds were incubated vertically in continuous light at 22°C. Seven day old seedlings were used for microscopy directly or after 48 h cold pretreatment on dark at 4°C.

Fluorescent and Confocal Microscopy
Microscopy was carried out using the following imaging systems: For Fig. 1a-d and Movie 1, a Leica DM5500 microscope fitted with a Photometrics Cascade II 512B EMCCD camera (Photometrics, UK) and equipped with a HCX PL APO x100 oil NA 1.4 - 0.7 objective (part no. 506220, Leica Microsystems). GFP was observed using filter cube part no. 41028 (Chroma Technology Corp, Rockingham, VT). For Fig. 1e-h and Movie 2, a Leica SP5 TCS upright confocal equipped with a 63x oil objective. The 488 nm laser line was used to visualize the GFP. Images have been contrast enhanced to aid visualization using ImageJ to yield no more than 0.5% saturated pixels.

Results and Discussion
The transgenic N. benthamiana lines used in this study have been previously selected for high level expression of the chimeric fluorescent protein A9-GUS-GFP, (14). The presence, at the N terminus of the GUS-GFP protein, of the signal peptide region from the A9 anther specific gene from Arabidopsis directs the reporter protein to the ER, where it enters in the secretory pathway. The accumulation of large amount of the
fluorescent protein in the ER and at the cell surface, allows for clear observation of the ER network and apoplast of the leaf cells using fluorescence microscopy but at the same time do not allow a detailed study of the interaction between different endomembrane compartments, due to the strong steady fluorescence (14). We assume that application of a cold pretreatment or photo bleaching of the samples could reduce the likelihood of saturated fluorescence signal derived from the excessive protein accumulation and will make possible more clear observations of the newly translocated or secreted fluorescent protein.

**Cold pretreatment**

Cold incubation of the samples prior to fluorescence microscopy were used successfully in several studies on mammalian cell cultures where it slows protein secretion and leads to the accumulation of the GFP tagged secretory proteins mainly in the Golgi, (12). In plants, the cold treatment of leaves expressing the signal peptide fused GFP variants targeted to the secretory pathway also appears to prevent trafficking from the ER, but the incubation at 4°C for a period longer than 12-18 h resulted in the resumption of secretion possibly due to cold acclimatization of the plant, (1). In the present study, the microscopy observation of cold pretreated seedlings, expressing the A9-GUS-GFP protein, shows very low level of GFP fluorescence in the leaf cells. Recovery of GFP fluorescence began in a leaf guard cell 20 minutes after transfer to room temperature. The overall observation of the leaf guard cell shows a widely distributed polygonal network of cortical ER and multiple bright Golgi stacks closely associated with the ER, (Fig. 1a). To analyze the dynamics of the ER- Golgi interaction, time lapse images were collected at 330 msec and viewed as a movie sequence presented in the supplemental data (SD: Movie 1). The movie shows highly mobile, A9-GUS-GFP labeled Golgi stacks which always remained closely associated with the ER tubules during their oscillation. Closer observation of the direction and trajectory of the individual stack motions along the ER tubules, (Movie 1, Fig. 1b-d) shows a range of movements including: i) rapid appearance of new ER tubules within the unchanged stronger fluorescent polygonal ER network, ii) Rapid stop-and-go movements of Golgi stacks along individual ER tubules, iii) direct tracking of Golgi stacks along the elements of ER polygonal network. The observed GFP fluorescent pattern reveal that the sub-cellular translocation of the chimeric A9-GUS-GFP protein within plant secretory pathway is associated with close, intensive and dynamic interaction between the ER and Golgi compartments. Several recent studies have demonstrated that ER and Golgi form a highly structured and dynamic complex involving export sites behaving as secretory units in the plant cell, (4, 6, 11). The data from the present study shows a similar manner of interaction of the A9-GUS-GFP-labeled ER and Golgi based on close dynamic association between the two compartments within putative export sites.

Fig. 1. Expression of chimeric A9/GUS/GFP protein in *N. benthamiana* leaf cells

(a-d) fluorescent microscopy of leaf guard cells from *in vitro* seedlings 20–30 min after incubation at room temperature following pretreatment on dark at 4°C for 48h. (b-d) images of three sequential frame captures from Movie 1 shows the movement of individual Golgi stack along a single ER strand. Arrows show a Golgi stack moving along the ER, tubule. Scale bar = 100 μm

(e-h) confocal laser scanning microscopy of leaf epidermal cell after photo bleaching showing secretion of the reporter protein and movement through the apoplast. (f-h) images of three sequential frame captures from Movie 2 shows the secretion and movement around the cell of large quantities of the reporter protein. Arrows show a large quantity of GUS/GFP moving around the cell after exocytosis. Scale bar = 100 μm
Photo-bleaching

Exocytosis is the final event for the proteins translocated through the secretory pathway. It involves the fusion of the secretory vesicle membrane with the plasma membrane and release outside of the cell of the vesicle cargo, (15). The chimeric A9-GUS-GFP protein is efficiently secreted from the N. bentamiana cells (14) and in leaf tissue it is accumulated in large quantities in the apoplast. This significantly hampers the observations of the exocytotic activity and mobility of the secreted reporter protein outside the cells. To study further the mode of secretion of the A9-GUS-GFP protein we used a photo-bleaching-based technique. Although high intensity laser bleaching does not abolish fluorescence from the accumulated reported protein, it was sufficiently reduced to allow observation of the secretion of the reporter protein to the apoplast. The movie prepared from time lapse images collected at 60 sec intervals and the sequential frame captures (SD: Movie 2, Fig. 1 e-h) show a result from exocytosis event where a larger amount of GUS-GFP reporter protein is secreted in the apoplast and further move between the adjacent cell.

The use of the signal peptides for production of recombinant proteins from plants and cell cultures require selection of transgenic lines highly expressing the protein of interest. At the same time the expressed protein has to be efficiently directed for secretion outside of the cell or targeted to specific cell compartment suitable for the applied protein purification procedure. The fusion of the protein of interest with GFP reporter protein and study of the sub-cellular localization and traffic of the expressed fluorescent chimeric protein could provide very useful information for optimization of the protein expression sub-cellular accumulation pattern. Quite often the high level of expression of GFP-fused proteins resulted in strong fluorescence in the cell and tissue compartments. It significantly hampers the fluorescent microscopy observations of the translocation of the GFP tagged protein because of the high fluorescent background from already accumulated fluorescent protein. Here we employ cold pretreatment and photo bleaching of the samples prior fluorescent microscopy in order to reduce the fluorescent background from accumulated GUS-GFP protein. The both treatment resulted in reducing of the fluorescent background to levels sufficient for a detail observation of ER/Golgi interaction after cold treatment and secretion of the fluorescent protein in apoplast after photobleaching. Employing of these pretreatment will allow efficient application of fluorescent microscopy for observation of the sub-cellular localization and traffic of GFP-tagged proteins of interest directed to ER following fusion with A9-signal peptide region.

Supplemented Data, (The movies could be seen using of QuickTime 7 software, http://www.apple.com/quicktime. The movies could be also access at http://bulgenom.abi.bg/, (13):

Movie 1. Movie sequence of time lapse images of leaf guard cells from cold pretreated N. benthamiana seedlings expressed A9/GUS/GFP. The images are collected at 330 msec.

Movie 2. Movie sequence of time lapse images of leaf cells from N. benthamiana seedlings expressed A9/GUS/GFP after photo bleaching for 2 min at maximal laser lower. The images are collected at 60 sec.

REFERENCES