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ORIGINAL PAPER

Expression of a glycosylated GFP as a bivalent reporter in exocytosis

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Abstract The complex-type N-linked glycans of plants differ markedly in structure from those of animals. Like those of insects and mollusks they lack terminal sialic acid(s) and may contain an α-(1,3)-fucose (Fuc) linked to the proximal GlcNAc residue and/or a β-(1,2)-xylose (Xyl) residue attached to the proximal mannose (Man) of the glycan core. N-glycosylated GFPs were used in previous studies showing their effective use to report on membrane traffic between the ER and the Golgi apparatus in plant cells. In all these cases glycosylated tags were added at the GFP termini. Because of the position of the tag and depending on the sorting and accumulation site of these modified GFP, there is always a risk of processing and degradation, and this protein design cannot be considered ideal. Here, we describe the development of three different GFPs in which the glycosylation site is internally localized at positions 80, 133, or 172 in the internal sequence. The best glycosylation site was at position 133. This glycosylated GFPgl133 appears to be protected from undesired processing of the glycosylation site and represents a bivalent reporter for biochemical and microscopic studies. After experimental validation, we can conclude that amino acid 133 is an effective glycosylation site and that the GFPgl133 is a powerful tool for in vivo investigations in plant cell biology.

Keywords GFP · Glycosylation · Protoplast · Secretion

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Introduction

Posttranslational modifications (PTMs) of proteins in eukaryotes include N- and O-glycosylation. The basic structure of N-linked glycans is similar in plants, animals, and yeasts; however, the complex-type N-linked glycans of plants differ markedly from those of mammals. Like those of insects and mollusks they lack terminal sialic acid(s). On the other hand, they may contain an α-(1,3)-fucose (Fuc) linked to the proximal GlcNAc residue and/or a β-(1,2)-xylose (Xyl) residue attached to the proximal mannose (Man) of the glycan core, similar to what is found in insects and mollusks (Staudacher et al. 1999). Plant-specific modifications have long been a major limitation to the extensive use of plant-made pharmaceuticals in human therapy (Saint-Jore-Dupas et al. 2007). In fact, these additional epitopes are thought to have antigenic and/or

allergenic properties. Little is known about O-linked glycans in plants. Recently, plants and algae have started to be used for the production of therapeutic proteins. More information is thus required on glycosylation in plants and its manipulation, if these therapeutic proteins are to be effectively used.

Processing of N-linked oligosaccharides in the secretory pathway requires the sequential action of numerous glycosidases and glycosyltransferases (Saint-Jore-Dupas et al. 2006). *N*-glycan processing enzymes are specifically distributed along membranes in the early secretory pathway, their fine sub-cellular compartmentation being ensured by both their trans-membrane domain (TMD) length and by signals in their cytoplasmic tails (Brandizzi et al. 2002).

N-glycosylated GFPs were used in previous studies to report on membrane traffic between the ER and the Golgi apparatus in tobacco epidermal cells. In one case, glycosylation of GFP was obtained within a ten-residue linker at the N-terminus of the protein (Batoko et al. 2000) and was further used as a fusion with membrane proteins (daSilva et al. 2005, 2006). More recently, Sohn et al. (2007) used a GFP with a high-mannose oligosaccharide linked to a site within the C-terminal vacuolar sorting determinant, previously produced in a related laboratory (Wilkins et al. 1990). In animal cells Meder et al. (2005) used a GFP with a 16-aa glycosylation tag from human rhodopsin (Bulbarelli et al. 2002).

All these published cases used glycosylated tags at the N- or C-termini of GFP. However, since trafficking signals are often localized in terminal (pro)peptides, an additional glycosylation site and the associated linker may affect sorting. In addition, termini are often processed, which can lead to the loss of the glycan. As a consequence glycosylation tags at the N- or C-terminus are not ideal to study trafficking and maturation of proteins.

Here, we describe the production of three different GFPs in which a N-glycosylation site was introduced into internal sequences that correspond to loops protruding from of the β-barrel structure of the GFP. The best glycosylation site was identified as the amino acid 133: gl133.

Materials and methods

Constructs

Glycosylation sites were engineered by PCR into the coding sequence for secGFP (plasmid pSGFP5, Di Sansebastiano et al. 1998). The mutagenic primers each included a nearby restriction site, which allowed easy reconstruction of the whole length coding sequence. For position 80 (gl80) the peptide sequence was changed from MKRHDFF to MK**N**H**T**FF (oligo p80f: atc ata tgg aag aac cac acc ttc, including an NdeI site), for position 133 (gl133), the sequence was changed from FKEDGNIL to FKE**N**G**S**IL (oligo p133f: aatc gat ttc aag gag aac gga agc atc ctc, including a ClaI site), for position 172 (gl172) the sequence was changed from EDGGV to E**N**D**S**GGV (oligo p172r: tga tca gcg agt tgc acg ccg ccg ctg tcg ttt tcg, including a BclI site).

The mutations were introduced into the pSGFP5 plasmid as NdeI-ClaI (gl80), ClaI-PstI (gl133) and NcoI-BclI (gl172) fragments. This produced the coding sequences for secGFPgl80, secGFPgl133, and secGFPgl172, respectively.

121T was described previously and corresponds to the truncated version of NtSYP121 previously named SP2 (Leucci et al. 2007). It was recently renamed 121T for clarity (Di Sansebastiano et al. 2006; Rehman et al. 2008), to be distinguished from other SYPs mutants.

Transient expression in protoplasts and in leaves

Transient expression in *Nicotiana tabacum* (cv SR1) epidermal leaf cells was performed as previously described (Brandizzi et al. 2002) using 3- to 4-week-old plants and *Agrobacterium tumefaciens* (strain GV3101 p2260) infiltration. The suspension of transformed agrobacteria was inoculated at a concentration of $OD_{600} = 0.3$ at the lower epidermis after making a small lesion to facilitate the infiltration. For plasmolysis, the tissue was submitted to 2% NaCl for 15 min prior to observation with confocal microscope.

Transient transformation of tobacco protoplasts was performed as previously described (Rehman et al. 2008) digesting leaves from 2- to 3-week-old plants grown at 26°C in continuous light. Leaves were surface-sterilized in a 1:5 bleach bath for 40 s and rinsed in distilled water. The transformation efficiency of the reported experiments was always above 40%.

Biochemical analysis of GFP

Transformed protoplasts were harvested by gentle centrifugation at 65 g with no break, diluting incubation medium with two volumes of W5 (NaCl 154 mM; $CaCl_2$ 125 mM; KCl 5 mM; glucose 5 mM). A fifth of the medium was saved for analysis (extra cellular fraction). Protoplasts were suspended in extraction buffer (1× TBS, proteinase inhibitors cocktail "Complete", Roche, Basel) with 2% SDS to obtain an intracellular fraction for direct SDS-PAGE: cells were lysed by three consecutive freeze–thaw cycles. Insoluble residues were removed by centrifugation for 5 min at 14,000*g*. Extra- and intracellular fractions were precipitated with 3 volumes acetone. Pellets were suspended in volumes proportional to the original sample for SDS-PAGE.

Western blotting, immunolabeling, pulse-chase, and immunoprecipitation were performed as previously described (Leucci et al. 2007).

GFP detection was obtained using anti GFP (Molecular Probes A6455) primary antibodies, with 1:10,000 dilution; anti-rabbit secondary antibodies coupled to peroxidase (SIGMA) were used.

Inhibition of *N*-linked glycosylation in vivo was performed with 5 mg/L tunicamycin (Sigma) added to the protoplasts immediately after transformation for the whole incubation period.

We used the endoglycosidase H (EndoH) deglycosylation kit (Boehringer Mannheim), according to the manufacturer's instructions, to remove high-mannose-type Asn-linked glycan chains from glycoproteins.

Pulse-chase and immunoprecipitation

For pulse-chase experiments, freshly transformed protoplasts were pulse-labeled 2 h after transformation (following the washing step) with 100 μCi mL^{-1} of a PRO-MIX (Amersham Biosciences) solution (70% ^{35}S-methionine and 30% ^{35}S-cysteine). Pulse labeling, chase, and immunoprecipitation were performed as detailed by Leucci et al. (2007) using the same antibodies used for Western-blotting at 1:1,000 dilution.

Statistical analysis

Data about the secretion index of the glycosylated and non-glycosylated secGFPgl133 reported in the text are averages from three independent experiments as specified. The band intensity was evaluated, after digital acquisition, with Kodak 1D 3.6 software. The significativity of the data was calculated by *t* test ($P < 0.05$).

Confocal microscopy

Protoplasts transiently expressing the various GFPs were observed by fluorescence microscopy in their culture medium at different times after transformation, using a confocal laser-scanning microscope LSM Pascal Zeiss. GFP was detected with the filter set for FITC (505–530 nm) while chlorophyll fluorescence was detected with the filter set for TRITC (>650 nm). For imaging in leaf epidermis we used a Leica TCS sp2 (Wetzlar, Germany). We observed the fluorescence 24–72 h after transient expression in tobacco epidermal cells from a small piece of leaf cut off from the plant and mounted in water between slide and cover slip. 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. That is, we ensured that the signal collected for one fluorophore remains unchanged when the laser line used to excite the second fluorophore is switched off. Images were mounted using Adobe Photoshop 7.0 software (Mountain View, CA).

Results

Preparation of glycosylated GFPs as reporters of processing events in the Golgi

To investigate the transit of chimerical proteins through the Golgi, we introduced N-glycosylation sites at different positions of the GFP sequence. The GFP structure forms of a β-barrel with 11 parallel β-strands and an internal α-helix on which the active fluorophore spontaneously forms. Both N- and C-termini are on the same side of the barrel. We selected three positions exposed on external loops either on the same side as the termini (gl80) or on the opposite side (gl133 and gl172), which had been described as tolerant to insertion (Abedi et al. 1998). The glycosylation site was engineered in the sequence of a secreted GFP, secGFP (Leucci et al. 2007) producing the following changed sequences: –MK**NHT**FF– for gl80, –KE**NGS**IL– for gl133 and –IE**NDS**GG– for gl172. In the ER, *N*-glycans are added to proteins and processed to a high-mannose-type glycan (5-9 Man). If the protein transits through the Golgi, the *N*-glycans can undergo various modifications that lead to complex-type *N*-glycans.

The three candidate glycosylated secGFP and the original non-glycosylated construct were transiently expressed in tobacco mesophyll protoplasts. Total protein extracts were then analyzed by SDS-PAGE. Glycosylation could be detected as an increased molecular weight; i.e., lower electrophoretic mobility on polyacrylamide gel than the non-glycosylated secGFP (Fig. 1). In order to confirm that the GFP is glycosylated, transformed protoplasts were incubated with tunicamycin, a strong *N*-glycosylation inhibitor. This pharmacological treatment should lead to a shift in band migration of glycoproteins. The results were different for the three tested glycosylation sites: while secGFPgl80 was totally glycosylated, secGFPgl133 was only partially glycosylated, and secGFPgl172 was not glycosylated at all (Fig. 1).

The distribution of the three glycosylated variants between the intracellular and extracellular fractions was analyzed, revealing that secGFPgl80 was not secreted at all while the other two forms were secreted similarly to the unmodified secGFP (Fig. 1).

We analyzed the transiently transformed protoplasts by confocal microscopy (Fig. 2). A fluorescence pattern similar to secGFP (Fig. 2a) was observed for secGFPgl133 (Fig. 2b) and secGFPgl172 (Fig. 2c). SecGFPgl80 was not

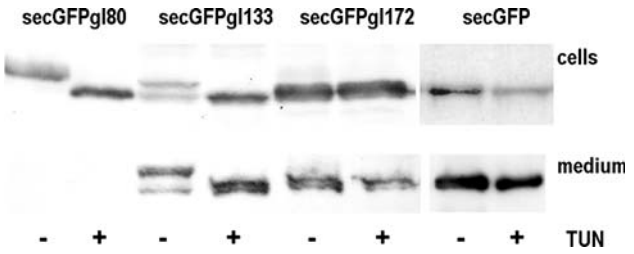

Fig. 1 Glycosylation and secretion of glycosylated GFPs. Western blot analysis of GFP glycosylation in intracellular (*cells*, *upper line*) and extracellular (*medium*, *lower line*). Inhibition of glycosylation by tunicamycin (*TUN*) reduces the apparent molecular weight of successfully glycosylated GFPs. SecGFPgl80 is exclusively accumulated in the intracellular fraction, while the other forms are secreted into the medium

fluorescent at all even after tunicamycin treatment (not shown), indicating that the mutation itself, rather than the presence of a glycan at this position was responsible for the absence of fluorescent signal. It was only possible to detect little fluorescence from secGFPgl80 by incubating transformed protoplasts for 2–8 h at 8°C prior to observation. The obtained fluorescence was in the endoplasmic reticulum, which was slightly perturbed by microtubular structure alteration caused by the cold treatment (Fig. 2d) (Schwarzerová et al. 2006). The same weak ER labeling after cold treatment was observed with the other secGFP variants such as secGFPgl133 (Fig. 2e), secGFPgl172, and the control secGFP (not shown).

We conclude that the glycosylation site mutation at position 80 disturbed protein trafficking but also interfered with proper folding, preventing the GFP from fluorescing efficiently. SecGFP172 fluoresces but unfortunately is not glycosylated while secGFPgl133 is partially glycosylated and fluoresces.

We then asked if secGFPgl133 was affected by the alteration of vesicular traffic due to the specific inhibition of SYP121 function. The SNARE SYP121 is required by a sub-population of specific secretory vesicles. Its dominant negative mutant 121T (formerly called SP2) inhibits specific exocytotic steps but does equally affect all secreted molecules (Leucci et al. 2007). The effect of 121T on secGFPgl133 sorting was similar to that described by Leucci et al. (2007), inducing the visible accumulation of GFP in vacuolar-like compartments associated to the ER (Fig. 2f).

Comparison of glycosylated and non-glycosylated GFPs

The partial glycosylation of the variant secGFPgl133 resulting in the presence inside the cell of both glycosylated and non-glycosylated GFP, was exploited to investigate further the specific effect of the glycan on trafficking efficiency. To detect a possible effect on the secretion kinetic a pulse-chase analysis was performed. Transiently transformed protoplasts were pulse labeled for 2 h and

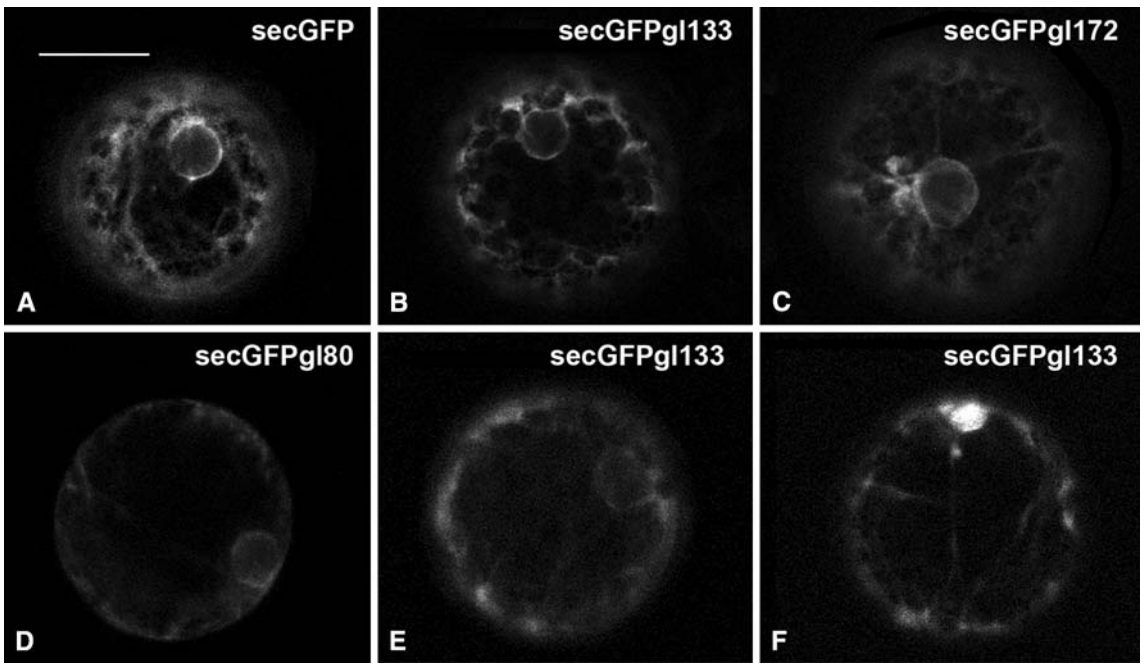

Fig. 2 Confocal images of tobacco protoplasts transiently expressing secGFP variants. **a** SecGFP 18 h after transformation; **b** secGFPgl133 18 h after transformation; **c** secGFPgl172 18 h after transformation; **d** secGFPgl80 fluorescence was visible in the ER only after 6-h incubation at 4–8°C following 12-h incubation after transformation; **e** secGFPgl133 after 6-h incubation at 4–8°C following 12 h of normal incubation; **f** secGFPgl133 accumulation pattern after 18-h co-expression with 121T. *Scale bar* 20 μm

chased up to 12 h. At different times, intra- and extracellular fractions were separated and analyzed by SDS-PAGE. The ratio of secreted versus retained fraction was then calculated for each of the two forms of secGFPgl133. The kinetics of secretion of the glycosylated and non-glycosylated forms was found to be identical (Fig. 3). After 12 h of chase, the secretion indexes of the glycosylated and non-glycosylated secGFPgl133 were not significantly different (1.7 ± 0.21 and 1.6 ± 0.19, respectively; $n = 3$; $P > 0.6$), indicating that glycosylation does not alter the rate of secretion.

We then further questioned the pathway used by both forms of secGFPgl133 by pulse-chase analysis of secretion in presence of 121T. We found that transport of the glycosylated and non-glycosylated secGFPgl133 was affected similarly by 121T (statistical significance: $P > 0.5$) as a similar shift in the secretion index was observed for the glycosylated (1.8 ± 0.12 fold reduction; $n = 3$) and the non-glycosylated (1.6 ± 0.12 fold reduction; $n = 3$) forms (Fig. 3).

Glycosylation on gl133 as an indicator for transit via the Golgi apparatus

In the Golgi apparatus, high-mannose-type *N*-glycans can undergo several modifications leading to complex-type *N*-glycans, which are resistant to endoglycosidase H (EndoH). EndoH resistance thus demonstrates the transit of a glycoprotein through the Golgi. EndoH sensitivity, however, is less informative, as not all *N*-glycans are modified even if they transited through the Golgi (Lerouge et al. 1998).

The secreted marker secGFPgl133 present in the incubation medium or in the intracellular soluble fraction was digested with EndoH. Interestingly, the extracellular glycosylated form was resistant to EndoH digestion while the intracellular glycosylated form was sensitive (Fig. 4).

Glycosylation at the gl133 site does not interfere with GFP subcellular localization

Since it is impossible to visualize fluorescence after secretion in protoplasts, we expressed secGFPgl133 in tobacco epidermal cells from leaves and we performed a western blot analysis 2 days after transformation. As shown in Fig. 5a, secGFPgl133 was fully glycosylated. We also treated the extract with EndoH and found secGFPgl133 to be resistant (Fig. 5b). This indicates that the secGFPgl133 is glycosylated in leaf epidermal cells as in protoplast, even better, with similar properties of this glycan.

We then observed the transformed cells under the microscope. Compared to the localization of secGFP in epidermal cells (Fig. 6a), secGFPgl133 was also massively

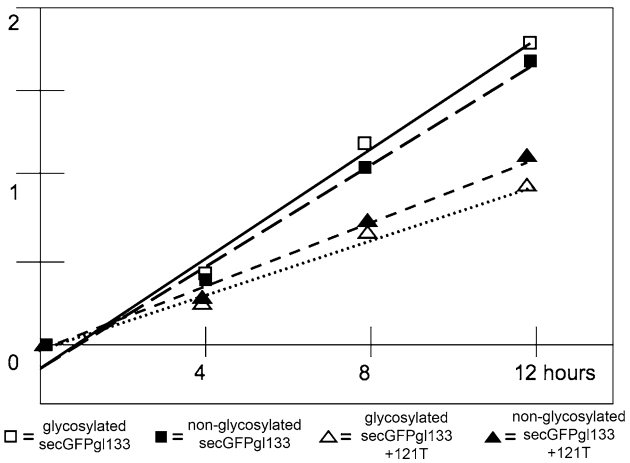

Fig. 3 Reduction of the secretion rate of glycosylated and non-glycosylated secGFPgl133 by the dominant negative SNARE derivative 121T. Pulse-chase labeling of tobacco protoplasts transiently expressing secGFPgl133 alone (*squares*) or co-expressing 121T (*triangles*); *open symbols* glycosylated, *full symbols* non-glycosylated. The figure represents the natural logarithmic plot of the secretion index (ratio between the extracellular and intracellular fractions, *Y* axis) derived from the single pulse-chase experiment closer to average values. Two hours after transformation, the protoplasts were pulse-labeled for 2 h and chased for the indicated time. The effect of 121T was similar on glycosylated and non-glycosylated forms and reproducible in three experiments. No statistical difference appeared significant. Slopes: *open squares* 0.315, *full squares* 0.2845, *open triangles* 0.191, *full triangles* 0.1585

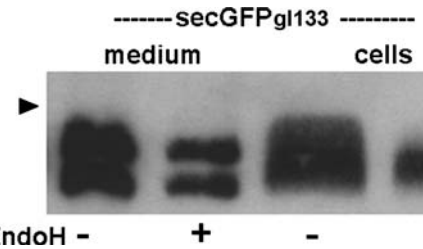

Fig. 4 EndoH sensitivity assay of glycans on secGFPgl133 transiently expressed in protoplasts. Soluble extracts from incubation medium or lysed protoplast (cells) after 18 h of transient expression were treated with EndoH and analyzed by western blotting. The *arrow* indicates the marker MW corresponding to about 32 kDa

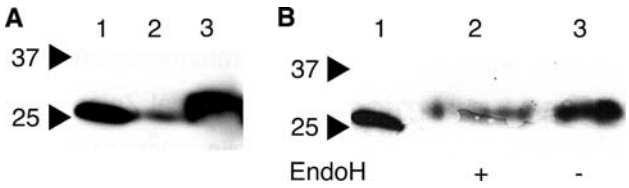

Fig. 5 EndoH sensitivity assay of glycans on soluble secGFPs transiently expressed in tobacco leaves. **a**, **b** Western blot analysis of a total protein extract from tobacco leaves expressing secGFPgl133 (*lanes A3*, *B2* and *B3*) or secGFP (*lane A2*). *Lanes A1* and *B1*: recombinant GFP. The *arrows* indicate marker MW in kDa

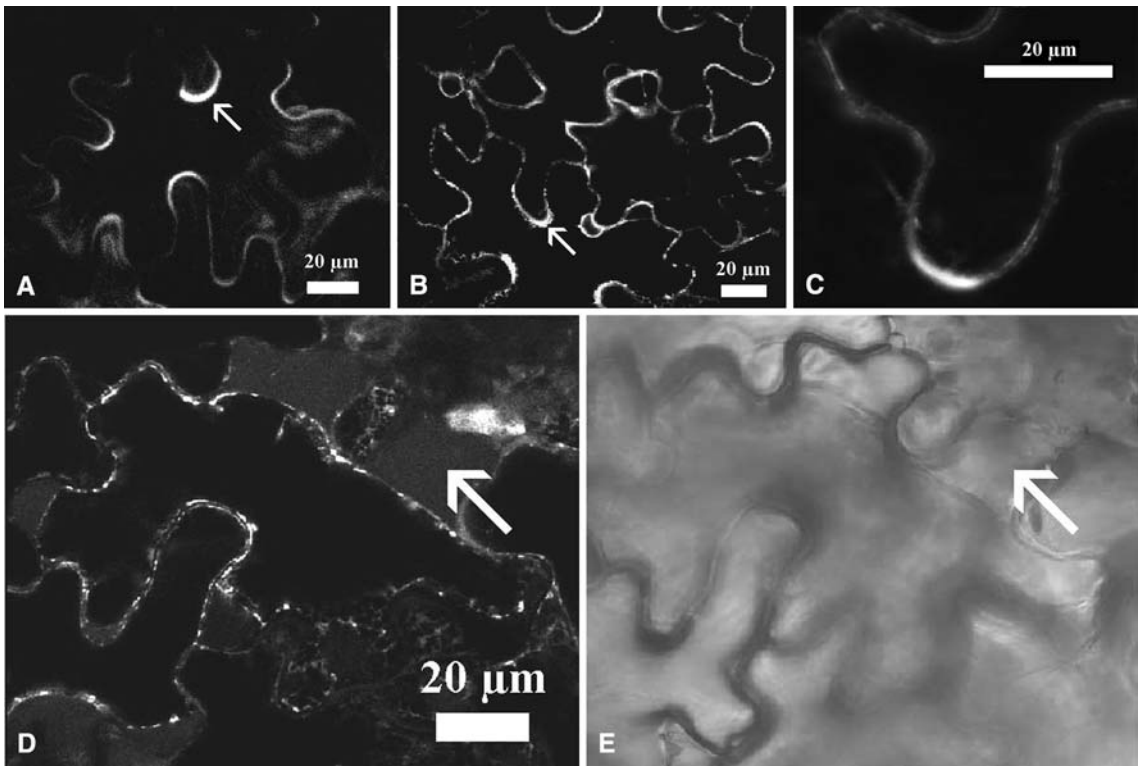

Fig. 6 Fluorescent pattern of secGFP variants in tobacco epidermal cells. **a** SecGFP distribution after 2 days of transient expression, the arrow evidences apoplastic accumulation; **b** secGFPgl133 distribution after 2 days of transient expression, the *arrow* evidences apoplastic accumulation; **c** higher magnification of secGFPgl133 fluorescent pattern; **d** fluorescent pattern of secGFPgl133 in plasmolysed epidermal cells; **e** bright field image of the same tissue area

secreted (Fig. 6b, c) as it is detected in the apoplast. Since we could also detect some fluorescence in the ER, we wanted to make sure that the peripheral staining was really due to secreted GFPgl133. For this, we treated the cell with 2% NaCl, and we looked at plasmolysed cells. As seen on Fig. 6d, e, GFPgl133 fluorescence is truly in the apoplast and periplasm (arrow).

Discussion

GFP was used as scaffold for post-translational modifications to monitor the glycosylation. Three glycosylation sites were engineered: at positions 80 (gl80), 133 (gl133), or 172 (gl172) of the protein, within three different loops on either faces of the β-barrel. The efficiency of glycosylation and the effects on localization were compared with the secreted reporter secGFP to choose the best site for further use. The gl80 site was efficiently glycosylated but caused the intracellular retention of the glycoprotein. The lack of fluorescence for secGFPgl80 suggested that mistargeting was due to misfolding. In fact, some fluorescence could only be recovered after incubation of cells at low temperature and was confined to the ER. These observations are all compatible with incorrect folding of the GFP.

The protein secGFPgl172 was not glycosylated and the use of this GFP variant was abandoned.

GFPgl133 behaved as a good marker in two different transient expression systems even if glycosylation was sometimes only partial. The fact that secGFPgl133 is fully glycosylated in epidermal cells and fluoresces fully rules out the possibility that only the non-glycosylated fraction was fluorescent in protoplast.

Inhibition of N-linked glycosylation by tunicamycin caused the upper band to disappear, confirming that the site was glycosylated. The presence of two forms of secGFPgl133 (glycosylated and non-glycosylated) in protoplasts can be explained by the saturation effect due to massive synthesis derived from the presence of multiple copy of the expression vector, directly introduced in the cell by PEG transformation. While it may be considered an undesired phenomenon, the partial glycosylation of GFP in protoplasts gave us the opportunity to compare directly the fate of the two forms (glycosylated and non-glycosylated), to reveal if the glycosylation modifies protein transport. Both forms of secGFPgl133 were secreted with equal kinetics and efficiency. A dominant negative mutant of

SYP121 (121T), which inhibits specific exocytic steps (Leucci et al. 2007), was used to inhibit the secretion of secGFPgl133. 121T inhibits exocytosis by a specific subpopulation of secretory vesicles and does not affect equally all secreted molecules, e.g., polysaccharide secretion is much less affected than protein secretion (Leucci et al. 2007). Pulse-chase analysis of secGFPgl133 secretion indicated that the rate of secretion of the glycosylated and non-glycosylated GFP are affected in a similar way. This effect indicated that the presence of a glycan did neither change the final destination of GFP nor the last step of exocytosis. The secretion index at 12 h was stable, with a SD of 0.2 but was higher at intermediate time intervals (highest SD = ±0.5 at 8 h interval; $n = 3$) probably because protoplasts were obtained independently, in different periods of the year, and because of sampling errors more relevant in the intracellular fraction. To facilitate data representation, we therefore produced Fig. 3 with data from one single experiment closer to average values (coincident at time 12 h). Nevertheless, the influence of glycosylation and of 121T co-expression on secretion of SecGFP was extremely reproducible for the three experiments performed. Indeed, the ratio of glycosylated form/non-glycosylated form was 1.03 ± 0.03 in control conditions and 0.96 ± 0.06 when 121T was co-expressed.

Moreover, this experiment indirectly confirmed that the glycan was not removed during sorting. Thus, the problem of de-glycosylation described previously (daSilva et al. 2006) with the N-term glycosylated GFP (Batoko et al. 2000) may be here overcome.

The EndoH sensitivity of the new glycosylated reporter was tested. It is known that before the action of mannosidase II in the Golgi apparatus, *N*-glycans are sensitive to hydrolysis by EndoH (Dupree and Sherrier 1998). It is not known where mannosidase II acts in the Golgi stack, but the medial-Golgi stacks are a likely location (Staehelin and Moore 1995; Dupree and Sherrier 1998). It was observed that the glycan of GFP extracted from the intracellular fraction was EndoH-sensitive, whereas the glycan of GFP extracted from the medium was EndoH-resistant.

The EndoH essay indicates that the gl133 glycosylation site is compatible with Golgi modifications and that the glycosylated secGFPgl133 passed through the Golgi apparatus to reach its final destination. The partial EndoH sensitivity of intracellular secGFPgl133, present in protoplasts, most probably corresponds to the presence of an ER pool of not yet exported glycoproteins.

Transient expression in tobacco leaves changed the efficiency of glycosylation. In the total extract from agro-infiltrated leaves all GFPgl133 appeared glycosylated. This may be explained by the lower rate of protein synthesis in epidermal cells compared to protoplasts. Such lower synthesis does not saturate the glycosyltransferases involved in the process. As observed in protoplasts, the glycosylated secGFPgl133 accumulated in leaves was entirely EndoH resistant. At the same time the result is in contradiction with the observation of fluorescence in the ER of epidermal cells at the time of extraction, and we expect this fluorescence is due to EndoH-sensitive glycosylated GFP. In fact, it is possible that the fraction of fluorescent GFP in the ER represents a minority of the whole GFP accumulated in the tissue and the corresponding EndoH-sensitive protein may not be detected on the western-blot. Similar situations in which the non-fluorescent GFP is the large majority of the protein accumulated were already described (Di Sansebastiano et al. 2004, 2007).

The transient expression system in leaves allowed the conclusion that glycosylation at gl133 site does not interfere with the subcellular localization since the glycosylated secGFPgl133 is sorted to the apoplast like secGFP.

Based on these results, GFPgl133 site can be considered a reporter with high potential in the study of protein sorting. This reporter may be used to study protein sorting in different species with a comparative approach (Di Sansebastiano et al. 2007; Verweij et al. 2008) as well as transmembrane protein topology (Di Sansebastiano et al. 2006).

GFPgl133 presents several advantages compared to other glycosylation tags attached to the N- or C-termini of GFP. With these other tagged GFPs it is always difficult to distinguish between the removal of the glycan or of the whole tag, since both cause a band shift in western blot analysis due to molecular weight changes.

In summary, we conclude that GFPgl133 has an efficient glycosylation site and is a powerful new tool for in vivo investigations in plant cell biology.

This construct may contribute to a refined knowledge of the sites of glycan modification within the secretory pathway of plants, which may open a wider spectrum of possibilities with important biotechnological applications (Petruccelli et al. 2006).

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