The N-terminal 77 amino acids from tobacco *N*-acetylglucosaminyltransferase I are sufficient to retain a reporter protein in the Golgi apparatus of *Nicotiana benthamiana* cells

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Abstract In order to investigate sequences of tobacco *N*-acetylglucosaminyltransferase I (GnTI), involved in targeting to and retention in the plant Golgi apparatus the cytoplasmic transmembrane stem (CTS) region of the enzyme was cloned in frame with the cDNA of the green fluorescent protein (gfp) and subsequently transiently expressed in *Nicotiana benthamiana* plants using a tobacco mosaic virus (TMV) based expression vector. Confocal laser scanning microscopy showed small fluorescent vesicular bodies in CTS-gfp expressing cells, while gfp alone expressed in control plants was uniformly distributed in the cytoplasm. The CTS-gfp fusion protein colocalised with immunolabelling observed by an antibody specific for the Golgi located plant Lewis a epitope. Furthermore, treatment with brefeldin A, a Golgi specific drug, resulted in the formation of large fluorescent vesiculated areas. These results strongly suggest a Golgi location for CTS-gfp and as a consequence our findings reveal that the N-terminal 77 amino acids of tobacco GnTI are sufficient to target to and to retain a reporter protein in the plant Golgi apparatus and that TMV based vectors are suitable vehicles for rapid delivery of recombinant proteins to the secretory pathway.

Key words: Tobacco *N*-acetylglucosaminyltransferase I; Plant Golgi targeting/retention and retention sequences

1. Introduction

In mammalian cells, glycosylation enzymes are located within the Golgi cisternae in the same sequence in which they act to modify oligosaccharide substrates (reviewed in [1]). The lack of sequence similarities between different glycosyltransferases suggests that multiple signals may be involved in determining their specific location (for a review see [2]). Essential signals for targeting to the Golgi compartment have been identified in the transmembrane domains (TMD) and surrounding sequences, the so called cytoplasmic transmembrane stem (CTS) regions of the enzymes. Although the Golgi retention mechanism(s) is (are) not known in detail it appears to be conserved to some extent as the Golgi targeting and retention signals of mammalian glycosyltransferases are functional in plants [3,4].

Expression of the reporter protein gfp (green fluorescent protein) has been achieved in a variety of plants. A major advantage of gfp is the maintenance of its fluorescence when fused to other proteins. This property has been exploited to trace fusion proteins in plant cells, as for instance in studies on the targeting of heterologous proteins to the plant ER and to the Golgi [3,5]. However, targeting and retention of endogenous plant Golgi proteins has to date not been investigated, since the first complete sequence of a plant Golgi resident protein was elucidated only recently [6].

For rapid expression of recombinant proteins in plants, virus based vectors have been found extremely useful. In particular a potato virus-X based vector was used for the expression of recombinant proteins to study targeting in plant cells [3,5].

To investigate whether the CTS region of the recently identified tobacco glycosyltransferase *N*-acetylglucosaminyltransferase I (GnTI) [6] contains Golgi targeting and retention signals, we made a CTS-gfp fusion and used a tobacco mosaic virus based expression system to transiently express this construct in *Nicotiana benthamiana* plants. Using biochemical fractionation and confocal laser scanning microscopy our results clearly illustrate that the subcellular location of the fusion protein is consistent with GnTI-CTS as being sufficient to target to and retain a reporter protein in the plant Golgi apparatus.

2. Materials and methods

2.1. Plant material, construction of the p4GD-CTS-gfp and inoculation of plants

N. benthamiana plants were cultivated in a controlled growth chamber with 22°C day and night temperature, 50% humidity and 16 h photoperiod.

To construct the fusion protein CTS-gfp, 231 nucleotides (corresponding to 77 amino acids of the N-terminal CTS region of tobacco GnTI) were amplified by PCR (standard PCR method, annealing temperature 56°C) using the primer combination 5′-CGCGTCGACATGAGAGGGTACAAGTTT and 5′-CGAGTCGACCTGACGCTTCATTTGTTC. Both primers contain a *Sal*I site (underlined), the start codon of GnTI is given in bold letters. The resulting PCR product was cloned into the TMV based vector p4GD-gfp [7] in frame with gfp resulting in the vector p4GD-CTS-gfp. The recombinant viral vectors, p4GD-CTS-gfp and p4GD-gfp and p4GD-PL (control TMV vector, no gfp insert) were linearized by *Sfi*I digestion and transcripts were made using a T7 transcription kit (Life Technologies). In vitro transcripts were used to mechanically inoculate *N. benthamiana* plants at a six leaf stage.

2.2. Isolation of plant microsomes

Subcellular fractionation was performed according to [8] with certain modifications. Two hundreds mg of infected leaves of *N. benthamiana* plants were cut in small pieces, incubated in 10 ml of buffer

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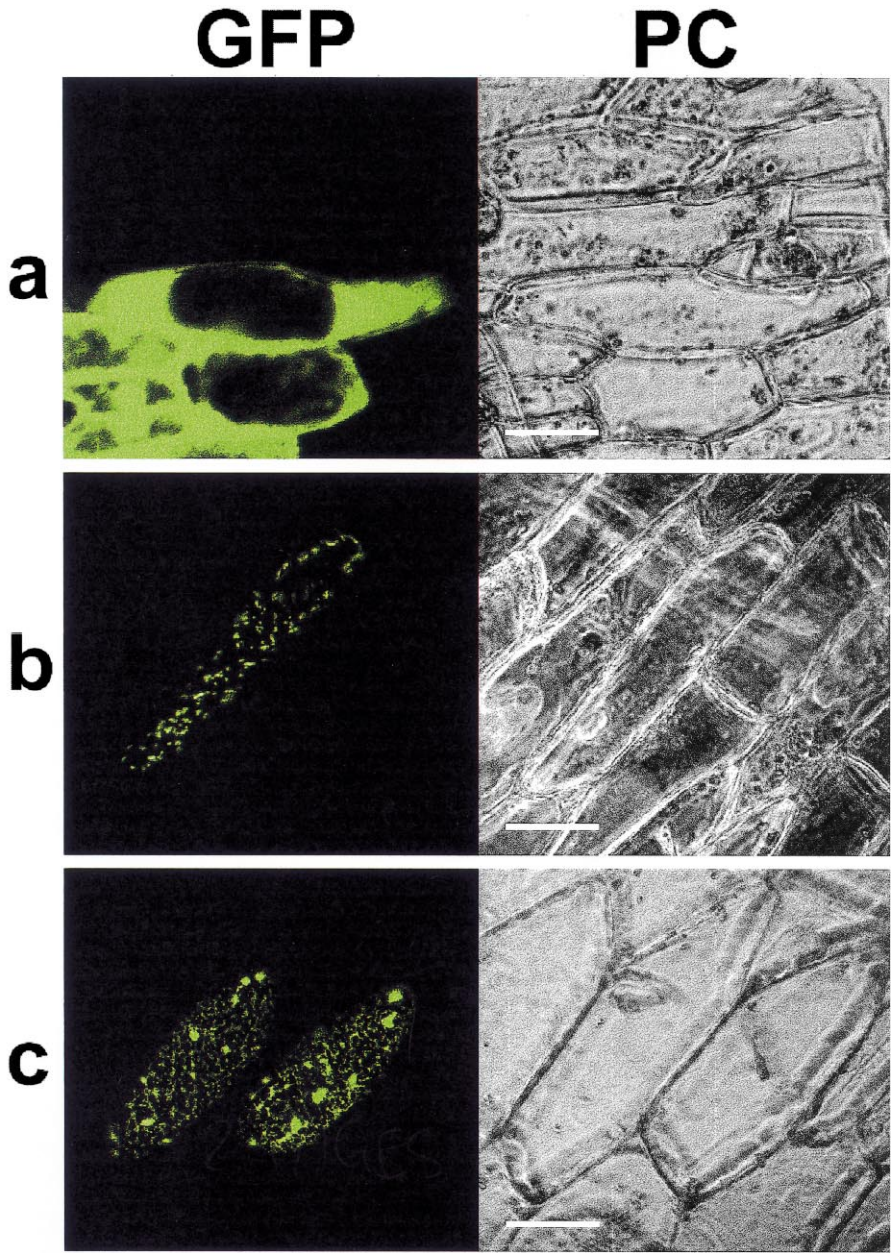

Fig. 1. Detection of gfp and CTS-gfp fusion protein in epidermal cells by confocal laser scanning microscopy. a: Homogenous labelling throughout the cytoplasm in p4GD-gfp infected epidermal cells. b: Punctate labelling throughout the cytoplasm in a p4GD-CTS-gfp infected epidermal cell. c: Disruption and clustering of CTS-gfp labelled structures in epidermal cells after brefeldin A treatment. GFP: Recording of gfp associated fluorescence. PC: Corresponding phase contrast view. Bars in a, b and c = 50 μm.

M (25 mM Tris/HCl, pH 7.2; 0,5 mM DTE; 1 mM EDTA; 0.5 mM PMSF; 250 mM sucrose) and disrupted by Ultraturrax (three 10 s periods, with 30 s of cooling on ice between each period). The crude extract was filtrated and centrifuged at $3000 \times g$ for 15 min at 4°C to remove cell debris, nuclei, and mitochondria. The supernatant was centrifuged at $100\,000 \times g$ for 1 h at 4°C to isolate the microsomal fraction. The microsomal pellet was resuspended in buffer M to obtain the same volume as the supernatant. The supernatant (cytoplasmic fraction) and the pellet (microsomal fraction) were monitored by fluorimetric measurements (HITACHI F2000). In contrast to the clear cytoplasmic fraction the microsomal fraction contained a significant amount of plant endogenous chromophores, e.g. chlorophyll. Gfp was excitated at 395 nm and gfp associated fluorescence was monitored at 509 nm (Table 1).

2.3. Tissue preparation for fluorescence microscopy

Epidermal tissue sections of leaf stalks of infected *N. benthamiana* plants were obtained manually using a flexible, double sided razor blade. The tissue was constantly kept in a moist atmosphere at room temperature and subjected directly to examination with confocal laser scanning microscopy (see below).

In order to test brefeldin A (BFA, Sigma) sensitivity of the CTS-gfp stained compartments, the respective epidermal tissue layers were incubated with 100 μg/ml BFA in water for 120 min at room temperature.

2.4. Colocalisation of the CTS-gfp fusion protein and Lewis a epitope by indirect in situ immunofluorescence

Indirect in situ immunofluorescence was performed according to [9,10] with modifications. All solutions were made in microtubule stabilising buffer (MTSB: 50 mM PIPES, 5 mM EGTA, and 5 mM $MgSO_4$, pH 6.9). Epidermal tissue sections from CTS-gfp expressing plants, prepared as described above, were fixed in 4% (v/v) formaldehyde for 60 min, followed by four washing steps in MTSB over

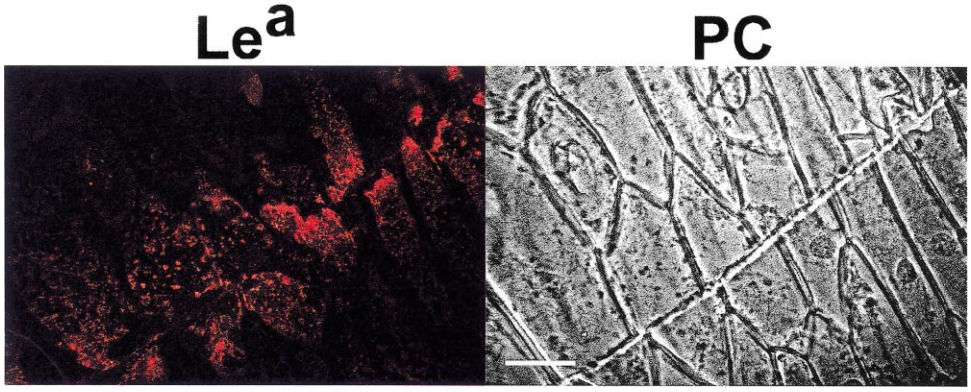

Fig. 2. Detection of Le^a epitope in epidermal cells by in situ indirect immunofluorescence. Punctate labelling pattern throughout the cytoplasm in cross-hatched epidermal cells was visualised by confocal laser scanning microscopy. Due to the staining procedure [9,10] a strong staining gradient of the Le^a epitope was obtained. The Le^a epitope was detected by Cy3 associated fluorescence recording. PC: Corresponding phase contrast view showing the cross-section through the cells. Bar = 50 μm.

60 min. In order to aid antibody penetration, the tissue was cross-hatched with a flexible, double sided razor blade. Permeabilisation was performed by 10% DMSO and 0.4% IGEPAL (Sigma) for 15 min. An additional blocking step was performed by incubating in 1% BSA for 15 min. Both antibody incubations were carried out for 60 min followed by three washing steps with the blocking solution after the primary incubation and with MTSB after the secondary incubation. As primary antibody a rabbit anti-Le^a monoclonal antibody [11] was used, the secondary antibody was a Cy3 conjugated sheep anti-rabbit antibody (Sigma). As anti-fade mounting reagent, CITIFLUOR was used (City University, London).

2.5. Microscopic imaging

Imaging was conducted on a Biorad MRC 600 confocal laser scanning microscope equipped with a Krypton/Argon mixed gas laser. Excitation of gfp was done at 488 nm, and excitation of the Cy3 fluorochrome was done at 568 nm using the dual channel filter set K1/K2. Usually, a ×40 objective was used, but for the double labelling imaging a ×100 oil immersion lens was chosen. Phase contrast illumination of the tissue sections was performed on the same sections. Images from the confocal system were imported into PaintShopPro 5.01 for colourisation.

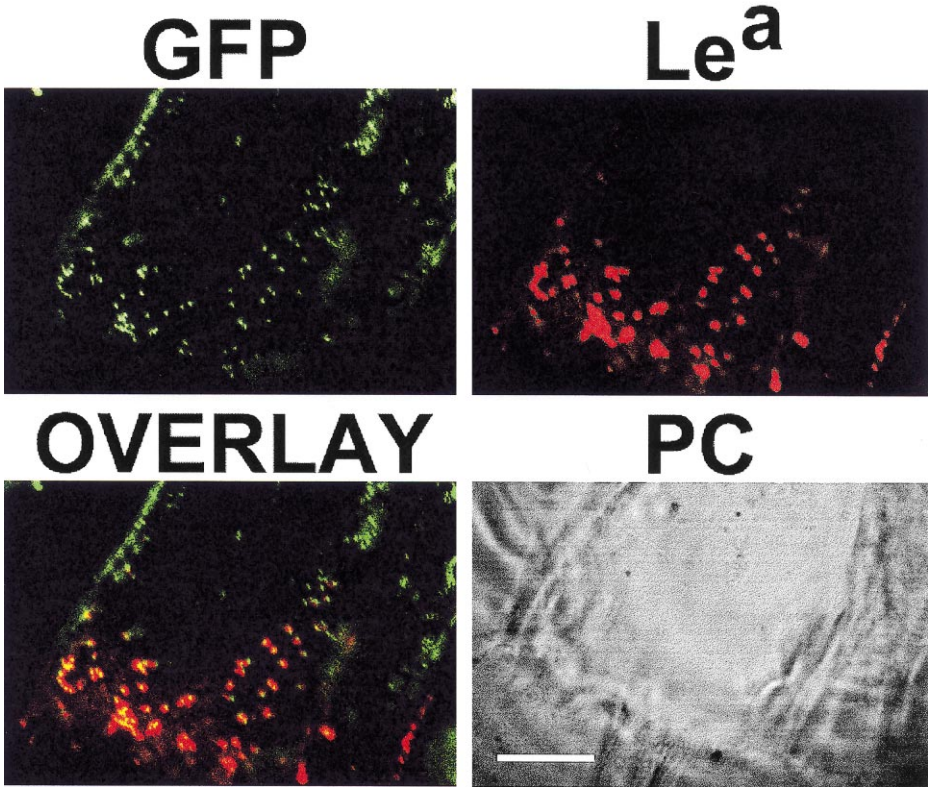

Fig. 3. Colocalisation of CTS-gfp fusion protein and Le^a epitope in an epidermal cell from *N. benthamiana* by confocal laser scanning microscopy. Le^a epitope was detected by in situ indirect immunofluorescence staining. Recordings of gfp and Cy3 associated fluorescence reveal an overlapping subcellular distribution of the CTS-gfp and the Le^a epitope in proximity of the antibody penetration sites which is substantiated by an overlay of the images. GFP: gfp associated fluorescence; Le^a: Le^a epitope detected by Cy3 fluorescence; PC: corresponding phase contrast image. Bar = 20 μm.

3. Results and discussion

3.1. Overexpression of gfp and CTS-gfp

In order to determine whether the CTS region of the tobacco GnTI is sufficient to target a recombinant protein to the plant Golgi apparatus an expression system based on tobacco mosaic virus [7] was used. A comparison of mammalian [12] and tobacco GnTI [6] suggests that the CTS region from tobacco GnTI contains at least 77 amino acids. Therefore, a CTS-gfp fusion (p4GD-CTS-gfp) or gfp alone (p4GD-gfp [7]) were transiently expressed in *N. benthamiana* plants using tobacco mosaic virus based expression vectors. p4DG-CTS-gfp and p4GD-gfp are identical vectors except that the latter does not contain the CTS region of GnTI. After 3–5 days post inoculation (dpi) of plants with TMV in vitro transcripts, bright green fluorescent areas indicative for gfp expression could be visualised upon illumination with ultraviolet (UV) light source (366 nm) on inoculated leaves. The viruses were capable of infecting and expressing gfp in plant tissues both locally in inoculated leaves and systemically throughout the entire plant. No differences between plants infected with the two constructs p4GD-gfp and p4GD-CTS-gfp were observed when plants were monitored by visual viral symptoms. However, upon visualising gfp in plants with UV light an increased level of fluorescence in p4GD-gfp infected plants was observed although the same amount of gfp was detected in Western blot analysis for both constructs (data not shown).

3.2. Subcellular localisation of recombinant expressed gfp and CTS-gfp

In order to determine the subcellular location of the two gfp constructs a biochemical fractionation from crude plant extracts was performed. The Golgi enriched membrane fraction was separated from the cytoplasmic fraction and subsequently analysed by spectrofluorimetric measurement (Table 1). The measurements indicated that the majority of gfp extracted from p4GD-gfp infected leaves is located in the cytoplasmic fraction whereas the majority of gfp extracted from p4GD-CTS-gfp infected leaves is located in the microsomal pellet. No gfp associated fluorescence was detected in extracts of plants infected with the control TMV vector (p4GD-PL [7]). These results demonstrate a membrane location of CTS-gfp.

In addition, the subcellular location of gfp and CTS-gfp was investigated by confocal laser scanning microscopy (Fig. 1). It is clearly visible that gfp in p4GD-gfp infected leaves is located in the cytoplasm of epidermal cells (Fig. 1a) whereas CTS-gfp expressing cells show the typical punctate staining pattern (Fig. 1b) with small fluorescent bodies, characteristic for Golgi compartments [13,14]. To verify that these structures actually represented Golgi stacks, their sensitivity to brefeldin A (BFA) was tested. This drug has numerous effects to the Golgi morphology such as clustering and/or vesiculation (for literature see e.g. [15]). Although BFA causes swelling of the endoplasmatic reticulum cisternae, unlike in mammalian cells, it does not induce the disassembly of Golgi stacks. Instead, BFA induces the formation of large clusters of Golgi stacks and the accumulation in the cytoplasm of very dense vesicles which contain large amounts of xyloglucan, the major hemicellulosic cell wall polysaccharide [15]. When epidermal tissue expressing CTS-gfp was incubated with brefeldin A the gfp labelling pattern appeared significantly different (Fig. 1c). A few large structures rather than numerous dispersed small punctate spots were detected. This further indicated that CTS-gfp is located within a BFA sensitive compartment, which is consistent with a Golgi location.

3.3. Colocalisation of CTS-gfp and Lewis a epitope

To identify unequivocally the intracellular location of CTS-gfp, cells expressing the fusion protein were stained with an antibody to the plant carbohydrate epitope Lewis a (Le^a [11]). Confocal laser scanning micrographs in plant cells showed immunocytochemical labelling of the Golgi apparatus and plasma membrane using plant Le^a specific antibodies [16]. (For images see: http://www.brookes.ac.uk/schools/bms/research/molcell/hawes/hawes.html.) In the present study in situ indirect immunofluorescence was carried out on epidermal cells of the *N. benthamiana* leaf stalks. As illustrated in Fig. 2, a punctate immunocytochemical labelling of the Le^a epitope was detected which is consistent with previous results [16]. In our study mechanical cross-sectioning of the tissue provided penetration sites for the antibodies leading to a Le^a staining gradient. Hence, enzyme treatment to allow antibody penetration causing distortions could be avoided. The colocalisation of CTS-gfp fusion protein with the Le^a epitope is shown in Fig. 3. A significant overlap between the staining obtained by CTS-gfp and Le^a is visible, as demonstrated by a superimposition of both fluorescence recordings.

In conclusion, the present data indicate that 77 amino acids from the CTS region of tobacco GnTI are sufficient to target to and retain a reporter protein in the plant Golgi apparatus. This is the first example of a plant derived Golgi targeting sequence. Hence, our results are the first insight into the so far poorly understood compartmentation of the N-glycan modification machinery in the plant Golgi apparatus. Furthermore, we demonstrated that a tobacco mosaic virus based expression system can be used as an effective and rapid strategy to direct recombinant proteins to the secretory pathway of plant cells.

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Table 1
Fluorimetric measurements of fractionated *N. benthamiana* leaf extracts infected with p4GD-gfp and p4GD-CTS-gfp

	Cytoplasmic fraction (clear-yellowish)	Microsomal fraction (green)
p4GD-gfp	166.1[a]	5.2[b]
p4GD-CTS-gfp	4.3[a]	33.3[b]

Gfp was excitated at 395 nm and emission recorded at 509 nm. Microsomal pellets were resuspended in the same volume as the supernatant (cytoplasmic fraction) and subjected directly to the measurements. Microsomal associated fluorescence is partially quenched due to contamination with endogenous plant chromophores (e.g. chlorophyll).
[a]Background fluorescence of cytoplasmic fraction (14.5) detected in p4GD-PL (negative control, TMV vector containing no gfp) infected plant extracts was substracted.
[b]Background fluorescence of microsomal fraction (0.5) detected in p4GD-PL (negative control, TMV vector containing no gfp) infected plant extracts was substracted.

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