

A functional antibody lacking *N*-linked glycans is efficiently folded, assembled and secreted by tobacco mesophyll protoplasts

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Summary

A potential drawback in the use of plants as an expression platform for pharmaceutical proteins such as antibodies is that plant-specific *N*-glycosylation can result in proteins with altered function and potential antigenicity. In many cases, the *N*-glycans are essential for the correct folding, assembly and transport of the recombinant proteins. We tested whether progressive removal of glycosylation sites had a detrimental effect on the synthesis, assembly and secretion of a plant-made immunoglobulin G, Guy's 13. Our results indicate that the plant secretory pathway can cope well with aglycosylated antibody chains. The immunoglobulin without *N*-linked glycans is correctly assembled and secreted by tobacco protoplasts. Capture enzyme-linked immunosorbent assay also shows that antigen-binding properties are unaffected. Our results therefore suggest one possible alternative to the engineering of a humanized glycosylation machinery in plants.

Introduction

Plants are an attractive system for the production of pharmaceutical proteins such as antibodies (Hiatt *et al.*, 1989; Ma *et al.*, 2003; Twyman *et al.*, 2003). The plant secretory pathway easily accommodates immunoglobulin (Ig) chains and is capable of folding, assembling and transporting them with high efficiency (Frigerio *et al.*, 2000; Hadlington *et al.*, 2003). Endoplasmic reticulum (ER) chaperones, in particular BiP, aid in the folding and assembly process, thus efficiently replacing their mammalian counterparts (Nuttall *et al.*, 2002). One of the perceived problems associated with Ig expression in plants is that the plant-specific protein *N*-glycosylation process differs from that in mammalian cells (Lerouge *et al.*, 1998). These differences in glycan structure have caused concern over the potential immunogenicity of plant-produced therapeutics (Cabanes-Macheteau *et al.*, 1999). However, Guy's 13, an IgG produced in transgenic plants, was shown not to be immunogenic in mice (Chargelegue *et al.*, 2000). Despite the limited evidence for immunogenicity currently available (Ramirez-Soto and Poretz, 1991), glycosylation still remains a

potential regulatory issue for the widespread therapeutic product expression in plants (Ma *et al.*, 2003). To alleviate these concerns, attempts have been made to address the problem of plant-specific glycosylation. Such strategies comprise the humanization of plant glycans by introducing mammalian glycosyltransferases (Bakker *et al.*, 2001) and the accumulation of Igs in their high-mannose glycosylated form in the ER (Gomord and Faye, 2004). One obvious alternative strategy is the removal of glycosylation sites from antibodies. However, although hybridoma-produced antibodies without *N*-glycans retain antigen-binding capacity, they are impaired in their ability to activate complement and to bind to the Fc receptor. Their clearance *in vivo* is also impaired (Nose and Wigzell, 1983). There are, however, a number of applications in which antibody effector properties are either not required (Presta *et al.*, 2001) or even undesirable (Friend *et al.*, 1999; Reddy *et al.*, 2000) and the production of aglycosylated antibodies is a practical alternative. However, a potential problem is that, in addition to affecting IgG function, *N*-glycosylation is also essential for the folding and assembly of antibodies, although its importance varies depending on the antibody

type (Rudd *et al.*, 2001; Krapp *et al.*, 2003). A very recent report has shown that an aglycosylated antibody against the epidermal growth factor receptor can be expressed in agroinfiltrated tobacco leaves, where it retains antigen-binding capacity (Rodriguez *et al.*, 2005). Here, we have studied the expression of a monoclonal IgG, Guy's 13 (Ma *et al.*, 1994), devoid of either or both of its *N*-glycosylation sites. The purpose was to study the effect of progressively removing the glycosylation sites from a heterologous protein on its expression in the plant secretory pathway. Our findings indicate that sugars are not required for correct expression and function, and that the molecular chaperone BiP is up-regulated in order to assist the folding of the aglycosylated molecule.

Results

We have recently shown that the intracellular fate of Ig chains is similar in transiently transfected tobacco mesophyll protoplasts and in stably transformed tobacco leaves (Hadlington *et al.*, 2003). Therefore, in this study, we used transient expression as a means to rapidly assess the effect of glycosylation site mutations. Initially, we studied the effect of the glycosylation inhibitor, tunicamycin, on the transport of Guy's 13 IgG. Tobacco mesophyll protoplasts were transfected with plasmids encoding both the γ heavy chain and the κ light chain of Guy's 13 IgG (Figure 1). Protoplasts were pulse

labelled for 1 h with ^{35}S -Promix and chased for up to 8 h, either in the presence or absence of tunicamycin. Cell homogenates and incubation media were immunoprecipitated with anti- γ antiserum, separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by fluorography (Figure 2). In untreated cells, both chains were secreted in the incubation medium during the chase, as reported previously (Frigerio *et al.*, 2000). This is indicative of correct IgG assembly and transport. A higher molecular weight band, which we have previously shown to be BiP (Nuttall *et al.*, 2002), was also coselected from the cell homogenates (Figure 2; open arrowhead). Treatment with tunicamycin resulted in increased mobility of the γ chain, indicating removal of its glycans (Figure 2; lanes 4–6). However, secretion of both light and heavy chains was not prevented by tunicamycin treatment. Rather, more antibody chains seemed to be secreted in the medium at the end of the pulse in the presence of the drug (Figure 2; cf. lanes 7 and 10). These data indicate that tunicamycin treatment does not negatively affect the intracellular fate of IgG. Another observation was that the expression levels of γ and κ chain were slightly reduced and slightly more BiP was immunoselected with the antibody chains in the presence of tunicamycin (Figure 2; cf. lanes 1 and 4: less antibody chains are detected in lane 4, but the amount of coselected

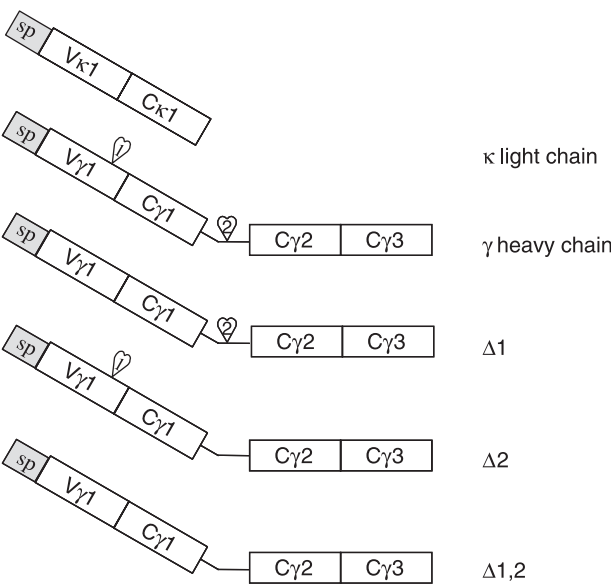


Figure 1 Schematic representation of the immunoglobulin chain constructs used in this study. All constructs were fused downstream of the cauliflower mosaic virus (CaMV) 35S promoter in the expression vector pDHA (see 'Experimental procedures'). C, constant domain; sp, signal peptide; V, variable domain. The hearts indicate the glycosylation sites in the heavy chains.

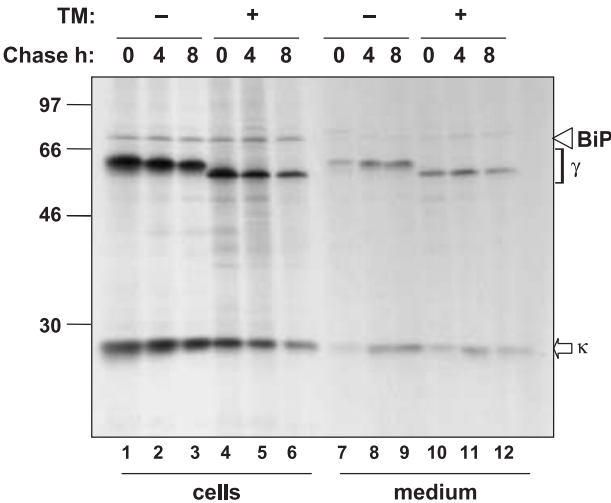


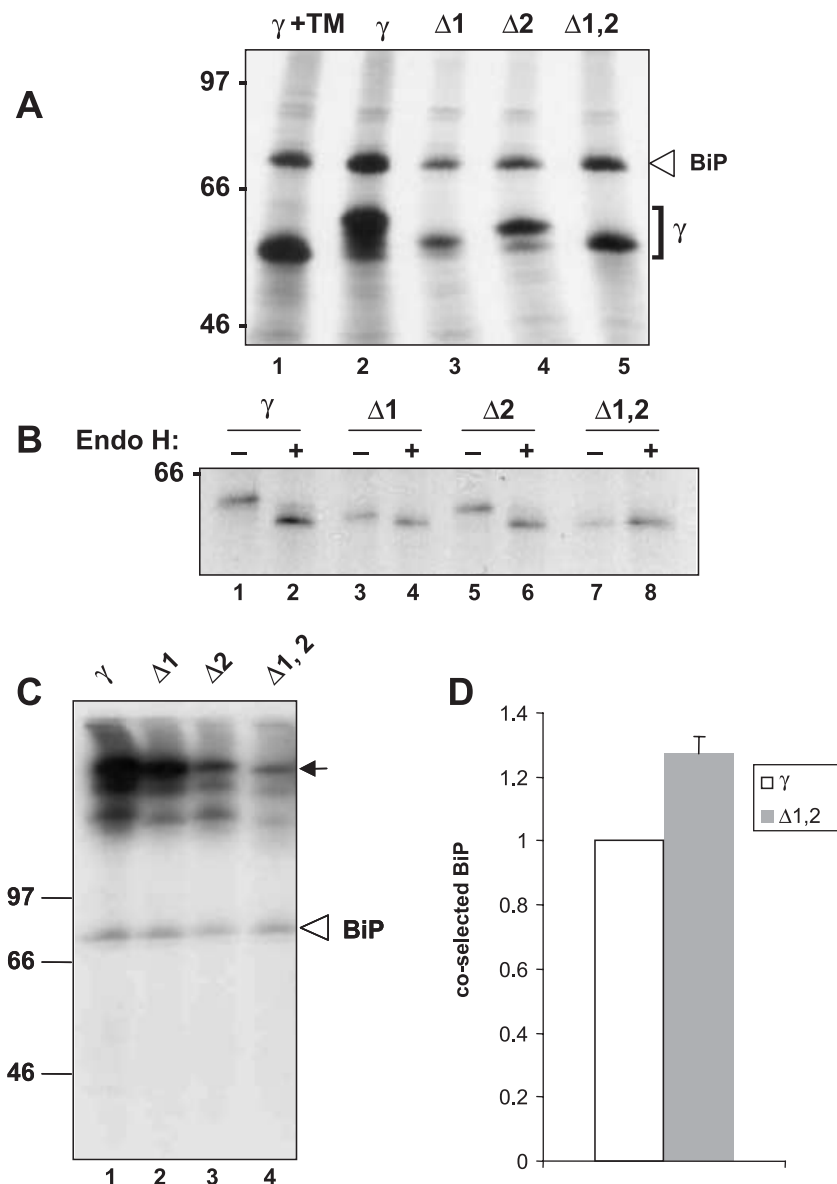
Figure 2 Inhibition of glycosylation with tunicamycin does not prevent immunoglobulin G (IgG) secretion. Tobacco mesophyll protoplasts were transfected with plasmids encoding Guy's 13 γ and κ chains. Cells were pretreated for 30 min with tunicamycin (TM+) or buffer (TM-) as a control, pulse labelled for 1 h with ^{35}S -Promix, and then chased for the indicated periods of time. Cell homogenates and incubation media were immunoprecipitated with anti- γ chain antiserum. Labelled polypeptides were visualized by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Numbers on the left, positions of the molecular mass markers in kilodaltons; open arrowhead, position of the BiP polypeptide; γ , position of the γ heavy chain; κ , position of the κ light chain.

BiP is the same as in lane 1). This can be ascribed to the induction of the unfolded protein response (UPR) by the drug (Martinez and Chrispeels, 2003). To avoid a general induction of the UPR by tunicamycin and to focus on the properties of the IgG glycans themselves, we generated a set of mutant heavy γ chains in which either or both glycosylation sites were inactivated by mutagenesis by substitution of the target asparagine residue with alanine (Figure 1). The γ chain contains an N-terminal signal peptide (SP), followed by the first variable (V γ 1) and constant (C γ 1) domains. These domains are linked to the C-terminal second and third constant domains (C γ 2 and C γ 3) by the IgG hinge region. The two potential *N*-glycosylation sites are present in the V γ 1 and hinge regions of the heavy chain (heart shapes in Figure 1; Cabanes-Macheteau

et al., 1999). The Δ 1 mutant was derived from the wild-type γ chain, with the V γ 1 glycosylation site being inactivated. The Δ 2 mutant (Figure 1) was again derived from the wild-type γ chain, with the hinge region glycosylation site being inactivated by mutagenesis. The Δ 1,2 mutant (Figure 1) was derived from the Δ 1 γ chain variant, with the hinge region glycosylation site also being inactivated by mutagenesis.

We transiently expressed the glycan mutant constructs in tobacco protoplasts to determine their fates. Protoplasts were pulse labelled for 1 h with ^{35} S-methionine and ^{35} S-cysteine, and then homogenized and subjected to immunoprecipitation with anti- γ chain antiserum. Figure 3A shows that all mutant chains are correctly expressed. Clear polypeptide bands corresponding to the different glycoforms of γ chain,

Figure 3 (A) Expression of wild-type and mutant γ chains. Protoplasts were transfected with plasmids encoding the stated γ chain glycan variants and pulse labelled for 1 h with ^{35} S-Promix. Cell homogenates were immunoprecipitated with anti- γ antiserum and polypeptides were visualized by reducing 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. TM, tunicamycin. (B) Samples as in (A) were incubated overnight in the presence (+) or absence (–) of endoglycosidase H (Endo H) before visualization by reducing 10% SDS-PAGE and fluorography. (C) γ chains lacking glycans are able to assemble with κ chains to form immunoglobulin G (IgG) tetramers. Protoplasts were transfected with plasmids encoding the stated γ chain glycan variants together with a plasmid encoding the κ chain. Protoplasts were pulse labelled for 1 h with ^{35} S-Promix and cell homogenates were immunoprecipitated with anti- γ antiserum. Labelled polypeptides were visualized by 10% SDS-PAGE under non-reducing conditions followed by fluorography. Numbers on the left, positions of the molecular mass markers in kilodaltons; thin arrow, position of the IgG tetramer; open arrowhead, position of the BiP polypeptide. (D) Absence of *N*-glycosylation of the γ heavy chain results in increased levels of interaction with BiP. Protoplasts expressing wild-type (γ) or Δ 1,2 γ chain were pulse labelled for 1 h. Cell homogenates were immunoprecipitated with anti- γ chain antiserum. Labelled polypeptides were visualized by 10% SDS-PAGE followed by fluorography. The intensities of γ chain and coimmunoselected BiP were measured by densitometry. The amount of BiP coselected with wild-type γ chain was assigned the reference value of unity. The graph shows the average of four independent experiments. Vertical bars indicate standard deviations.



together with BiP (open arrowhead), were selected by the antiserum (Figure 3A). Removal of either or both glycosylation sites resulted in increased electrophoretic mobility of the γ chain. Wild-type (γ) and $\Delta 2$ heavy chains appear as doublets, with the lower band having the same mobility as the tunicamycin-treated band (Figure 3A; cf. lane 1 with lanes 2 and 4). This reflects incomplete glycosylation of the newly synthesized heavy chains, as observed previously (Nuttall *et al.*, 2002). As heavy chains accumulate in the ER in the absence of light chain (Nuttall *et al.*, 2002), we predict both *N*-glycans to be of the high-mannose type and therefore to be susceptible to digestion with endoglycosidase H (Endo H). To further demonstrate that both sites are used in plants (Cabanès-Macheteau *et al.*, 1999) and that sequential mutagenesis successfully prevented the usage of these sites, we subjected the immunoprecipitated samples to Endo H digestion (Figure 3B). Treatment with Endo H resulted in increased mobility of wild-type γ chain and of the single glycosylation site mutants (Figure 3B; lanes 2, 4 and 6). As expected, the mobility of the double mutant was not affected by Endo H treatment and was comparable with that of the deglycosylated wild-type and single mutant chains (Figure 3B; lanes 7 and 8). Although both single glycosylation site mutants have a slower electrophoretic mobility than the double mutant (Figure 3A,B), it appears that inactivation of the first site results in a higher gel mobility shift of the γ chain than does inactivation of the second site (Figure 3A; cf. lanes 3 and 4; Figure 3B; cf. lanes 3 and 5). It is possible that the position of each glycan affects the proteins' accessibility to SDS, thus resulting in the slightly different mobility of the two single mutant chains.

To confirm that γ chain variants lacking glycans were still able to assemble with κ light chains, we transfected protoplasts with plasmids encoding the γ chain glycan variants, together with the κ chain, and pulse labelled them for 1 h with ^{35}S -Promix. Cell homogenates were immunoprecipitated with anti- γ antiserum, separated by SDS-PAGE under non-reducing conditions and visualized by fluorography (Figure 3C). High molecular weight forms (approximately 180 kDa) corresponding to tetrameric IgG are visible for all glycan variants (Figure 3C). A band of the expected molecular weight for BiP was also coselected in all lanes at a uniform intensity. The intensity of the tetrameric IgG band is slightly lower in the $\Delta 2$ and $\Delta 1,2$ mutants than in the wild-type or $\Delta 1$ forms. This reduction in synthesis is probably due to fluctuations in the levels of transient expression of specific constructs. It is worth noting that the intensity of the BiP band that is coimmunoprecipitated with the glycosylation mutant constructs remains the same as the glycosylated constructs (Figure 3C). Therefore, as observed in Figure 2, more BiP

seems to be associated per IgG molecule in these variants, in particular in $\Delta 1,2$, suggesting an up-regulation of BiP. We analysed this in more detail by transfecting protoplasts with plasmid encoding the γ heavy chain in both wild-type and $\Delta 1,2$ forms. After pulse labelling for 1 h, we subjected the samples to immunoprecipitation with anti- γ chain. We analysed the amount of BiP coselected by densitometry and normalized it for the amount of immunoprecipitated heavy chain. Figure 3D is the result of four independent experiments and shows that coselection of BiP with aglycosylated heavy chain is increased about 1.3-fold compared to wild-type heavy chain.

Having established that mutant IgG can assemble correctly, we tested whether the intracellular fate was altered by the absence of one or both glycans. We transfected tobacco protoplasts with wild-type or mutant γ chains, together with κ light chains, and subjected them to pulse-chase analysis (Figure 4). In order to visualize only the assembled heavy and light chains, we used anti- γ chain antibodies for immunoprecipitation. Therefore, the κ light chain polypeptides which are coselected represent the proportion of light chain that is assembled with γ chain (Hadlington *et al.*, 2003). Secretion of both γ and κ chains occurred after 4 or 8 h of chase (Figure 4; lanes 15 and 16). Taking into account the aforementioned fluctuations in overall protein synthesis for different constructs during transfection, secretion levels of the γ chain mutants were comparable with those of the wild-type IgG (Figure 4). We therefore conclude that inactivation of *N*-glycosylation sites has a negligible effect on the intracellular fate of the γ chain.

We have shown so far that the removal of glycosylation sites on the γ heavy chain does not inhibit folding, assembly or secretion of the Ig chains. However, the absence of glycans could have a negative effect on antigen binding. To test this possibility, we transfected protoplasts with plasmids encoding the γ chain glycan mutants and κ chain. The incubation media were collected, serially diluted and the amount of secreted antibody was quantified by capture enzyme-linked immunosorbent assay (ELISA) using κ chain antibody for capture, γ chain antibody for detection and a dilution series of a purified IgG (MOPC31C) as a standard (data not shown). Protoplast media corresponding to equal amounts (50 ng/mL) of secreted IgG were then used in an antigen-binding ELISA (Figure 5). Samples were added in a two-fold dilution series to plates coated with the *Streptococcus mutans* SA I/II antigen (Russell and Lehner, 1978). Incubation media isolated from mock-transfected (mock) protoplasts and 50 ng/mL of a nonspecific IgG (MOPC31C) were used to determine the background absorbance. All secreted antibodies containing

Figure 4 Mutation of γ chain *N*-glycosylation sites does not alter the intracellular fate of immunoglobulin G (IgG). Protoplasts were transfected with plasmids encoding the indicated γ chain glycan variants together with a plasmid encoding the κ chain. Protoplasts were subsequently pulse labelled for 1 h with ^{35}S -Promix, and chased for 4 and 8 h. Cell homogenates and incubation media were immunoprecipitated with anti-IgG antiserum and labelled polypeptides were visualized by 15% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Numbers on the left, positions of the molecular mass markers in kilodaltons; TM, tunicamycin-treated γ chain sample.

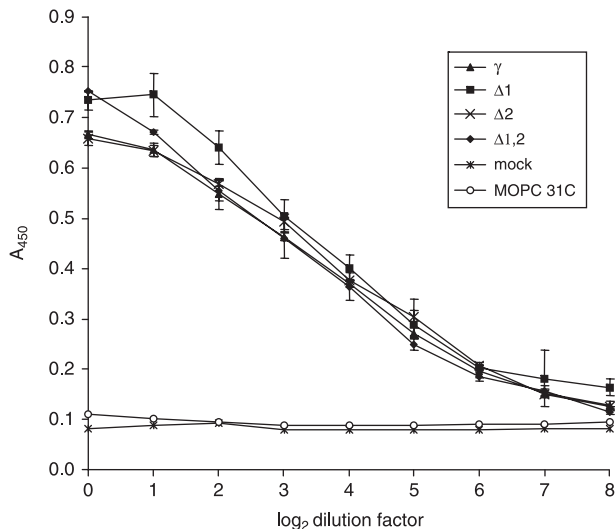
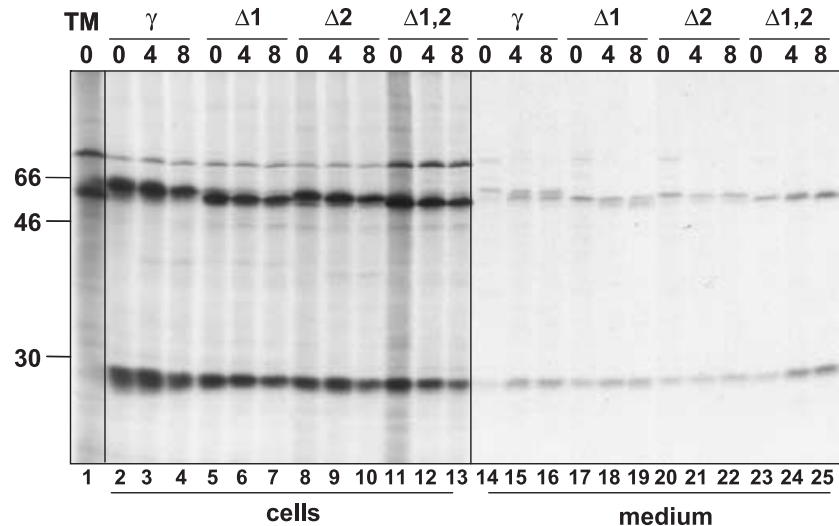


Figure 5 Immunoglobulin Gs (IgGs) containing γ chain glycosylation mutants retain their antigen-binding capacity. Protoplasts were mock transfected with empty plasmid (mock) or plasmids encoding the stated γ chain variants, together with a plasmid encoding the κ chain. After a 16 h recovery period from transfection, incubation medium samples were collected and secreted IgG was quantified by capture enzyme-linked immunosorbent assay (ELISA). Media corresponding to 50 ng/mL of secreted antibody were used for antigen-binding ELISA on plates coated with the streptococcal SA I/II antigen, as described previously (Ma *et al.*, 1994). The data shown are the average of two independent experiments. Bars represent standard deviations.

glycosylation mutant heavy chains clearly showed antigen-binding properties which were comparable with those of the wild-type, fully glycosylated IgG (Figure 5). We therefore conclude that progressive removal of glycosylation sites has no negative effect on the antigen-binding capacity of a plant-made IgG.

Discussion

We have tested the effect of removing glycosylation sites on a plant-made Ig, Guy's 13 (Ma *et al.*, 1994). A pharmacological approach was initially attempted, which involved the treatment of transgenic plants expressing IgG with the *N*-glycosylation inhibitor, tunicamycin. This drug had little influence on IgG secretion, although slightly reduced expression levels and up-regulation of BiP (i.e. the same amount of BiP coimmunoselected with lower amounts of γ chain) suggested the induction of the UPR by tunicamycin (Kozutsumi *et al.*, 1988). UPR is typified by an inhibition of protein synthesis and an up-regulation of ER-resident chaperones (Kozutsumi *et al.*, 1988; Martinez and Chrispeels, 2003). To focus on the function of *N*-glycans on the fate of the IgG molecule alone, we generated γ chain mutants with inactivated glycosylation sites. Such an approach has been tested previously for several Igs in mammalian cells (Sidman *et al.*, 1981; Taylor and Wall, 1988). The Guy's 13 γ heavy chain has two potential glycosylation sites, one in the first variable domain and another in the hinge region (Cabanes-Macheteau *et al.*, 1999). We generated mutants to inactivate either or both sites. Transient transfection of these constructs revealed that the expression and secretion of these mutant Igs appeared to be unaffected. It also appeared that both sites were normally glycosylated in tobacco protoplasts. We have shown that removal of either or both glycosylation sites does not affect the capacity of the γ chain to assemble with its companion κ light chain. This was shown both by the presence of high molecular weight forms corresponding to IgG tetramers under non-reducing conditions and by coimmunoprecipitation of stoichiometric amounts of κ chain

with anti- γ chain antiserum. These results clearly indicate that the *N*-linked glycans are not necessary for IgG folding and assembly in the plant ER and for secretion of the assembled molecules. The major concern over glycan removal is the possible loss of antigen-binding function; however, ELISAs revealed that γ chains lacking glycans were still able to bind to the streptococcal SA I/II antigen. Indeed, the binding affinity of these mutants was comparable with that of IgG molecules incorporating the wild-type γ chain. It therefore appears that *N*-glycans are not essential for antigen binding and, consequently, the activity of Guy's 13 IgG. Our results are in accordance with findings obtained when expressing IgG in *Escherichia coli* (Simmons *et al.*, 2002). Polycistronic bacterial expression constructs can lead to the successful expression of assembled, functional antibody molecules, although the extent of antibody assembly is limited in comparison with the plant expression system. Moreover, plant expression has the additional advantage of allowing for secretion of the assembled antibody, thus greatly increasing the ease of purification (Drake *et al.*, 2003).

The removal of glycans has been shown previously to reduce interactions with the Fc γ receptor (Krapp *et al.*, 2003). This is of limited importance in this case, as Guy's 13 does not usually elicit an immune response for its activity (Chargelegue *et al.*, 2000). More generally, this lack of effector function does not constitute a problem for several applications of antibodies where only antigen-binding capacity is required (Presta *et al.*, 2001; Presta, 2003).

There also remains the possibility that the removal of the *N*-glycans could expose new epitopes on the antibody, which may in themselves result in immunogenic reactions. The generation of transgenic plants followed by antigenicity trials would determine whether this is in fact the case. However, it is clear from this study that the removal of *N*-glycans from the Guy's 13 γ heavy chain has a limited effect on the stability and fate of the molecule, indicating that the plant quality control system can cope well with this modification.

Experimental procedures

Transgenic plants

Transgenic *Nicotiana tabacum* cv. Xanthi plant lines expressing assembled IgG and IgA/G under the control of the cauliflower mosaic virus 35S promoter have been described previously (Ma *et al.*, 1994). For protoplast transfection experiments, wild-type *N. tabacum* cv. Petit Havana SR1 was used. Plants were grown in axenic conditions under a 12 h light–dark regime.

Recombinant DNA

Guy's 13 γ heavy chain was cloned into transient expression vector pDHA (Tabe *et al.*, 1995). Guy's 13 κ light chain was cloned into transient expression vector pSK3 (Hadlington *et al.*, 2003). Mutations of the putative glycosylation sites of the heavy chain were produced by replacing the target asparagine residue with alanine, using the 'Quickchange' *in vitro* mutagenesis system (Stratagene, La Jolla, CA, USA). Mutations were introduced into the wild-type heavy chain sequence using the following oligonucleotides: for $\Delta 1$: 5'-ACTGTAGACAAT-TCCGCCACCTCAGCCTACA-3' and 5'-TGTAGGCTGAGGT-GGCGGAATTGTCTACAGT-3'; for $\Delta 2$: 5'-GAGCAGCTCAA-CAGCGTTTTCCGCTCAGTCAG-3' and 5'-CTGACTGAGCG-GAAAACGCTGTTGAGCTGCTC-3'. $\Delta 1,2$ was obtained with the $\Delta 2$ oligonucleotides using $\Delta 1$ heavy chain as a template.

Protoplast transfection

Protoplasts prepared from axenic leaves of tobacco were subjected to polyethylene glycol-mediated transfections exactly as described by Pedrazzini *et al.* (1997). Forty micrograms of each plasmid were used to transform 10^6 protoplasts in 1 mL. After transfection, cells were incubated for 16 h at 25 °C before metabolic labelling or ELISA.

In vivo labelling of protoplasts and analysis of expressed polypeptides

Pulse-chase experiments and immunoprecipitation were performed exactly as described previously (Hadlington *et al.*, 2003). Digestion of immunoprecipitated proteins with Endo H (Roche Diagnostics, Lewes, UK) was performed as described previously (Hadlington *et al.*, 2003).

Antisera

Rabbit polyclonal anti-mouse IgG (whole molecule) was purchased from Sigma (UK). Rabbit polyclonal anti-tobacco BiP (Pedrazzini *et al.*, 1997) was a gift from Alessandro Vitale (CNR, Milan, Italy). Goat anti-mouse IgG1 γ chain and goat anti-mouse IgG1 κ chain were purchased from Southern Biotechnology Inc. (Birmingham, AL).

ELISA

For capture ELISA, 50 μ L of incubation medium from transfected protoplasts was added in a two-fold dilution series to plates coated with anti- κ IgG antibody; 1 μ g/mL of the

purified IgG1 MOPC31C was used as a standard for quantification. After binding for 2 h at 37 °C, bound IgG was detected with peroxidase-conjugated anti- γ chain antibody.

For antigen-binding ELISA (Ma *et al.*, 1994), protoplast incubation medium corresponding to 50 ng/mL of secreted antibody was added in a two-fold dilution series to plates coated with the *Streptococcus mutans* SA I/II antigen. Detection of bound IgG was with peroxidase-conjugated anti- γ chain antibody.

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