

Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core- $\alpha(1,3)$ -fucose residues

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Summary

Plant-based expression systems are attractive for the large-scale production of pharmaceutical proteins. However, glycoproteins require particular attention as inherent differences in the N-glycosylation pathways of plants and mammals result in the production of glycoproteins bearing core-xylose and core- $\alpha(1,3)$ -fucose glyco-epitopes. For treatments requiring large quantities of repeatedly administered glycoproteins, the immunological properties of these non-mammalian glycans are a concern. Recombinant glycoproteins could be retained within the endoplasmic reticulum (ER) to prevent such glycan modifications occurring in the late Golgi compartment. Therefore, we analysed cPIPP, a mouse/human chimeric IgG1 antibody binding to the β -subunit of human chorionic gonadotropin (hCG), fused to a C-terminal KDEL sequence, to investigate the efficiency of ER retrieval and the consequences in terms of N-glycosylation. The KDEL-tagged cPIPP antibody was expressed in transgenic tobacco plants or *Agrobacterium*-infiltrated tobacco and winter cherry leaves. N-Glycan analysis showed that the resulting plantibodies contained only high-mannose (Man)-type Man-6 to Man-9 oligosaccharides. In contrast, the cPIPP antibody lacking the KDEL sequence was found to carry complex N-glycans containing core-xylose and core- $\alpha(1,3)$ -fucose, thereby demonstrating the secretion competence of the antibody. Furthermore, fusion of KDEL to the diabody derivative of PIPP, which contains an N-glycosylation site within the heavy chain variable domain, also resulted in a molecule lacking complex glycans. The complete absence of xylose and fucose residues clearly shows that the KDEL-mediated ER retrieval of cPIPP or its diabody derivative is efficient in preventing the formation of non-mammalian complex oligosaccharides.

Keywords: antibody, endoplasmic reticulum, glycan, glycoprotein, KDEL, recombinant.

Introduction

Plants are emerging as competitive systems for the large-scale production of recombinant therapeutic proteins (Giddings *et al.*, 2000; Twyman *et al.*, 2003). The many advantages of plants include their high intrinsic safety due to the absence of human and animal pathogens, their capacity to correctly process, fold and assemble complex hetero-multimeric proteins such as antibodies (Hiatt *et al.*, 1989), the ease of

production scale-up and their cost-effectiveness. Recently, we reported the transient expression and purification of cPIPP, a mouse/human chimeric antibody specific for the β -subunit of human chorionic gonadotropin (hCG). This protein was expressed in tobacco leaves (*Nicotiana tabacum* cv. Petite Havana SR1) using vacuum-assisted agroinfiltration (Kathuria *et al.*, 2002). Given the fact that hCG plays a crucial role in the implantation of the embryo to initiate pregnancy (Fishel *et al.*, 1984), a recombinant antibody with high specificity

for hCG has a significant potential for pregnancy testing and application as a contraceptive (Talwar *et al.*, 1994). Moreover, anti-hCG antibodies may also be used for imaging and the targeted delivery of radiation or drugs to tumours producing hCG or its β -subunit (Acevedo *et al.*, 1997; Hameed *et al.*, 1999; Kido *et al.*, 1996; Okamoto *et al.*, 2001).

Although therapeutic glycoproteins can be produced in plants, inherent differences in the glycosylation pathways of plants and animals need specifically to be addressed. Plants synthesize complex N-linked glycans containing a core Man₃GlcNAc₂ bearing two terminal GlcNAc (N-acetylglucosamine) residues, similar to those found in mammals. However, this core is substituted in plants by a β (1,2)-xylose residue (core-xylose), Lewis^a epitopes and an α (1,3)-fucose residue (core- α (1,3)-fucose). The core-xylose and core- α (1,3)-fucose epitopes are absent from mammalian cells and are known to constitute carbohydrate epitopes that can be recognized by allergen-reactive IgE (Aalberse *et al.*, 1981; Faye and Chrispeels, 1988; Garcia-Casado *et al.*, 1996; Van Ree *et al.*, 2000; Wilson and Altmann, 1998). Furthermore, immunization of goats (Kurosaka *et al.*, 1991), rabbits (Faye *et al.*, 1993) and rats (McManus *et al.*, 1988) with plant glycoproteins can elicit the production of core-xylose- and/or core- α (1,3)-fucose-specific antibodies. In this context, we have recently demonstrated that such glyco-epitopes are also able to elicit an immune response in humans (Bardor *et al.*, 2003).

For immunotherapy requiring large quantities of repeatedly administered antibodies, the immunogenicity of non-mammalian glycans is a concern. Various strategies have been employed either to prevent the formation of plant N-glycans on recombinant proteins, or to produce plant glycoproteins harbouring mammalian-like N-glycans (Lerouge *et al.*, 2000). These strategies include the use of plant mutants with a modified N-glycosylation pathway, as well as the co-expression of human glycosyltransferases to humanize the plant N-glycosylation pathway (Bakker *et al.*, 2001; Lerouge *et al.*, 2000). Deglycosylated antibodies can be used to avoid any immune response directed against non-human glycans. However, deglycosylated antibodies lack effector functions (Nose and Wigzell, 1983), and their half-life and serum stability are dramatically reduced.

Upstream from the modifications occurring in the Golgi apparatus, the processing of N-linked glycans in eukaryotes starts in the endoplasmic reticulum (ER) with a conserved set of glycan modifications yielding high-mannose-type N-glycans. In this paper, we describe the N-glycan structure of recombinant anti- β -hCG (PIPP) antibodies expressed in tobacco and winter cherry (*Physalis alkekengi*) with or without the C-terminal KDEL ER retrieval sequence. The results show that

the expression of an antibody fused to the KDEL tag in plants results in the production of plantibodies containing high-mannose-type oligosaccharides that are common to plants and mammals, and therefore are potentially devoid of glycan-related immunogenicity in humans.

Results

Production and purification of chimeric antibodies

Chimeric antibodies with and without KDEL were purified using protein A affinity chromatography and analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the size, purity and integrity of the protein. The purification of cPIPP without the KDEL tag from agroinfiltrated tobacco leaves is shown as an example (Figure 1). It should be noted that the relative amounts of the antibody chains in the extract and the flowthrough are too low (0.02%–0.1% of total extracted protein) to be detected on the Coomassie-stained polyacrylamide gels. All plant-derived cPIPP proteins, from either transient or stable expression in tobacco and winter cherry, showed the expected sizes for the antibody heavy and light chains. We selected an additional plant species to show that the results obtained in tobacco are not unique to that species. Winter cherry was chosen because it has been identified previously as a suitable expression host for agroinfiltration (R. Sriraman *et al.*, unpublished data). Proteins obtained from transgenic tobacco plants showed no degradation on SDS-PAGE gels or in Western blots (Figure 2). However, degradation products containing the

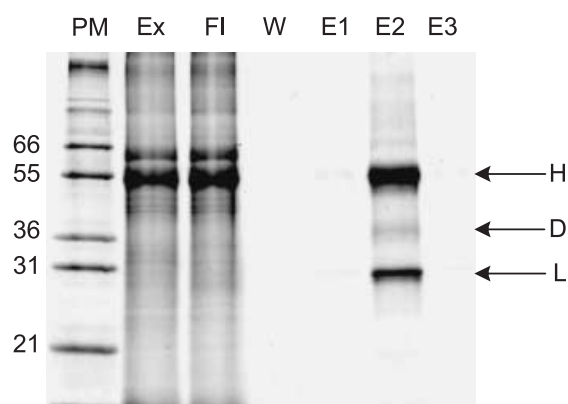


Figure 1 Purification of cPIPP (without KDEL) from co-infiltrated tobacco leaves by protein A affinity chromatography. Proteins were separated on a 12% polyacrylamide gel and stained with Coomassie brilliant blue. Molecular masses for protein marker bands are expressed in kilodaltons. PM, protein marker; Ex, leaf extract; FI, flowthrough; W, wash fraction; E1, E2, E3, elution fractions; H, chimeric heavy chain; L, chimeric light chain; D, chimeric heavy chain degradation products.

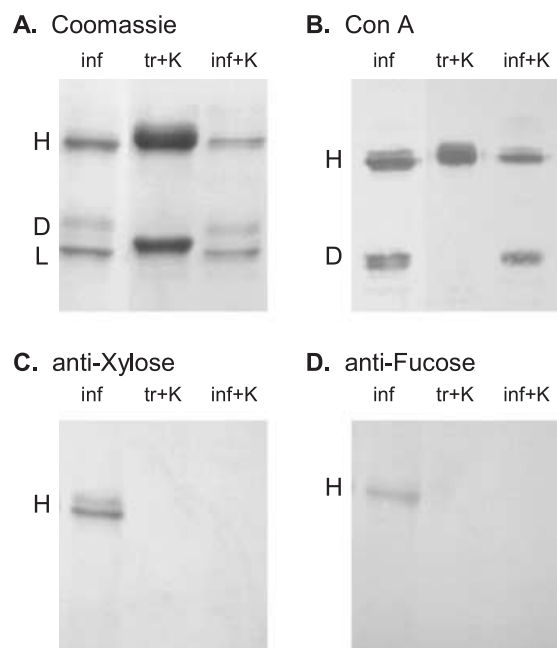


Figure 2 Gel electrophoresis and Western blot analysis of purified cPIPP antibodies produced in tobacco and winter cherry. (A) Coomassie brilliant blue staining; (B) affindetection using concanavalin A (Con A); (C) immunodetection using core- β (1,2)-xylose antibodies; and (D) immunodetection using core- α (1,3)-fucose antibodies. inf, cPIPP without a KDEL tag purified from agroinfiltrated tobacco leaves; tr + K, cPIPP + KDEL purified from transgenic tobacco plants; inf + K, cPIPP + KDEL purified from agroinfiltrated winter cherry leaves; H, chimeric heavy chain; L, chimeric light chain; D, chimeric heavy chain degradation products.

Fc part of the heavy chain were detected in preparations of the antibodies purified from agroinfiltrated leaves from both tobacco and winter cherry. The fragments reacted both with the antihuman IgG₁-Fc-specific serum (data not shown) and with concanavalin A (Con A) (Figure 2B).

Presence of an N-linked glycosylation site in the heavy chain variable domain (V_H) of cPIPP

A consensus sequence for N-linked glycosylation at Asn⁵⁸ was identified within the variable domain of the PIPP heavy chain (Figure 3A). This site is absent in the germ-line gene (IgHV1s61) (Lefranc, 2001), which shows the greatest sequence identity to the recombined PIPP antibody gene, and has been acquired by mutation of Asn⁶⁰ $\rightarrow$ Ser⁶⁰ generating the consensus motif NYS. Glycosylation within the Fab region is observed in about 30% of serum antibodies (Rademacher *et al.*, 1986). A homology-based model of cPIPP Fv indicated that the N-glycosylation site at Asn⁵⁸ is exposed on the surface (Figure 3C). This glycosylation apparently does not

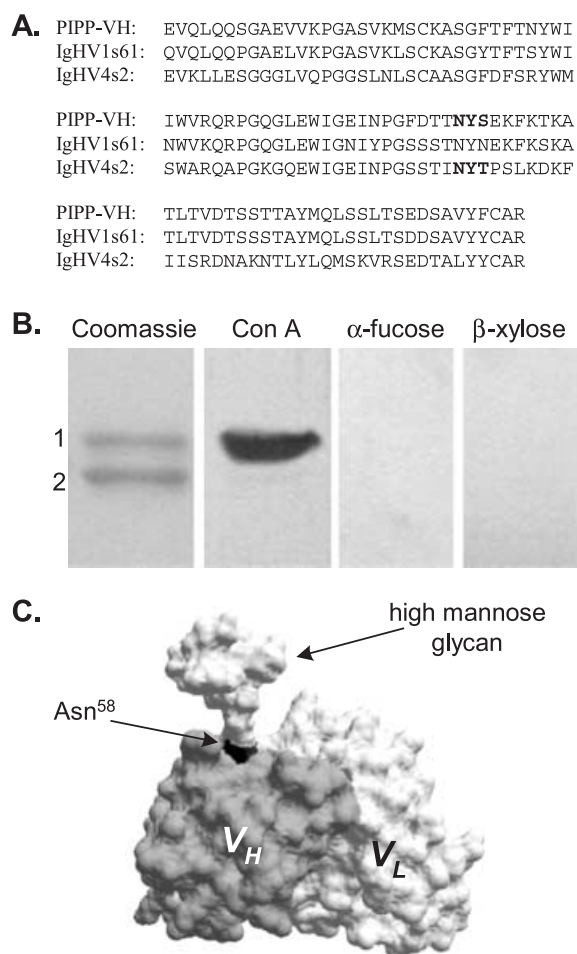


Figure 3 The PIPP heavy chain variable domain (V_H) contains a glycosylation site. (A) Sequence alignment of PIPP V_H with the most similar mouse germ-line gene IgHV1s61 and the mouse germ-line gene IgHV4s2, which has a natural glycosylation site at Asn⁵⁸. (B) Affino- and immunodetection of PIPP diabody-KDEL purified from transgenic tobacco. The glycosylated diabody (1) has a lower electrophoretic mobility than the glycan-free diabody (2) and is detected by Con A, but not by β -xylose- and α -fucose-specific antibodies. (C) An illustration of PIPP Fv with high-mannose-type N-glycan at Asn⁵⁸ derived by homology-based modelling (Guex *et al.*, 1999).

interfere with the structural integrity of the antibody and a consensus sequence for N-glycosylation at Asn⁵⁸ is present in both functional germ-line genes of V_H family 4 (IgHV4s1 and IgHV4s2). This supports the observation that glycosylation does not disrupt the structure and folding of the V_H domain. Asn⁵⁸ is part of complementary determining region (CDR) H2 and is located close to the antigen-binding site of PIPP. Whether the binding properties are affected by glycosylation is currently under investigation.

The PIPP diabody fused to a KDEL tag was stably and transiently expressed in tobacco plants (Kathuria *et al.*, 2002). As determined by Coomassie brilliant blue staining, approximately

20%–30% of the plant-derived PIPP diabody is glycosylated (Figure 3B, band 1). The glycosylated diabody has lower electrophoretic mobility and migrates as a distinct band. Only this band is detected by Con A, while the majority of the diabody (Figure 3B, band 2) does not bind Con A. The glycosylated diabody is not detected by anti-core-fucose- and xylose-specific antibodies (Figure 3B), indicating that KDEL-mediated ER retrieval is also efficient for this homodimeric molecule. The extent of glycosylation does not differ between the transiently and stably produced proteins (data not shown).

N-Glycan analysis of chimeric PIPP plantibodies and diabodies

Glycan structural analysis was carried out to investigate the effect of the C-terminal KDEL ER retrieval sequence on the N-glycosylation of cPIPP. The initial analysis was performed using Western blotting and affinodetection with Con A (Faye and Chrispeels, 1985), and by immunodetection using antibodies specific for plant N-glycan epitopes, i.e. core-xylose and core- $\alpha(1,3)$ -fucose (Faye *et al.*, 1993). As shown in Figure 2(B), the heavy chains of the KDEL-tagged cPIPP antibodies purified from transgenic tobacco plants, agroinfiltrated tobacco or winter cherry leaves were affinodetected by Con A, a plant lectin specific for the mannose core as well as other high-order N-linked glycans (Bryce *et al.*, 2001). A second protein band of ~36 kDa, reacting with Con A and corresponding to a degradation product of the antibody heavy chain, was also observed in protein preparations from transient expression experiments. In contrast with the strong affinodetection with Con A, the KDEL-tagged antibody heavy chain did not react with core-xylose or core- $\alpha(1,3)$ -fucose glycan-specific antibodies (Figure 2C,D), indicating the absence of glycans undergoing late Golgi maturation. Matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF-MS) analysis of oligosaccharides isolated from the KDEL-tagged cPIPP antibody produced in transgenic tobacco plants showed a mixture of ions that were assigned to $(M + Na)^+$ adducts of high-mannose-type N-glycans (Man-6 to Man-9; Figure 4C). Consistent with the immunoblotting experiments, no xylose-containing or $\alpha(1,3)$ -fucose-containing N-glycans were detected in the mass spectrum. A similar N-glycosylation profile was obtained with the ER-retained cPIPP antibody transiently expressed in winter cherry, indicating that the effect of the KDEL tag on the N-glycosylation of the cPIPP antibody is not particular to tobacco alone (data not shown).

To determine whether the N-linked glycans of the cPIPP antibody are processed into mature oligosaccharides during

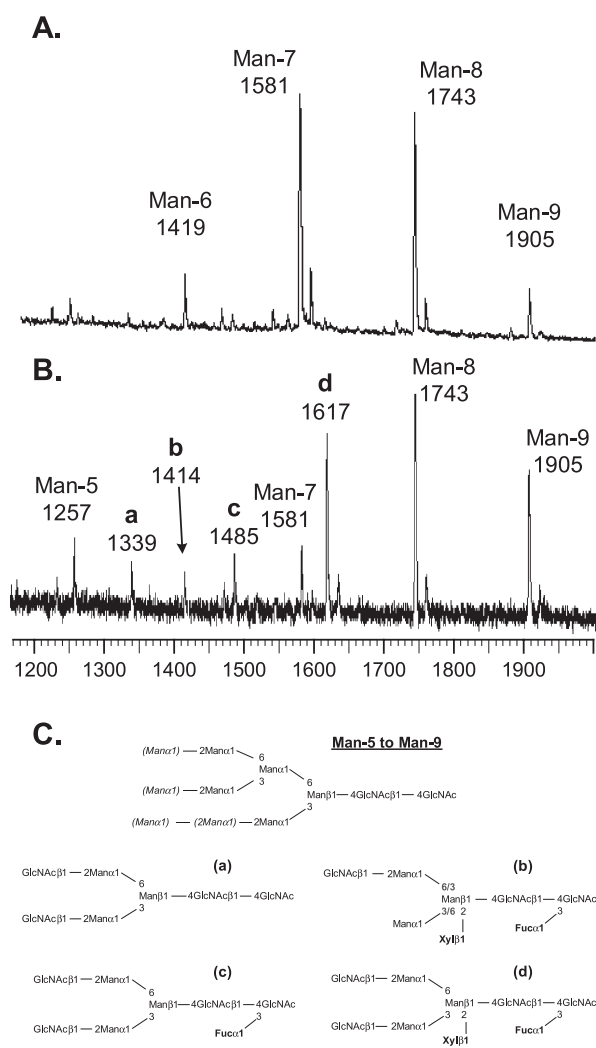


Figure 4 Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra of N-linked glycans isolated from: (A) KDEL-tagged cPIPP antibody purified from transgenic tobacco leaves and (B) cPIPP antibody without a KDEL tag transiently expressed in agroinfiltrated tobacco leaves. (C) Structures of high-mannose-type (Man-5 to Man-9) and complex-type (a–d) N-linked glycans isolated from cPIPP antibodies.

the transport of the protein through the Golgi, a cPIPP antibody without a KDEL tag was transiently expressed in tobacco leaves by agroinfiltration. The resulting antibody was analysed by Western blot. As shown in Figure 2(C,D), the antibody heavy chain reacted with antibodies specific for core-xylose and core- $\alpha(1,3)$ -fucose epitopes, two Golgi-specific glycan maturations. Weak signals were obtained with antibodies directed against Lewis^a, which indicates that such epitopes are present in very low amounts on the heavy chains (data not shown). Furthermore, compared with the KDEL-tagged antibody, the MALDI-TOF-MS showed additional ions assigned to the mature N-linked glycans (a–d), some of

them (b–d) bearing core-xylose and/or core- $\alpha(1,3)$ -fucose (Figure 4B,C). Although some major ions detected correspond to high-mannose-type Man-8 and Man-9, the identification of mature oligosaccharides demonstrates that the N-linked glycans of the cPIPP antibody are accessible to Golgi enzymes and are processed into complex N-glycans in the Golgi during the secretion of the plantibody. As no additional bands were detected in the blots, we can be confident that no significant impurities were present in the samples and that the majority of the glycans were thus released from the antibody heavy chain.

Discussion

In plants, as in other eukaryotes, the processing of N-linked glycans within the ER is highly conserved. The oligosaccharide precursor $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ is processed into Man-8/Man-9 high-mannose-type N-glycans by the elimination of the three terminal glucose units, a reaction catalysed by glucosidases I and II, followed by removal of a single mannose residue by an ER-mannosidase (Lerouge *et al.*, 1998). The N-glycosylation of reticuloplasmins relies on the efficiency of the recycling machinery between the ER and the Golgi apparatus. For instance, N-glycans from calreticulins, purified either from spinach (Navazio *et al.*, 1996) or maize (Pagny *et al.*, 2000), were identified as Man-8 and Man-8/Man-9, respectively, as expected for strict localization within the ER. More recently, calreticulin from *Liriodendron tulipifera* was found to bear complex-type N-glycans in addition to oligomannosides, indicating that this reticuloplasmin is not fully retained in the ER and can be processed in the Golgi stacks (Navazio *et al.*, 2002). It has also been shown previously that the addition of H/KDEL sequences to the C-terminus of a recombinant protein is sufficient for retention in the plant ER (Frigerio *et al.*, 2001; Gomord *et al.*, 1997). The efficiency of ER retention in plants is still a matter of debate. Recent results (Frigerio *et al.*, 2001) have indicated that KDEL-tagged phaseolin is efficiently retained in the ER, as shown by the absence of late Golgi maturations. The recombinant protein was found to be sensitive to endoglycosidase H when fused to a KDEL signal, which indicates the absence of core-xylose and core-fucose. In contrast, Pagny *et al.* (2000) have shown that recombinant glycoproteins fused to HDEL undergo N-glycan modifications, such as $\alpha(1,3)$ -fucosylation and $\beta(1,2)$ -xylosylation. The different results in these experiments were obtained using different reporter glycoproteins and different ER retrieval signals (HDEL vs. KDEL). Therefore, it is not yet possible to conclude whether the efficiency of the recycling machinery is related to the sequence of the ER retention signal, the protein or the production system.

In this study, we looked at the N-glycosylation of a therapeutically relevant glycoprotein retained in the ER by means of a C-terminal KDEL tag. The heavy and light chains of cPIPP were each tagged with a KDEL sequence and, as a consequence, the fully assembled H_2L_2 antibody carried four retrieval signals. We showed that the resulting plantibody contained only high-mannose-type N-glycans, demonstrating that fusion of KDEL to both the heavy and light chains allows the production of plant-derived antibodies that are devoid of core- $\alpha(1,3)$ -fucose and core-xylose. The presence of Man-6 and Man-7, in addition to the expected Man-8/Man-9, on the KDEL-tagged cPIPP antibodies shows that the plantibody has been partially trimmed by α -mannosidase I, an enzyme located in the *cis*-Golgi, which sequentially converts the Man-8 high-mannose-type N-glycans into Man-5 by elimination of $\alpha(1,2)$ -mannose residues (Nebenfuhr *et al.*, 1999). This observation is consistent with the model of KDEL-mediated ER retrieval by binding to the KDEL receptor in the *cis*-Golgi network, followed by retrograde transport to the ER. Consequently, modifications of the glycans by enzymes localized in the ER and *cis*-Golgi network (such as α -mannosidase I) are observed, while modifications by enzymes located in the Golgi stacks and the *trans*-Golgi network, such as $\alpha(1,3)$ -fucosyl- and $\beta(1,2)$ -xylosyltransferase (Fitchette *et al.*, 1994; Pagny *et al.*, 2000), are expected to be absent from KDEL-tagged proteins that are efficiently retrieved.

By showing the presence of xylose and fucose residues in the untagged antibody, we were able to prove that this protein is secretion-competent and does not contain other intrinsic signals that would mediate ER targeting or retention. Thus, ER retrieval of the KDEL-tagged antibodies is exclusively a result of the KDEL tag itself. The fusion of a KDEL tag to the PIPP diabody also results in efficient retention of the molecule in the ER. No late Golgi modifications were detected in the glycosylated form of the protein. Although experiments have not been carried out to demonstrate that the Asn⁵⁸-linked N-glycan is accessible to Golgi enzymes, homology-based modelling (Guex *et al.*, 1999) suggests that glycans at Asn⁵⁸ are exposed and thus are likely to undergo maturation in the Golgi apparatus. Furthermore, analysis of the mouse monoclonal antibody (mAb) Guy's13 produced in tobacco showed that carbohydrates attached to Asn⁷⁴ are matured into complex-type glycans (Cabanes-Macheteau *et al.*, 1999).

Proteins purified after transient expression from tobacco and winter cherry show partial degradation of the heavy chain, whereas those purified from transgenic plants are entirely intact. The degradation most probably results from higher or different proteolytic activity in the agroinfiltrated leaves. Transgenic plants are not subject to the same kind of

injury and stress. Therefore, we argue that the general physicochemical environment is better in transgenic leaves than in agroinfiltrated leaves. Despite the presence of degradation products, the quantity and quality of the antibodies were sufficient to perform glycan analysis. The presence of non-mature glycans, such as Man-8 and Man-9 ER-specific modifications, on untagged cPIPP antibodies transiently expressed in tobacco leaves can be related to a considerable fraction of proteins '*en route*', as this plantibody was purified 72 h after *Agrobacterium* infiltration. Consistent with this, it has been reported recently that developing leaves contain up to 30% high-mannose glycans, compared with older and more mature leaves which contain up to 10% of high-mannose glycans (Elbers *et al.*, 2001). Therefore, glycan analysis performed for glycoproteins obtained from transient expression after *Agrobacterium* infiltration probably reflects the dynamic state of the N-glycosylation process in plants rather than the end-point.

Two recent reports have described the N-glycan structures of KDEL-tagged mAbs expressed in transgenic tobacco (Ko *et al.*, 2003; Ramirez *et al.*, 2003). Ko *et al.* (2003) produced an anti-rabies human mAb. The ER retrieval of this protein was not entirely efficient and about 10% of the antibodies were shown to possess complex-type glycans. In addition, the pool of high-mannose-type glycans was shifted towards lower oligomers, with Man-7 as the major species. The serum clearance of the plant-derived antibody, carrying mainly high-mannose glycans, was faster than that of the control antibody produced in a murine/human hybridoma cell line. Nevertheless, the plant-derived antibody was able to protect hamsters from a lethal dose of coyote rabies street virus. Ramirez *et al.* (2003) found an even higher proportion of complex-type glycans in a mouse mAb directed against a hepatitis B virus surface antigen. It remains unclear whether the observed differences between these reports and our data are related to the intrinsic properties of the different antibodies, the plant expression host, the sequence context and length of the KDEL tag, or the different numbers of KDEL tags present in the assembled molecules. We used an extended version of the KDEL retrieval signal that also contains two restriction sites and a Gly₄Ser linker motif (VDGGGGSAAARGSEKDEL). The primary intention was to ensure that the C-terminal KDEL tag could be accessed efficiently to enable efficient binding to the KDEL receptor. Previously, we had observed efficient ER retrieval of an anti-tobacco mosaic virus (TMV) bispecific scFv in transgenic tobacco (Fischer *et al.*, 1999), and therefore we opted to use the same tag again for this study. Whether both the light and heavy chains need to carry a KDEL tag for efficient ER retrieval is an interesting question that is currently under investigation. However, the results obtained for the

KDEL-tagged homodimeric diabody show that two well-exposed KDEL tags are equally sufficient for efficient ER retrieval, suggesting that length and sequence context might be more important factors.

The production of antibodies with all biological functions intact requires specific N-glycosylation of the Fc domain (Jefferis, 2001). This has been evaluated by measuring the effector functions of antibodies having various Fc domain N-glycan structures. These N-glycans were either obtained by enzymatic modification using exoglycosidases (Mimura *et al.*, 2000), or by expressing the antibodies in Chinese hamster ovary (CHO) cell glycosylation mutants (Wright and Morrison, 1994, 1998). The antigen-binding properties of the antibody are not affected by the absence or the structure of the glycan. The effector functions, however, depend on the glycosylation of the Fc domain (Wright and Morrison, 1994). An IgG antibody carrying Man-5 has been purified after expression in the CHO *Lec1* mutant that lacks N-acetylglucosaminyltransferase I. This antibody had lower effector activities and a reduced *in vivo* half-life than the IgG produced in wild-type CHO cells (Wright and Morrison, 1994, 1998). In addition, the (Man-5)-IgG showed a higher capacity to activate the alternative pathway of the complement system by interacting with the mannan-binding lectin, which, when activated inappropriately, might cause inflammation. For antibodies requiring effector functions for their therapeutic use, additional experiments need to be conducted to investigate whether plantibodies carrying high-mannose-type N-glycans are suitable for human therapy.

For the immunoneutralization of hCG, a free circulating antigen, effector functions are neither necessary nor desirable, making the engineering of a plant-produced variant of cPIPP by removal or modification of the glycans more amenable for therapeutic use. The agroinfiltration system should help significantly in the development and production of glycovariants of cPIPP in particular and of other recombinant glycoproteins in general.

Experimental procedures

Generation of chimeric anti-hCG antibody genes for expression in plants

Mouse/human chimeric anti-hCG plant expression constructs for the heavy and light chain genes were generated by replacing the mouse light and heavy chain constant domain sequences with their human counterparts, kappa and IgG1, respectively, using splice overlap extension polymerase chain reaction (SOE-PCR) (Kathuria *et al.*, 2002). The recombinant

antibody genes were cloned in separate plant expression vectors, as described previously (Vaquero *et al.*, 1999). The C-terminal KDEL tag was added so that the antibodies could be retrieved to the ER (Denecke *et al.*, 1992). The chimeric antibodies secreted into the apoplast had no C-terminal KDEL tag. The nucleotide sequence of the C-terminus of cPIPP KDEL for the chimeric heavy and light chain constructs is shown below. The sequence of the KDEL signal is underlined, restriction sites (*Sal*I, *Not*I and *Xho*I) are shown in lower case, and stop codons are shown in italic: 5'-CAG AAG AGC CTC TCC CGT TCT CCG GGT AAA gtc gac GGA GGT GGA GGT TCT gcg gcc gcT CGT GGA TCT GAG AAA GAT GAG CTC TAA Act cga gGG GTA G-3'. The corresponding amino acid sequence is VDGGGGSAAARGSEKDEL. The constructs lacking KDEL for both the heavy and light chains were obtained by *Sal*I-*Xho*I double digestion of the KDEL construct and religation of the compatible restriction sites. A TAG stop codon, three nucleotides downstream of the *Xho*I site, terminated translation.

Diabody construction

The variable domains of the heavy and light chains were amplified by PCR. The PCR products were gel purified and cloned into the phage display vector pHen4-II. The diabody was constructed by joining the variable domains using an eight-amino acid linker (5'-GGCTCCACCTCAGCGGCGCGCCA-3'). The recombinant PIPP diabody gene was expressed in *Escherichia coli* and screened for binding to hCG, followed by subcloning in the plant expression vector pSSH1 as described previously (Kathuria *et al.*, 2002; Vaquero *et al.*, 1999).

Transient expression of chimeric antibodies in *N. tabacum* and *P. alkekengi*

Tobacco (*N. tabacum* cv. Petite Havana SR1) and winter cherry (*P. alkekengi*) plants were grown in a greenhouse in DE73 standard soil at 25 °C with a 16-h photoperiod. Transformation, *Agrobacterium tumefaciens* culture, and transient expression after vacuum-assisted infiltration with recombinant *A. tumefaciens* were carried out as described previously (Kapila *et al.*, 1997; Kathuria *et al.*, 2002; Vaquero *et al.*, 1999).

Generation of transgenic tobacco plants expressing ER-retained diabody and chimeric antibody constructs

Tobacco plants (*N. tabacum* cv. Petite Havana SR1) were regenerated after transformation of leaf discs with recombinant *A. tumefaciens* carrying the plant expression constructs

of the diabody or heavy and light antibody chains for ER retrieval. Regenerated plants were screened for the accumulation of the appropriate recombinant proteins, and lines with the highest levels were selfed to obtain non-segregating lines. To obtain plants producing the assembled chimeric antibody, a non-segregating line expressing the chimeric antibody light chain was crossed with a non-segregating line expressing the chimeric antibody heavy chain. The resulting F1 population accumulated both antibody chains and produced functional chimeric antibody, which was purified using protein A affinity chromatography. The biological activity of the plant-expressed diabody and chimeric antibody was confirmed using *in vitro* and *in vivo* bioassays, as described elsewhere (Kathuria *et al.*, 2002).

Protein extraction and purification

Soluble proteins were extracted from leaves using a Waring blender and 2 mL of extraction buffer (5 mM β -mercaptoethanol, 20 mM ascorbic acid and 1% polyvinylpyrrolidone in phosphate-buffered saline, pH 6.0) per gram of leaf material. The cell debris was removed by centrifugation (15 000 *g*, 30 min, 4 °C). The pH of the supernatant was adjusted to 8.0 and 500 mM NaCl was added to reduce nonspecific binding to the purification matrix. After incubation at 4 °C for 1–2 h, the extract was again centrifuged at 7500 *g* for 30 min, filtered through Whatman paper and applied to the column. Proteins were purified following manufacturers' instructions. Following elution from the matrices, samples were extensively dialysed against phosphate-buffered saline containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM β -mercaptoethanol.

Western blot analysis

Purified plantibodies were resolved on 15% SDS-PAGE gels, transferred on to a nitrocellulose membrane and blocked overnight at room temperature with 3% (w/v) gelatin dissolved in Tris-buffered saline (TBS). Affinodetection with Con A (Faye and Chrispeels, 1985) and immunodetection with anti-xylose and anti-fucose antibodies were carried out as described previously (Faye *et al.*, 1993).

N-Glycan analysis by MALDI-TOF-MS

N-Glycans were isolated from recombinant cPIPP, expressed with and without the KDEL tag, as described previously (Cabanes-Macheteau *et al.*, 1999). Those with the tag were isolated from transgenic tobacco plants and agroinfiltrated

winter cherry leaves, while those without were isolated from agroinfiltrated tobacco leaves. The N-glycans were then desalted on a non-porous, graphitized carbon-black column prior to the MALDI-TOF experiments (Bakker *et al.*, 2001). MALDI-TOF mass spectra of N-glycans were acquired on a Micromass ToF Spec E mass spectrometer equipped with a nitrogen laser. Mass spectra were obtained in reflector mode using 2,5-dihydroxybenzoic acid as the matrix (Bakker *et al.*, 2001).

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