

# Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like *N*-glycan structure

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## Summary

A common argument against using plants as a production system for therapeutic proteins is their inability to perform authentic human *N*-glycosylation (i.e. the presence of  $\beta$ 1,2-xylosylation and core  $\alpha$ 1,3-fucosylation). In this study, RNA interference (RNAi) technology was used to obtain a targeted down-regulation of the endogenous  $\beta$ 1,2-xylosyltransferase (*XylT*) and  $\alpha$ 1,3-fucosyltransferase (*FucT*) genes in *Nicotiana benthamiana*, a tobacco-related plant species widely used for recombinant protein expression. Three glyco-engineered lines with significantly reduced xylosylated and/or core  $\alpha$ 1,3-fucosylated glycan structures were generated. The human anti HIV monoclonal antibody 2G12 was transiently expressed in these glycosylation mutants as well as in wild-type plants. Four glycoforms of 2G12 differing in the presence/absence of xylose and core  $\alpha$ 1,3-fucose residues in their *N*-glycans were produced. Notably, 2G12 produced in *XylT/FucT*-RNAi plants was found to contain an almost homogeneous *N*-glycan species without detectable xylose and  $\alpha$ 1,3-fucose residues. Plant-derived glycoforms were indistinguishable from Chinese hamster ovary (CHO)-derived 2G12 with respect to electrophoretic properties, and exhibited functional properties (i.e. antigen binding and HIV neutralization activity) at least equivalent to those of the CHO counterpart. The generated RNAi lines were stable, viable and did not show any obvious phenotype, thus providing a robust tool for the production of therapeutically relevant glycoproteins in plants with a humanized *N*-glycan structure.

**Keywords:** 2G12 anti-HIV antibody, *Nicotiana benthamiana*, plant *N*-glycosylation, recombinant proteins, RNAi glyco-engineering.

## Introduction

Several studies have demonstrated that plants can produce efficiently complex mammalian proteins, such as monoclonal antibodies (mAbs; for recent reviews, see Stoger *et al.*, 2002, 2004). The conserved secretory pathway between plants and mammals provides the post-translational modifications and correct folding needed to assemble functional antibodies. One advantage of using plants as an expression system is their ability to perform *N*-glycosylation similar to mammalian cells. Although plants are able to synthesize an *N*-glycan core structure identical to that of mammalian cells (GnGn), the

terminal residues differ, mainly because plant complex *N*-glycans lack  $\beta$ 1,4-galactose (and sialic acid) and core  $\alpha$ 1,6-fucose; however, they carry instead  $\beta$ 1,2-xylose and core  $\alpha$ 1,3-fucose, which are absent in mammals. Typically, an *N*-glycan profile from a plant-derived mAb consists mainly of complex *N*-glycans carrying  $\beta$ 1,2-xylose and core  $\alpha$ 1,3-fucose residues (for example, Cabanes-Macheteau *et al.*, 1999; Bakker *et al.*, 2001; Bardor *et al.*, 2003; Schähs *et al.*, 2007). Although it seems that the natural lack of core  $\alpha$ 1,6-fucose may provide an advantage for a possible therapeutic use (for example, Shields *et al.*, 2002), unwanted *in vivo* side-effects of the non-mammalian sugars  $\beta$ 1,2-xylose and core

$\alpha$ 1,3-fucose cannot be excluded, in particular when mAbs are intended to be used for systemic application. The immunogenicity of these *N*-glycan epitopes is well documented and their role in allergy has not yet been clarified (for a recent review, see Altmann, 2007). RNA interference (RNAi) has proven to be a valuable tool for the elimination of core  $\alpha$ 1,3-fucosyltransferase (FucT) and  $\beta$ 1,2-xylosyltransferase (XylT) expression (Cox *et al.*, 2006), the two enzymes responsible for the attachment of  $\beta$ 1,2-xylose and core  $\alpha$ 1,3-fucose. The surprisingly high efficiency of this strategy led to the production of a mAb with a single *N*-glycan species (GnGn) in the aquatic plant species *Lemna minor*. More importantly, the effector activities of antibodies with such a glycan-optimized profile were significantly enhanced compared with their Chinese hamster ovary (CHO)-derived homologues (Cox *et al.*, 2006). Thus, it seems that the lack of core  $\alpha$ 1,3-linked fucose on a plant-made antibody has the same positive effect on its antibody-dependent cellular cytotoxicity activity as the removal of  $\alpha$ 1,6-fucose on CHO-derived antibodies (Shields *et al.*, 2002).

Because of several unique features of human immunodeficiency virus (HIV), immunogens able to elicit neutralizing antibodies to a broad range of primary HIV-1 isolates have not yet been found. Nevertheless, a cocktail of three mAbs broadly neutralizing different HIV-1 strains exhibits *in vivo* inhibitory viral activity in patients (Trkola *et al.*, 2005). The main antiviral effect of this cocktail was attributable to 2G12, making this antibody particularly interesting. Currently, recombinant 2G12 used for *in vitro* and *in vivo* studies is produced in CHO cells (for example, Armbruster *et al.*, 2002). Recently, we reported the expression of 2G12 in the model plant *Arabidopsis thaliana* and a glycosylation mutant thereof (XT/FT k.o.), synthesizing complex *N*-glycans without xylose and core  $\alpha$ 1,3-fucose (Strasser *et al.*, 2004a). Although *N*-glycans from wild-type *A. thaliana* 2G12 exhibit mainly xylosylated and core  $\alpha$ 1,3-fucosylated *N*-glycans (GnGnXF), 2G12 derived from the XT/FT k.o. line carries a homogeneous glycan species (GnGn) devoid of plant-specific glycan epitopes (Schähs *et al.*, 2007). *Arabidopsis thaliana*- and CHO-derived 2G12 exhibit no differences in electrophoretic mobility and antigen-binding capacity. However, no further characterization of *A. thaliana*-derived 2G12 was reported, mainly because of the relatively low expression levels and low biomass. Functional analyses are particularly important as, for unknown reasons, another anti-HIV antibody (2F5) produced in tobacco cells revealed a significant decrease in neutralization activity compared with the CHO-derived counterpart (Sack *et al.*, 2007). Although recent progress has been made in enhancing the expression levels of recombinant proteins in

*A. thaliana* seeds (De Jaeger *et al.*, 1999; Van Droogenbroeck *et al.*, 2007), this is currently not the species of choice if a large amount of recombinant proteins is needed (for example, detailed functional *in vitro* and *in vivo* studies). Tobacco and related species have been proven to meet many of the requirements needed for a rapid, efficient and cost-effective production platform. In particular, *Nicotiana benthamiana* is widely used for the efficient expression of recombinant proteins. Recent advances in expression technologies have enabled the production of recombinant proteins at extremely high levels in this plant species (Marillonnet *et al.*, 2004). These results, together with a relatively fast growth rate and reasonable growth conditions, make this plant species particularly economically interesting.

In this study we modulated the *N*-glycan composition in *Nicotiana benthamiana* using RNAi constructs to obtain a targeted downregulation of the expression of endogenous *FucT* and *XylT* genes. These glycosylation mutants were used to produce various 2G12 glycoforms differing in the presence/absence of plant-specific glycan epitopes. All plant-derived 2G12 glycoforms were investigated for their structural and functional integrity using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), antigen binding and virus neutralization activity.

## Results

### Generation of *N. benthamiana* glycosylation mutants

To obtain a targeted down-regulation of endogenous *XylT* and *FucT* genes, two binary constructs with appropriate RNAi sequences were used to transform *N. benthamiana* wild-type plants (Figure 1a). Independent transgenic *XylT*- and *FucT*-RNAi lines were screened for the presence/absence of the respective products: *N*-glycans with xylose or core  $\alpha$ 1,3-fucose residues, respectively. Protein extracts from RNAi lines were subjected to Western blot analyses using xylose- and core  $\alpha$ 1,3-fucose-specific antibodies, respectively (Figure 1b). Various staining intensities were obtained, indicating the presence of different amounts of xylose and fucose residues, respectively. One *XylT*-RNAi line (line 1, named  $\Delta$ XT) and one *FucT*-RNAi line (line 14, named  $\Delta$ FT), which exhibited no or very weak staining with the corresponding antibody, were brought to a homozygous stage and used for crossing. The progeny thereof were subjected to Western blotting using anti-horseradish peroxidase (anti-HRP) antibodies, which recognize  $\beta$ 1,2-xylose- and core  $\alpha$ 1,3-fucose-containing

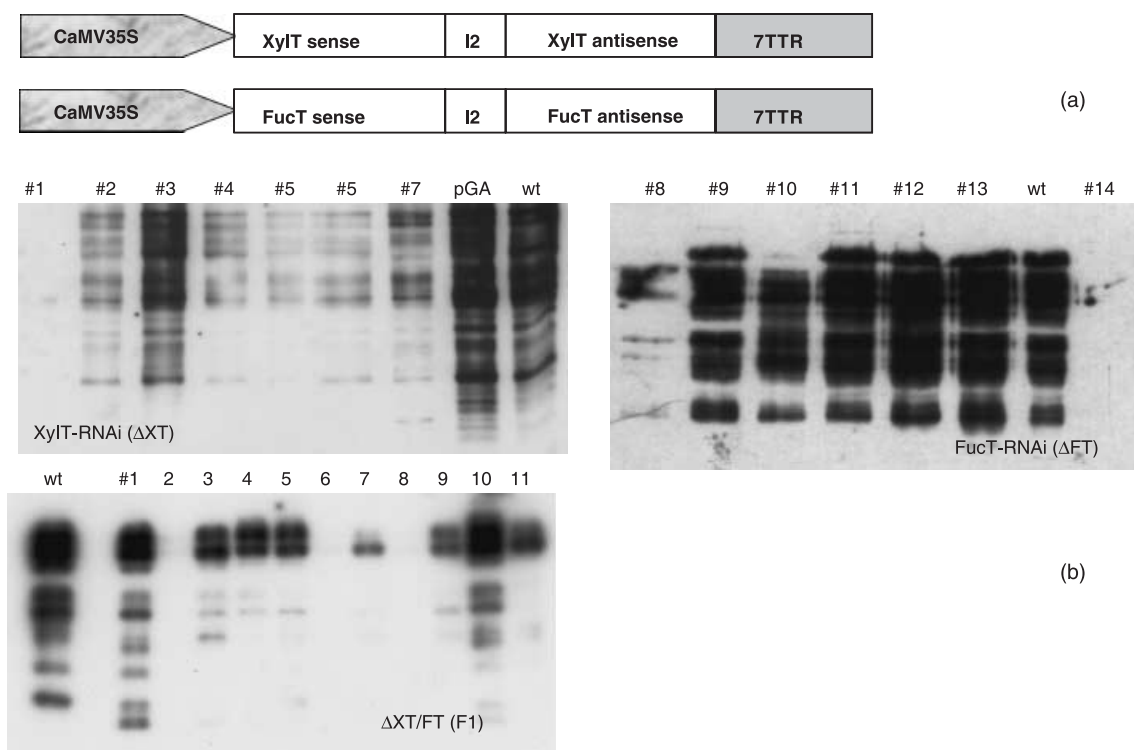


**Figure 1** (a) XylT- and FucT-RNAi constructs [RNA interference constructs targeting the expression of endogenous  $\beta$ 1,2-xylosyltransferase (*XylT*) and  $\alpha$ 1,3-fucosyltransferase (*FucT*) genes] used for *Nicotiana benthamiana* transformation. CaMV35S, cauliflower mosaic virus 35S promoter; I2, intron; 7TTR, transcriptional termination sequence. A detailed description of the cloning procedure and length of *XylT* and *FucT* sense–intron–antisense constructs is given in ‘Experimental procedures’. (b) Western blot analysis of total protein extracts derived from XylT- and FucT-RNAi transformed *N. benthamiana* leaves using anti-xylose (in  $\Delta$ XT lines) and anti-fucose (in  $\Delta$ FT lines) specific antibodies.  $\Delta$ XT/FT, total proteins extracted from progeny of crossed  $\Delta$ XT and  $\Delta$ FT. The presence/absence of xylose and fucose was detected with anti-horseradish peroxidase (anti-HRP) antiserum. Numbers indicate the different transformed lines, pGA, *N. benthamiana* transformed with empty binary vector; wt, non-transformed plants.

structures (Wilson and Altmann, 1998). Several plantlets of this F<sub>1</sub> generation did not exhibit any staining signal (Figure 1b), indicating the lack of both *N*-glycan epitopes. One line (line 6) was grown to maturity and used for further propagation. Although selfed progeny (F<sub>2</sub>) and F<sub>3</sub> seedlings still exhibited a heterogeneous staining pattern in immunoblotting using anti-HRP antibodies, F<sub>4</sub> progeny showed a uniform negative staining (data not shown), indicating the stable down-regulation of both *XylT* and *FucT*. This line was named  $\Delta$ XT/FT. In addition, xylose and fucose reduction was stable for at least three generations in  $\Delta$ XT and  $\Delta$ FT single RNAi lines, respectively. All RNAi lines were viable and did not exhibit any obvious phenotype under standard growth conditions.

#### *N*-Glycan analysis of total endogenous proteins from *N. benthamiana* wild-type and RNAi lines

To reveal the exact *N*-glycan composition of the total endogenous proteins, wild-type and all three glycosylation mutants of *N. benthamiana* were subjected to matrix-assisted laser desorption ionization-time of flight/time of flight mass

spectrometry (MALDI-TOF/TOF MS) analysis (Figure 2). As expected, the mass spectrum of wild-type plants revealed that the vast majority of the *N*-glycan species carried xylose and  $\alpha$ 1,3-fucose residues (over 80%). In contrast, the *N*-glycan composition in  $\Delta$ XT and  $\Delta$ FT exhibited a significant reduction of xylose and fucose, respectively. Surprisingly, the specific down-regulation was more pronounced in the  $\Delta$ XT, where only traces of one xylose-carrying glycoform were detected (GnGnXF: approximately 3%). However, significant amounts of  $\alpha$ 1,3-fucose carrying glycoforms were present in the  $\Delta$ FT (about 20% of MMXF and GnGnXF). The *N*-glycan composition of the  $\Delta$ XT/FT line exhibited a profile with complex-type *N*-glycans virtually lacking xylose and a significant reduction of  $\alpha$ 1,3-fucose (Figure 2).

#### Expression and purification of immunoglobulin G (IgG) glycoforms

$\Delta$ XT,  $\Delta$ FT,  $\Delta$ XT/FT and wild-type *N. benthamiana* plants were used to transiently express 2G12, a human IgG1 mAb. A binary vector that carries the two cDNAs of 2G12 light and

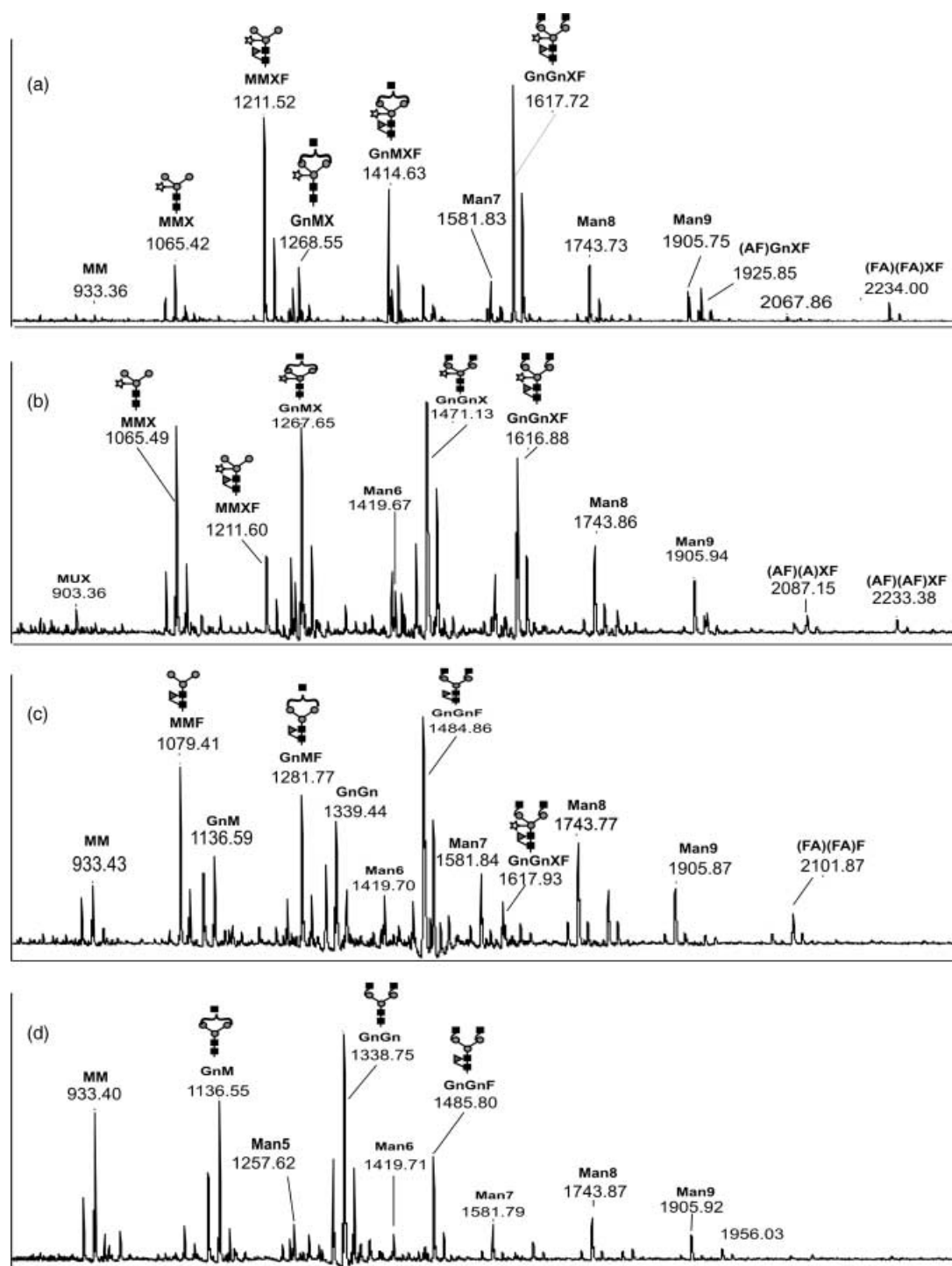


**Figure 2** Matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry (MALDI-TOF/TOF MS) of oligosaccharides from endogenous proteins extracted from *Nicotiana benthamiana* leaves: (a) wild-type; (b)  $\Delta$ FT; (c)  $\Delta$ XT; (d)  $\Delta$ XT/FT. The labelled peaks represent (M + Na)<sup>+</sup> ions. Other peaks are potassium adducts of the same glycans. (See <http://www.proglycan.com> for an explanation of N-glycan abbreviations.)

heavy chain cloned in tandem (Schähs *et al.*, 2007) was used to agroinfiltrate *N. benthamiana* plants. Three days after infiltration, the leaves were initially monitored for 2G12 expression by immunoblotting using anti-human IgG

antibodies. Two bands at positions 55 and 25 kDa, the expected size of the heavy and light chains, respectively, were detected (data not shown), demonstrating the functional expression of the two cDNAs. On average, an expression level

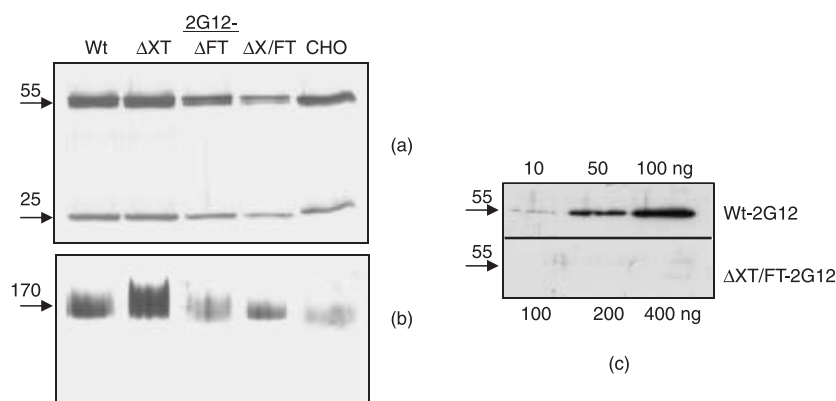


**Figure 3** Silver-stained sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of purified 2G12 derived from wild-type *Nicotiana benthamiana* (Wt), different RNA interference (RNAi) lines and Chinese hamster ovary (CHO) cells under reducing (a, 50 ng) and non-reducing (b, 200 ng) conditions. (c) Western blot analysis of the heavy chain from purified wild-type 2G12 (Wt-2G12) and ΔXT/FT-2G12 using anti-horseradish peroxidase antiserum. Please note that different amounts of 2G12 were loaded.

of about 0.5% of total soluble proteins was detected, which corresponds to 110 μg of 2G12 per gram of leaf material. Subsequently, agroinfiltrated leaf material was used to purify 2G12 by protein A affinity chromatography, yielding 50 μg of purified 2G12 per gram of biomass. In this respect, it should be mentioned that neither the infiltration process nor any purification step was systematically optimized. SDS-PAGE analysis of purified 2G12 revealed the presence of two bands that correlated with the expected molecular masses of the two chains (Figure 3a). An additional weak band was detected slightly below the band for the heavy chain, representing (LC-ESI-MS data of the glycopeptides) an unglycosylated form of the heavy chain. No further bands, degradation products or free heavy or light chains were visible in reducing and non-reducing gels, indicating the purity and structural integrity of all plant-produced 2G12 glycoforms (Figure 3a,b).

### N-Glycan analyses of 2G12 glycoforms

To determine the exact *N*-glycan composition of CHO- and plant-derived 2G12 glycoforms, the corresponding heavy chains, excluding the unglycosylated fraction, were subjected to LC-ESI-MS. Because of incomplete trypsin digestion of the heavy chain, two glycopeptides, which contain the carbohydrate attached to the side-chain asparagine-297, but differ in four amino acids (482.3 Da), were present. The absence of xylose and fucose residues on *N*-glycans can be monitored by a decrease in the mass of the respective peaks (132 mass units for xylose and 146 mass units for fucose).

The mass spectrum of wild-type 2G12 exhibited two major peaks for both glycopeptides (Figure 4b, Table 1), which were assigned to the structure GnGnXF. This *N*-glycan species encompassed over 68% of all assigned structures. Only two other structures (GnMXF and GnUXF) slightly exceeded a share of 5% of the total *N*-glycan structures. Noteworthy, that over 90% of all structures carried xylose and core α1,3-

**Table 1** Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) analysis of the glycosylated Fc peptides EEQYN297STYR and TKPREEQYN297STYR from *Nicotiana benthamiana* wild-type (wt) 2G12, ΔXT-2G12, ΔFT-2G12 and ΔXT/FT-2G12

| <i>N</i> -glycan | Relative abundance (%) <sup>*</sup> |      |      |        |
|------------------|-------------------------------------|------|------|--------|
|                  | wt                                  | ΔXT  | ΔFT  | ΔXT/FT |
| GnGn             | 1.9                                 | 20.8 | 5.8  | 80.6   |
| GnGnF            | 2.1                                 | 39.5 | 1.2  | < 1    |
| GnGnX            | 5.5                                 | 1.3  | 41.8 | < 1    |
| GnGnXF           | 67.6                                | 10.4 | 19.4 | < 1    |

<sup>\*</sup>Numbers are the sum of the relative abundances of a specific glycoform found on each of the two immunoglobulin G (IgG) glycopeptides. The data were deduced from the LC-ESI-MS analyses illustrated in Figure 4.

fucose residues. The mass spectra of ΔXT- and ΔFT-2G12 exhibited two major peaks identified as GnGnF and GnGnX, respectively (Figure 4c,d, Table 1). However, in both lines, GnGnXF was also found: 20% in ΔFT-2G12 and 10% in ΔXT-2G12. This result demonstrates a significant decrease, but not complete elimination, of xylose and fucose residues in the respective RNAi lines. For so far unknown reasons, interestingly, an increased level of GnGn structures was detected in ΔXT-2G12. By far the most abundant *N*-glycan structure present in ΔXT/FT-2G12 was GnGn (80%; Figure 4e, Table 1). Surprisingly, and in contrast with the *N*-glycan structure of total endogenous proteins, no xylose- and fucose-containing *N*-glycan structures were detected. As traces of xylose- and fucose-containing glycoforms could not be excluded, there was a need to estimate at what level plant-specific glycoforms could be detected using the method described here. A serial dilution of wild-type 2G12 in CHO-2G12 was analysed, and revealed no detection of plant-specific *N*-glycans when the wild-type 2G12 content was below 5% (data not shown). To further confirm this result, an immunoblot

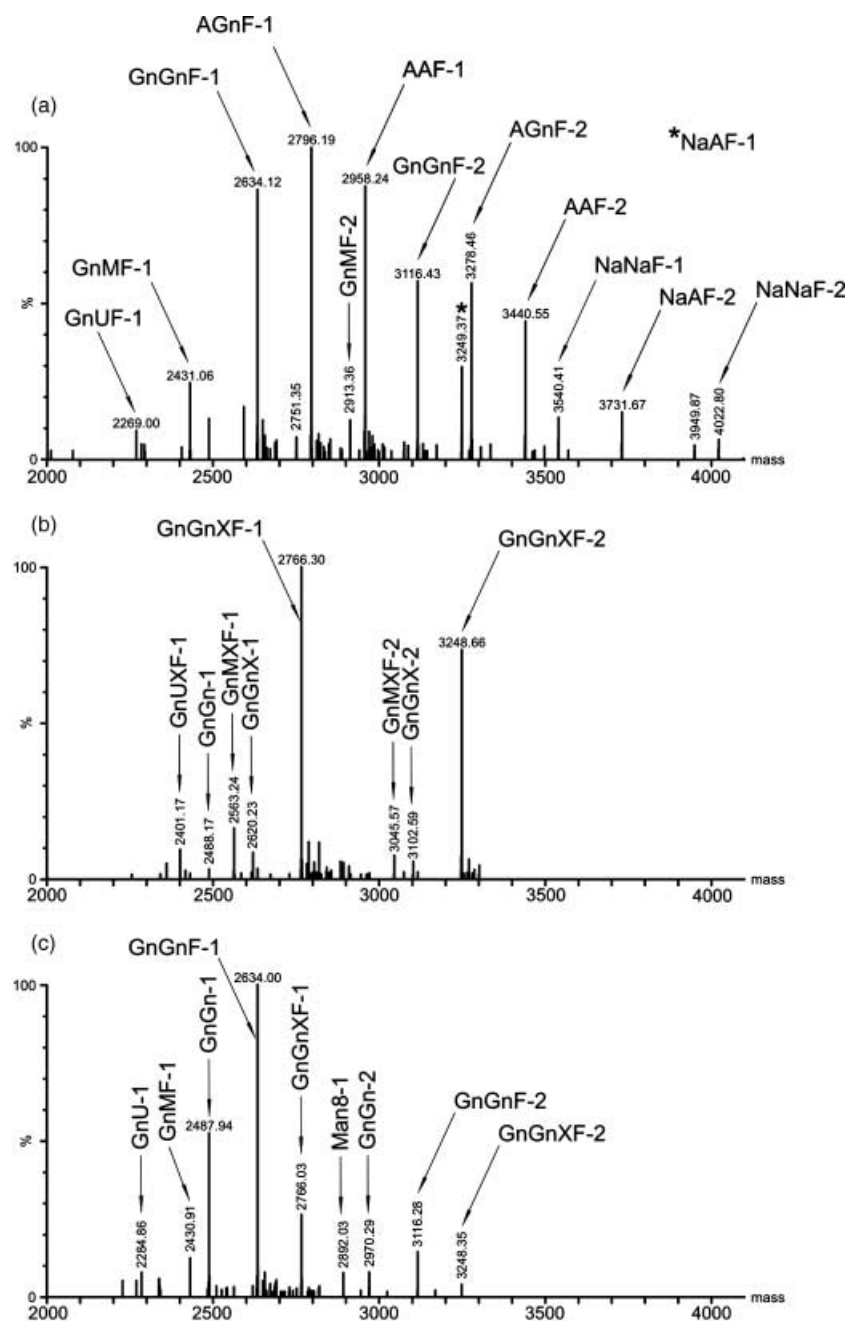


**Figure 4** Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of glycosylated peptides (1, EEQYNSTYR; 2, TKPREEQYNSTYR) containing asparagine-297 (Asn-297) from the 2G12 Fc region. Glycopeptides derived from Chinese hamster ovary (CHO)-2G12 (a), *Nicotiana benthamiana* wild-type 2G12 (b),  $\Delta$ XT-2G12 (c),  $\Delta$ FT-2G12 (d),  $\Delta$ XT/FT-2G12 (e). (See <http://www.proglycan.com> for an explanation of *N*-glycan abbreviations.) For CHO-2G12,  $\Delta$ XT-2G12 and  $\Delta$ FT-2G12, 500 ng of heavy chain was used; for  $\Delta$ XT/FT-2G12, 1  $\mu$ g.

using anti-HRP antiserum, which efficiently recognizes plant-specific *N*-glycan structures, was carried out (Figure 3c). Although anti-HRP recognizes 10 ng of wild-type 2G12, no reaction was obtained with  $\Delta$ XT/FT-2G12, even when 400 ng was loaded (Figure 4c). This result indicates a detection limit of plant-specific *N*-glycans below 2.5%, and demonstrates an efficient elimination of both xylose and core  $\alpha$ 1,3-fucose in  $\Delta$ XT/FT-2G12. *N*-Glycan analysis of CHO-2G12 revealed a mixture of structures containing either no (GnGnF), one (AGnF, GnAF) or two (AAF) terminal galactose residues and minor amounts of glycopeptides with terminal sialic acid

(NaAF, NaNaF). Interestingly, all detected glycoforms contained a fucose residue  $\alpha$ 1,6-linked to the innermost *N*-acetylglucosamine (GlcNAc).

#### Functional analyses of 2G12 glycoforms

In order to test the functional integrity of the 2G12 glycoforms, specific antigen-binding and HIV neutralization assays were carried out. In previous studies, it has been shown that 2G12 recognizes an epitope within the glycoprotein 120 (gp120) subunit of the HIV envelope glycoprotein 160 (gp160) (Trkola

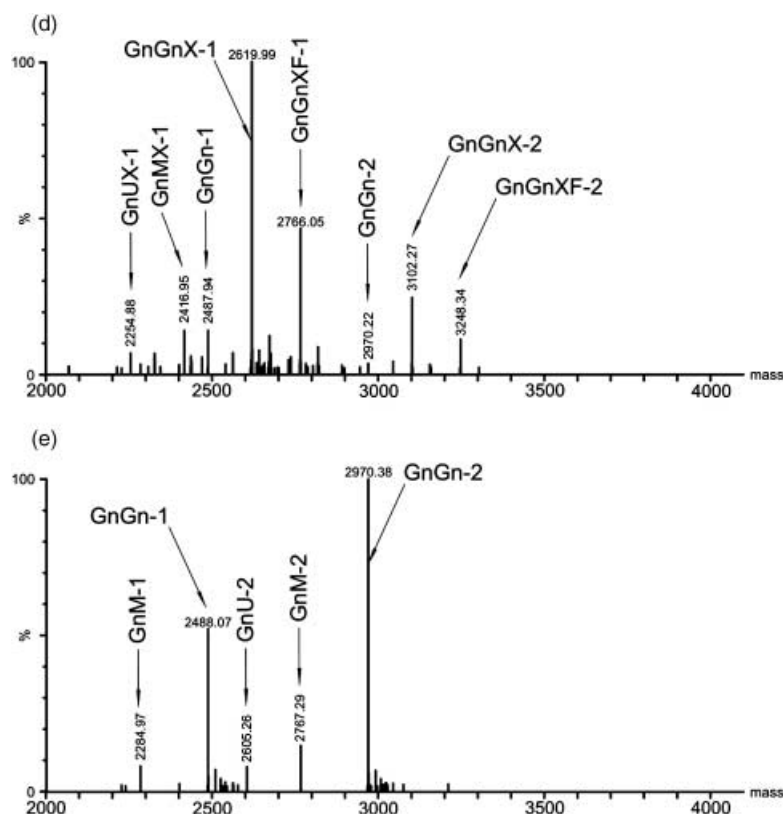


Figure 4 Continued

**Table 2** Analysis of functional integrity of 2G12 glycoforms. Antigen-binding capacity was detected by a specificity enzyme-linked immunosorbent assay (ELISA) coated with purified human immunodeficiency virus (HIV) glycoprotein 160 (gp160). *In vitro* HIV-1 neutralization activity of 2G12 glycoforms was detected by a syncytium inhibition assay using AA-2 cells

| 2G12 glycoform | Antigen binding (%) ( $\pm$ SD) | IC <sub>50</sub> ( $\mu$ g/mL) | IC <sub>50</sub> ( $\mu$ g/mL) | IC <sub>50</sub> ( $\mu$ g/mL) | Average IC <sub>50</sub> (SD) ( $\mu$ g/mL) |
|----------------|---------------------------------|--------------------------------|--------------------------------|--------------------------------|---------------------------------------------|
| Wild-type      | 117 ( $\pm$ 17)                 | 5.3                            | 1.3                            | 1.6                            | 2.7 (2.2)                                   |
| $\Delta$ XT    | 115 ( $\pm$ 16)                 | 4.4                            | 1.9                            | 1.9                            | 2.7 (1.4)                                   |
| $\Delta$ FT    | 138 ( $\pm$ 20)                 | 4.4                            | 0.6                            | 6.3                            | 3.8 (2.9)                                   |
| $\Delta$ XT/FT | 130 ( $\pm$ 19)                 | 3.1                            | 2.2                            | 2.2                            | 2.5 (0.5)                                   |
| CHO            | 100 ( $\pm$ 15)                 | 18                             | 4.4                            | 12.6                           | 11.7 (6.8)                                  |

CHO, Chinese hamster ovary; IC<sub>50</sub>, 50% inhibitory concentration; SD, standard deviation.

*et al.*, 1996). In order to test the antigen binding of plant-produced 2G12 glycoforms, a specificity enzyme-linked immunosorbent assay (ELISA), using purified gp160, was carried out. Compared with CHO-2G12, the binding capacity of the plant-derived antibodies was 100% or slightly above (Table 2), demonstrating that the antigen-binding capacity was at least as efficient as that of the CHO-derived protein.

Finally, the ability of plant-derived 2G12 glycoforms to neutralize HIV was examined using a syncytium inhibition assay (Table 2). All plant-derived 2G12 glycoforms exhibited relatively uniform 50% inhibitory concentration (IC<sub>50</sub>) values. No significant differences were observed between the four glycoforms during three independent virus neutralization

assays using three different 2G12 batches. Notably, in all tests, CHO-2G12 displayed a three- to fourfold greater IC<sub>50</sub> value.

Our results demonstrate that all plant-derived 2G12 glycoforms are fully functional antibodies and exhibit activities at least comparable with their CHO-produced counterpart.

## Discussion

The feasibility of glyco-engineering in *N. benthamiana*, a plant species commonly used for the high-level expression of recombinant proteins, has been demonstrated. For this, three glycosylation mutants were generated using RNAi-targeted

down-regulation of the endogenous *XylT* and *FucT* genes. Silencing was stable and plants were viable and did not show an obvious phenotype under laboratory conditions. More importantly, the human mAb 2G12 against HIV was efficiently expressed, secreted and assembled in all three glycosylation mutants, as well as in wild-type *N. benthamiana*. Although the *N*-glycans of wild-type 2G12 were mainly decorated with xylose and core  $\alpha$ 1,3-fucose residues,  $\Delta$ XT- and  $\Delta$ FT-2G12 exhibited a significant decrease in the respective glycan residues. Interestingly,  $\Delta$ XT/FT-2G12 carried a major complex *N*-glycan species with undetectable plant-specific carbohydrate modifications. The absence of xylosylated and core  $\alpha$ 1,3-fucosylated *N*-glycan structures was confirmed by MS analysis and immunoblotting of purified  $\Delta$ XT/FT-2G12. To roughly estimate the detection limits of the methods used, a serial dilution wt-2G12 in CHO-2G12 was analysed with MS, and revealed a detection limit below 5% (data not shown). Furthermore, plant-specific carbohydrates were recognized by anti-HRP antibodies when only 10 ng of wild-type 2G12 was used. No reaction was obtained using 400 ng of  $\Delta$ XT/FT-2G12, indicating that, if any glycan epitopes were present, the level was below the detection limit of 2.5%. The fact that neither xylose nor  $\alpha$ 1,3-fucose residues were detected in  $\Delta$ XT/FT-2G12 is surprising, as *N*-glycans of total endogenous proteins, as well as 2G12 expressed in single RNAi lines, did not exhibit a complete elimination of the *N*-glycan epitopes. This was particularly true for  $\alpha$ 1,3-fucose, for which amounts of about 20% were still present on total endogenous proteins in corresponding RNAi lines as well as in  $\Delta$ FT-2G12. These results suggest that an incomplete down-regulation of *XylT* and *FucT* activity is sufficient to allow the generation of IgGs carrying a glycan-optimized structure. One explanation of the relatively low down-regulation of *FucT* compared with *XylT* could be the presence of several copies of *FucT* genes, as described for other plant species (Strasser *et al.*, 2004a; Bondili *et al.*, 2006). Another unexpected observation was the presence of a significant portion of non-glycosylated heavy chain. However, this does not seem to influence antigen binding and HIV neutralization. This is not surprising as the importance of *N*-glycosylation status mainly lies in the *in vivo* activities of IgGs (for example, antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity). Possible *in vivo* effects of this incomplete glycosylation remain to be investigated.

The presence of a major *N*-glycan species in  $\Delta$ XT/FT-2G12 (GnGn) indicates that the strategy applied in this study has, beside the elimination of potentially immunogenic *N*-glycan residues, the additional benefit of producing a monoclonal antibody with a widely homogeneous *N*-glycan profile. This

may serve as a tremendous advantage over CHO-produced 2G12, where a variety of glycoforms are present. Notably, nearly all glycan structures of CHO-2G12 carry  $\alpha$ 1,6-fucose, a glycan residue which negatively interferes with the effector activities of IgGs (Shields *et al.*, 2002; Shinkawa *et al.*, 2003; Ferrara *et al.*, 2006). In this respect, the absence of plant-specific *N*-glycans on  $\Delta$ XT/FT-2G12 may not only eliminate potential negative side-effects, when systemically applied in humans, but may also positively influence effector functions, as recently described for other plant-derived glycan-optimized IgGs (Cox *et al.*, 2006; Schuster *et al.*, 2007).

All plant-derived 2G12 glycoforms were indistinguishable from the CHO-produced counterpart with regard to electrophoretic mobility and IgG assembly, and showed at least the same antigen-binding capacity. Moreover, for all plant-produced 2G12 glycoforms, HIV neutralization activity, as determined by syncytium inhibition assays, was at least the same as that of the CHO-derived protein. This is particularly interesting as, recently, another HIV antibody produced in tobacco cells exhibited a significantly reduced HIV neutralization activity compared with the CHO-derived counterpart (Sack *et al.*, 2007).

Therefore, in this study, it has been demonstrated that glyco-engineered *N. benthamiana* may serve as a robust and efficient expression platform for the production of human mAbs without detectable plant-specific *N*-glycan residues.  $\Delta$ XT/FT and the single RNAi lines, together with the recently developed viral-based expression vectors (Marillonnet *et al.*, 2004; Giritich *et al.*, 2006), which allow the generation of high titres of IgG (0.5 g of IgG per kilogram of fresh leaf biomass; Giritich *et al.*, 2006), will pave the way to the transfer of knowledge gained in the laboratory to a clinical trial scale, where grams of purified IgGs are needed.

## Experimental procedures

### RNAi constructs

For the *XylT*-RNAi construct, a  $\beta$ 1,2-xylosyltransferase fragment was amplified from *N. benthamiana* leaf cDNA using the forward primer XT24 (5'-TATATGTCGACTCTAGATTAGCAATGAAGAGCAAGTA-3') and reverse primer Tom-XT23 (5'-AGCAGCCAAGACTCCTCAAAT-3'), which were designed on the basis of sequence homology to already known (for example, Strasser *et al.*, 2004a; Bondili *et al.*, 2006) or annotated *XylT* sequences in the database. The polymerase chain reaction (PCR) product (304 bp) was *SalI/BamHI* digested and ligated into the *SalI/BamHI* sites of cloning vector puc18XTI2 (Strasser *et al.*, 2007) to create puc18Xsi. The antisense fragment was obtained by PCR using the primers XT25 (5'-TATATGAATTCTAGAT TAGCAATGAAGAGCAAGTA-3') and XT26 (5'-ATTGCGGTACCGCATAAGACCCCTCCA-3'), and cloned into the *EcoRI/KpnI* site of puc18Xsi to create puc18Xsias.

Subsequently, the 'sense-intron-antisense' cassette was excised by *Xba*I digestion and cloned into *Xba*I linearized plant expression vector pGA643. *FucT* cDNA (414 bp) was amplified by PCR using the primers NbFT1 (5'-TTATGGTACCGGATCCTTGGCAGCGGCTTTCATTT-3') and NbFT2 (5'-AATTGGTACCGGATCCATCAGATGGGCCCTCAAAC-3'), also designed on the basis of sequence homology with already known *FucT* genes (for example, Strasser *et al.*, 2004a; Bondili *et al.*, 2006), and cloned into the *Bam*HI site of puc18XTI2 to create puc18Fsi. The antisense fragment was amplified by PCR using primers NbFT2 and NbFT4 (5'-TTATGGTACCTCTAGATTGGCAGCGGCTTTCATTT-3'), and cloned into the *Kpn*I digested vector puc18Fsi to generate puc18Fsias. Subsequently, the 'sense-intron-antisense' cassette was excised by *Xba*I digestion and cloned into *Xba*I linearized plant expression vector pGA643.

### Screening of transgenic plants

Leaf disc transformation was used to transform *N. benthamiana* plants with appropriate RNAi constructs (Strasser *et al.*, 2004b). Kanamycin-resistant plants were screened by PCR to confirm the genomic insertion of the RNAi constructs. Total soluble proteins of transgenic plants were extracted in phosphate-buffered saline (PBS) (w/v) and subjected to Western blot analysis as described previously (Strasser *et al.*, 2004a) using rabbit anti-xylose and anti-fucose antiserum (1 : 500). Crossed F<sub>1</sub> and further selfed progeny were screened using rabbit anti-HRP antiserum (1 : 15 000; Sigma-Aldrich, St Louis, MO, USA). As detection antibody, HRP-conjugated goat anti-rabbit IgG (1 : 100 000; Sigma-Aldrich) was used. Peroxidase detection was carried out with Super Signal West Pico Chemiluminescent Substrate Kits (Pierce, Rockford, IL, USA).

### Preparation of N-linked glycans and MALDI-TOF/TOF MS analysis

The preparation and purification of glycans derived from total endogenous leaf proteins were performed as described by Strasser *et al.* (2004a). Mass spectrometric analysis of endogenous glycans was carried out with a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) in reflector mode at positive ionization. 2,5-Dihydroxybenzoic acid (DHB) [2% (w/v) DHB in 50% (v/v) acetonitrile in H<sub>2</sub>O] was used as matrix.

### Agroinfiltration and 2G12 purification

*Agrobacterium* strain GV3101::pMP90RK containing 2G12 binary vector, described recently (Schähs *et al.*, 2007), was grown overnight [optical density (OD), 1.0–1.5] and diluted in infiltration buffer [50 mg D-glucose 10 mL, 50 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), 2 mM Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O, 0.1 mM acetosyringone] to an OD of 0.2. Using a 1-mL syringe without a needle, *Agrobacterium* suspensions were injected through a small puncture into fully expanded leaves of 6–8-week-old *N. benthamiana*. For purification, 30 g of infiltrated leaf material was homogenized in liquid nitrogen, resuspended in 60 mL of pre-cooled extraction buffer (1 × PBS, pH 6.0) and incubated for 15 min at 4 °C. Insoluble material was removed by centrifugation at 21 000 *g* for 30 min at 4 °C. The supernatant was filtered and sedimented as above. Subsequently, an additional

isoelectric precipitation by reducing the pH to 4.5 (1 M HCl) was carried out. The supernatant was centrifuged for 15 min at 4 °C. The supernatant obtained was brought to pH 6.0–7.0 with 1 M KOH and applied to a disposable column (Bio-Rad, Hercules, CA, USA) packed with 1 mL rProtein A Sepharose Fast Flow (GE Healthcare, Freiburg, Germany). IgG was eluted with 100 mM glycine, pH 2.5. The elution fractions were immediately adjusted to pH 7.0 by the addition of 1 M tris(hydroxymethyl)aminomethane (Tris).

### 2G12 quantification and antigen-binding assay

To quantify and determine the antigen-binding specificity of plant-derived 2G12, a sandwich ELISA was carried out. The IgG concentration and gp160 binding were determined by coating ELISA plates with a polyclonal antibody 'goat anti-human IgG' (γ-chain, Sigma; for quantification) or with 100 ng of purified gp160 (provided by Polymun Scientific, GmbH, Vienna, Austria). 2G12 samples were diluted to a concentration of about 200 ng/mL. Subsequently, a 1 : 2 serial dilution was carried out (eight dilutions) and 50 µL was transferred per well. Recombinant 2G12 purified from CHO culture supernatants (provided by Polymun Scientific) was used as standard, and applied identically to plant-derived 2G12. Plates were incubated for 1 h at room temperature (gentle shaking) and, after washing, a polyclonal antibody 'goat anti-human IgG' (κ-chain) conjugated to alkaline phosphatase (1 : 1000, Sigma) was added. After intensive washing, bound alkaline phosphatase was detected by *p*-nitrophenylphosphate substrate solution using an ELISA reader at 405 nm, with 620 nm as reference wavelength. All analyses were carried out in duplicate, and a four-parameter polynomial was applied for curve fitting and data analysis.

### N-Glycan analysis of 2G12 by LC-ESI-MS

In order to allow a highly reliable identification of glycan structures, together with rapid and sensitive quantification, *N*-glycan analysis of 2G12 glycoforms was carried out by LC-ESI-MS of tryptic glycopeptides (Schuster *et al.*, 2007; Van Droogenbroeck *et al.*, 2007). Briefly, the heavy chain (500 ng for ΔXT- and ΔFT-2G12, and 1 µg for ΔXT/FT-2G12) of SDS-PAGE-separated, purified 2G12 was excised from the gel, S-alkylated and digested with trypsin. The tryptic peptides and glycopeptides were trapped on an Aquasil C18 pre-column (30 × 0.32 mm, Thermo Electron; Thermo Scientific, Waltman, MA, USA) using water as the solvent, and then separated on a Biobasic C18 column (100 × 0.18 mm, Thermo Electron) with a gradient of 5%–50% acetonitrile containing 0.1 M formic acid. Positive ions of *m/z* = 200–2000 were monitored with a Q-TOF Ultima Global mass spectrometer (Waters, Milford, MA, USA). Identification and glycoform quantification were performed on the summed and deconvoluted spectrum of the glycopeptide elution region.

### Western blotting of wild-type 2G12 and ΔXT/FT-2G12

Purified wild-type 2G12 and ΔXT/FT-2G12 were separated by 10% SDS-PAGE under reducing conditions, transferred to nitrocellulose membrane and incubated with rabbit anti-HRP (1 : 15 000; Sigma-Aldrich). Detection was carried out with HRP-conjugated goat anti-rabbit IgG (1 : 100 000; Sigma-Aldrich) and Super Signal West Pico Chemiluminescent Substrate Kits (Pierce).

## HIV neutralization assay

To test the ability of 2G12 to neutralize HIV-1, a syncytium inhibition assay was carried out (Trkola *et al.*, 2005). Syncytium inhibition was assessed using AA-2 cells as indicator cell line with syncytium formation as read-out. Briefly, 10 twofold serial dilutions of antibodies (starting concentration, 50 µg/mL) in polybrene-containing cell culture medium (5 µg/mL; Sigma) were pre-incubated with  $10^2$ – $10^3$  50% tissue culture infective dose (TCID<sub>50</sub>) per millilitre of HIV-1 for 1 h at 37 °C before the addition of AA-2 cells. The cells were incubated for 5 days before the assessment of syncytium formation. Experiments were performed with eight replicates per dilution step. The presence of at least one syncytium per well was considered as indication for HIV-1 infection. IC<sub>50</sub> was calculated according to the method of Reed and Muench (1938). All assays included a virus titration of the inoculum to confirm its infectious titre.

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