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Biochemical and functional characterization of anti-HIV antibody–ELP fusion proteins from transgenic plants

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Summary

The stability and recovery of recombinant proteins expressed in plants are improved by fusion to elastin-like peptides (ELPs). In order to test the suitability of ELP for the production of pharmaceutical proteins, transgenic plants were created that individually expressed the light and heavy chains of the broadly neutralizing anti-human immunodeficiency virus type 1 (anti-HIV-1) monoclonal antibody 2F5, which is being evaluated as a microbicide component. The antibody chains were expressed both with and without a C-terminal ELP fusion. Crossing these plants in all combinations resulted in transgenic lines producing the full antibody in four formats, with ELP on either the light or heavy chains, on both or on neither. Characterization of the affinity-purified antibodies by surface plasmon resonance spectroscopy showed that the kinetic binding parameters were identical to those of a Chinese hamster ovary (CHO) cell counterpart lacking ELP. *N*-Glycan analysis showed that all four derivatives contained predominantly oligo-mannose-type *N*-glycans and that the ELP fusions had no significant effect on *N*-glycan structure. It was concluded that ELP fusion to the light chain, heavy chain or both chains of a plant-derived antibody had no adverse affects on protein quality, but had a positive impact on the yield. ELP fusions do not interfere with folding, assembly, trafficking in the secretory pathway or post-translational modification, but enhance stability whilst at the same time simplifying recovery.

Introduction

Human monoclonal antibodies (mAbs) play an essential role in cancer therapy (Reichert and Valge-Archer, 2007) and in the treatment of other diseases (Saketkoo and Espinoza, 2006). Innovative recombinant DNA technologies have allowed the design of recombinant antibodies relevant in human (reviewed in Carter, 2006) and veterinary (reviewed in Floss *et al*., 2007) medicine, but novel therapies for major human diseases, such as acquired immunodeficiency syndrome (AIDS), are desperately needed. The annual AIDS epidemic update revealed that 33.2 million people were living with human immunodeficiency virus (HIV) in 2007 (www.unaids.org), making AIDS one of the major global health challenges of the 21st century. Unfortunately, an effective HIV vaccine is still not in sight, and therefore passive immunotherapy has regained considerable attention for both the treatment of infected individuals and the prevention of transmission (Check, 2003). Research has focused on mucosal microbicides for the prevention of HIV transmission (Weber *et al*., 2005; McGowan, 2006). The establishment and distribution of safe, effective, acceptable and affordable products that allow women to block HIV-1 transmission has been advanced by the Alliance for Microbicide Development

(www.microbicide.org). Amongst many other reagents, mAbs, including the broadly neutralizing human anti-HIV-1 antibody 2F5 (Purtscher *et al*., 1994), are being evaluated as microbicide components.

The 2F5 antibody binds to the highly conserved linear epitope ELDKWA located at the C-terminus of the C heptad of gp41 (Binley *et al*., 2004). This antibody has been evaluated in animal and clinical trials for its ability to treat and prevent HIV-1 (Mehandru *et al*., 2004). In combination with other broadly neutralizing antibodies, protection has been conferred against intravenous (Mascola *et al*., 1999), intravaginal (Mascola *et al*., 2000) and oral (Baba *et al*., 2000) challenge with simian-human immunodeficiency virus (SHIV) in rhesus macaques. A phase I open-label trial has shown that 2F5 is safe, non-toxic and non-immunogenic (Armbruster *et al*., 2002), and efficiency has been indicated in some patients when 2F5 is combined with the neutralizing antibodies 2G12 and 4E10 (Armbruster *et al*., 2004), although the effects have been attributed mostly to 2G12. Because of the enormous global demand for HIV microbicides, new and improved production systems for these antibodies must be developed (Peterson *et al*., 2006). The increasing number of infected individuals in developing countries means that large-scale microbicide production must be established at the lowest possible cost.

In recent years, plant-based production systems for human therapeutic proteins have been developed with particular emphasis on high-yield expression and reduced production costs (reviewed in Ma *et al*., 2003; Fischer *et al*., 2004; Stoger *et al*., 2005a). Plant-derived antibodies have already been used as components of vaginal microbicides in a mouse genital herpes model (Zeitlin *et al*., 1998). Fusion protein technology has been applied to increase the accumulation of recombinant target proteins in transgenic plants (Gil *et al*., 2006; Obregon *et al*., 2006; Van Droogenbroeck *et al*., 2007). In particular, fusion to elastin-like peptides (ELPs) has been shown to enhance the expression of spider silk proteins (Scheller *et al*., 2004; Patel *et al*., 2007), human interleukin-10 and murine interleukin-4 (Patel *et al*., 2007) and single-chain variable fragment (scFv) antibodies (Scheller *et al*., 2006).

Synthetic ELPs are designed around the characteristic VPGVP motif found in native mammalian elastins (Li and Daggett, 2002), utilizing repeats of the pentapeptide VPGXG, where X can be any amino acid except proline (Meyer and Chilkoti, 1999). These polypentapeptides undergo an inverse temperature-dependent transition from a structurally disordered state to a more ordered state in which intramolecular contacts between non-polar regions of ELPs are formed after the displacement of water (Li *et al*., 2001; Li and Daggett, 2003). The purification of ELP fusion proteins produced in bacteria and plants has been achieved by a simple procedure called 'inverse transition cycling' (Meyer and Chilkoti, 1999; Lin *et al*., 2006), which makes it possible to recover spider silk elastin from kilograms of tobacco leaves using simple centrifugation (Scheller *et al*., 2004) or microfiltration steps (Ge *et al*., 2006).

Although it is clear that ELP fusion can significantly increase recombinant protein accumulation and simplify purification by inverse transition cycling, it remains to be shown whether or not ELPs interfere with the quality control mechanisms of the host cell or influence post-translational modification, factors which could impact on the suitability of ELP technology for the production of therapeutic proteins. Recently, high-resolution surface plasmon resonance analysis has shown that 2F5 derived from transgenic tobacco BY2 suspension culture cells exhibits excellent functional properties comparable with those of its counterpart produced in Chinese hamster ovary (CHO) cells (Sack *et al*., 2007). However, the accumulation levels were much lower than those observed for other antibodies, preventing further exploration of plant-based production for this important molecule.

The aim of the present study was to examine whether the fusion of ELP to a heteromultimeric protein, such as an antibody, would place increased demands on the host cell protein folding and transport machinery. In addition, it was investigated whether ELP fusion would increase the accumulation of the 2F5 antibody. A detailed structural and functional characterization was performed to elucidate the effect of the fusion on folding, assembly and post-translational modification. Transgenic plants expressing individual 2F5 light and heavy chains, with and without ELP fusion, were generated, together with four different full-size antibody derivatives obtained after crossing. The structural integrity and assembly were studied by reducing and non-reducing sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of antibody preparations purified by protein A. The absolute antigen binding activity and binding kinetics were determined quantitatively by surface plasmon resonance spectroscopy and compared with those of the CHO-produced 2F5 product. Post-translational modifications were studied using mass spectrometry to characterize the *N*-glycans attached to the heavy chain asparagine-297 (Asn297) residue. The first experiments for the enrichment of recombinant antibody–ELP fusions in tobacco leaf extracts by inverse transition cycling are presented. The implications of our findings on the therapeutic potential of ELP fusion proteins are discussed.

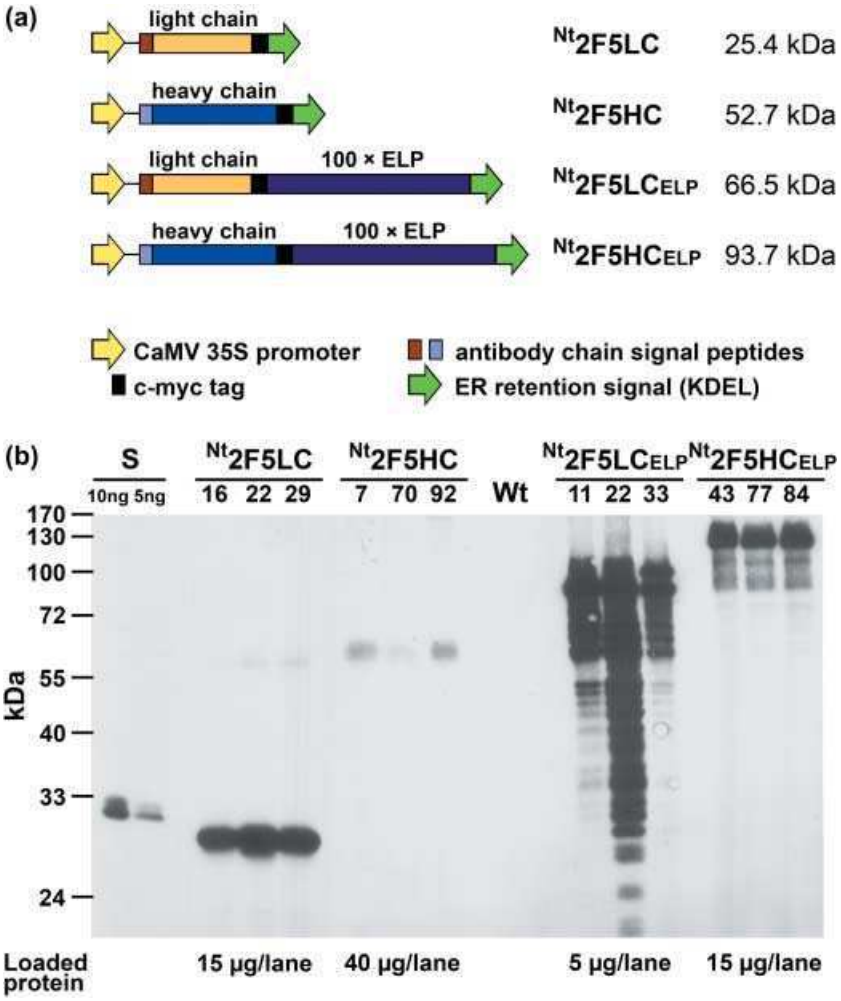

Figure 1 Accumulation of 2F5 light and heavy chain variants in transgenic tobacco plants. (a) Schematic presentation (not to scale) of the expression cassettes and designation of the individual antibody chain variants. The molecular masses of the mature chains are shown. Expression is driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter. Endoplasmic reticulum retrieval is mediated by a C-terminal KDEL tag. 100 × ELP, 100 repeats of the pentapeptide VPGXG; HC, antibody heavy chain; LC, antibody light chain. (b) Western blots of leaf extracts of individual T_0 plants separated by reducing 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted and immunodetected using an anti-c-myc antibody. S, c-myc standard; Wt, 20 µg protein per lane of wild-type *Nicotiana tabacum* cv. SNN.

Results

Generation of transgenic tobacco plants expressing 2F5 antibody derivatives

cDNAs encoding the 2F5 light and heavy chains (including their native signal sequences) and corresponding ELP fusions (100 × ELP; Scheller *et al.*, 2004) were introduced into plant expression vectors containing a constitutive promoter [cauliflower mosaic virus (CaMV) 35S], the c-myc tag and the KDEL signal for retention in the endoplasmic reticulum (ER) (Figure 1a). The functionality of all binary vectors was verified by transient agro-infiltration (Kapila *et al.*, 1997; Vaquero *et al.*, 1999) and detection of the recombinant proteins by Western blot (data not shown). For each construct (^{Nt}2F5LC, ^{Nt}2F5HC, ^{Nt}2F5LCELP, ^{Nt}2F5HCELP), about 100 kanamycin-resistant T_0 plants (Table 1) were generated by *Agrobacterium*-mediated leaf disc transformation and screened for high recombinant protein accumulation by Western blot with an antibody against c-myc (Figure 1b). As previously observed in other experiments (Shimazu *et al.*, 2003; Ge *et al.*, 2006), the ELP fusion proteins had a slightly lower electrophoretic mobility than expected. The presence of ELP substantially increased the accumulation of both the light and heavy chains in the ER of tobacco leaf cells, compared with the chains lacking ELP. In addition, the individual 2F5 light chains (with and without ELP) accumulated to higher levels than

Table 1 Designation of transgenic tobacco plants expressing individual antibody chains

Transgene	Generated plants	Positive plants*
^{Nt}2F5LC	95	74
^{Nt}2F5HC	100	55
^{Nt}2F5LCELP	100	65
^{Nt}2F5HCELP	93	44

*Plants with detectable transgene expression analysed by Western blot.

Table 2 Crossings yielding transgenics producing full-size antibody variants

Parental line	^{Nt}2F5LC	^{Nt}2F5LCELP
^{Nt}2F5HC	^{Nt}2F5	^{Nt}2F5LELP
^{Nt}2F5HCELP	^{Nt}2F5HELP	^{Nt}2F5ELP

their heavy chain counterparts, as often observed (Law *et al.*, 2006). The degradation products of the 2F5 light chain–ELP fusion (Figure 1b) appear more abundant than is actually the case, because the blot was overdeveloped in order to visualize the significantly weaker 2F5 heavy chain signals.

Seeds of T_0 plants with high levels of the recombinant antibody chains were germinated on kanamycin-containing medium, and transgenic lines showing 3 : 1 segregation, consistent with a single locus insertion, were used as parents for the crosses (Table 2). In the resulting progeny, T_2 lines accumulating fully assembled antibodies were identified for all possible combinations of the four antibody components (i.e. ^{Nt}2F5, ^{Nt}2F5LELP, ^{Nt}2F5HELP and ^{Nt}2F5ELP). This was achieved using a protein L/protein A sandwich enzyme-linked immunosorbent assay (ELISA) (Figure 2a) and Western blot with an anti-Fc antibody (Figure 2b). As anticipated from the T_1 generation, the antibodies with ELP fusion accumulated to the highest levels. Interestingly, the light chain–ELP fusion also increased the accumulation of the heavy chain (Figure 2b; ^{Nt}2F5LELP lines 23, 27, 25 and ^{Nt}2F5 lines 24, 15, 25). This co-stabilization was considered as a further indication that the antibody chains were assembled *in planta*. The different recombinant antibody–ELP variants (^{Nt}2F5LELP, ^{Nt}2F5HELP and ^{Nt}2F5ELP) accumulated to 0.3%, 0.2% and 0.6% of total soluble protein (TSP), respectively. ^{Nt}2F5 was accumulated to about 0.1% TSP. Relevant Western blots are presented in the 'Supplementary material' (Figure S4). The antibody–ELP fusion proteins were quantified relative to the CHO cell counterpart lacking ELP; the amount of the total fusion protein is underestimated.

Purification of plant-derived antibodies

All four 2F5 variants were purified from tobacco leaves by protein A affinity chromatography, followed by extensive dialysis and ultrafiltration. The purified antibody preparations were separated by non-reducing 6% SDS-PAGE and identified by Western blot using a human Fc-specific antibody for detection (Figure 2c). In all four cases, the electrophoretic

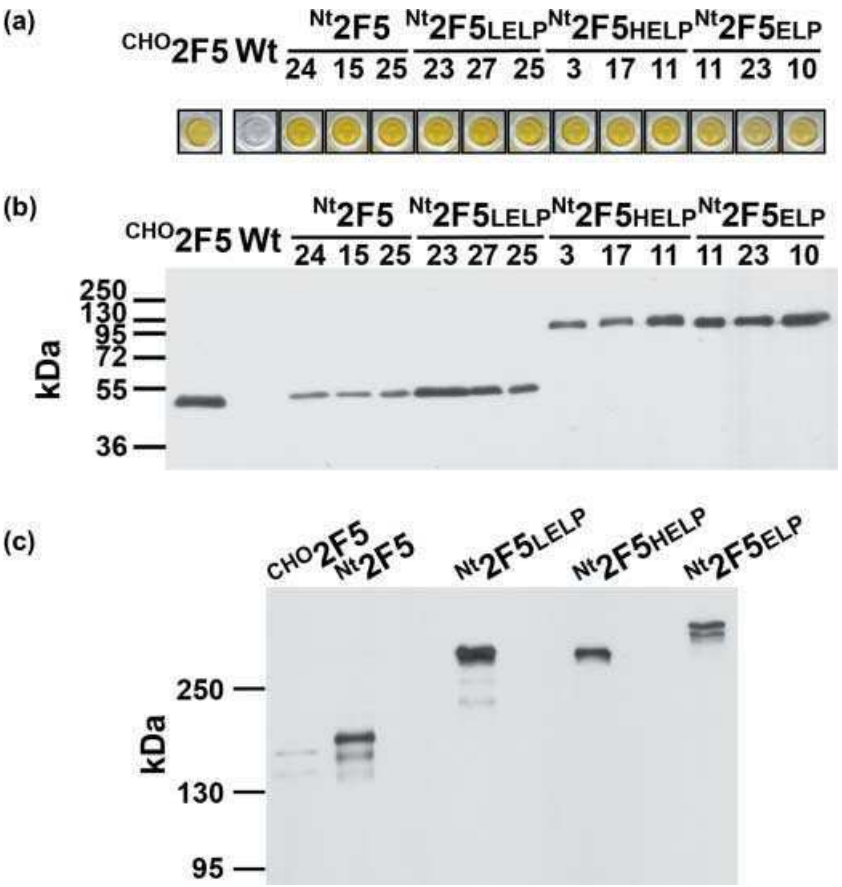

Figure 2 Expression and assembly of 2F5–elastin-like peptide (ELP) fusions produced in plants. (a) Assembly of light and heavy chain variants detected in leaf extracts of different T_2 tobacco plants of one cross by protein L/protein A sandwich enzyme-linked immunosorbent assay (ELISA). (b) Western blot analysis of T_2 plants of one cross: 10 μg protein per lane was separated by reducing 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted and analysed with anti-human Fc-specific immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP), followed by ECL detection. ^{CHO}2F5, 34 ng per lane. (c) Western blot analysis of affinity-purified 2F5 derivatives from plants. Proteins were separated by non-reducing 6% SDS-PAGE, and blotted and analysed with anti-human Fc-specific IgG conjugated to HRP, followed by ECL detection. ^{CHO}2F5, 2 ng per lane; Wt, *Nicotiana tabacum* cv. SNN.

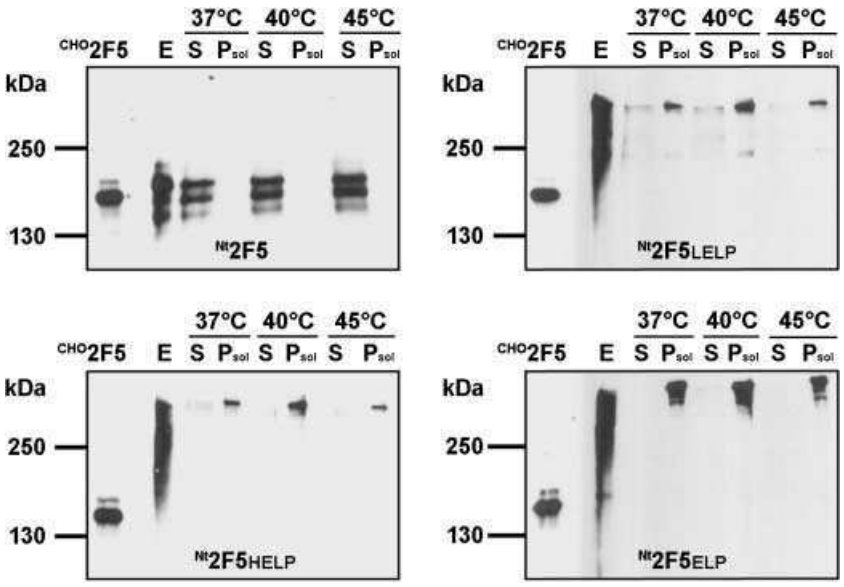

Figure 3 Western blot analysis of enrichment of antibody–elastin-like peptide (ELP) fusion proteins by inverse transition cycling (ITC). Proteins were separated by non-reducing 6% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted and analysed with anti-human Fc-specific immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP), followed by ECL detection. ^{CHO}2F5, 2 ng per lane; E, leaf extract containing 2 M NaCl; Psol, solubilized pellet; S, supernatant after centrifugation at a given temperature. Identical volumes of leaf extracts and supernatants were applied. The pellets were dissolved in 100 mM Tris-HCl. The amount applied to the gel reflects 50% of the amount applied from crude leaf extract and supernatant.

mobilities corresponded to the assembled antibody; no free heavy chains or heavy chain–ELP fusions were observed. These results clearly show that the antibody derivatives are efficiently assembled *in planta*. The fusion of either the light or heavy chain, or both, to 100 × ELP evidently does not interfere with assembly.

Furthermore, the antibody–ELP variants were enriched in tobacco leaf extracts by inverse transition cycling based on their temperature-dependent phase transition. The plant-expressed 2F5 antibody without ELP was included as a control. Different temperatures (37, 40 and 45 °C) were used for the precipitation of the ELP aggregates. The supernatant (S) and the solubilized pellet (Psol) were analysed by Western blot (Figure 3). As expected, ^{Nt}2F5 was detected in leaf extracts and in the supernatant. No protein was found in the solubilized precipitate after centrifugation. The corresponding bands of the antibody–ELP fusions were present in the leaf extracts and signals were obtained for the solubilized pellets. From 4 mL of each leaf extract, 200 μL of protein solution was obtained and only 1 μL was applied to the gel. The strongest signals were obtained for the three ELP fusions (^{Nt}2F5LELP, ^{Nt}2F5HELP and ^{Nt}2F5ELP) after precipitation at 40 °C. For ^{Nt}2F5LELP, weak signals were detected in the supernatant when the ELP aggregates were precipitated at 37 and 40 °C. The optimum temperature for the purification of the 2F5 antibody–ELP fusions seems to be around 40 °C.

N-Glycan analysis

The four affinity-purified and assembled 2F5 variants were separated by non-reducing SDS-PAGE, excised from the gel and their *N*-glycan profiles were analysed by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS), operated in positive ion mode. The *N*-glycan profiles of the four antibody derivatives were very similar with no qualitative and only minor quantitative differences. All preparations contained predominantly oligo-mannose-type (OMT) *N*-glycans with Man7 being the most abundant glycoform (Figure 4). Man8 and Man6 were present in smaller amounts and only traces of Man9 and Man5 were detected. Complex type (CT) *N*-glycans were found in all preparations, showing that KDEL-mediated ER retrieval was not entirely efficient. The relative abundance of all OMT *N*-glycans (Man9–Man5) was 67% for ^{Nt}2F5HELP, 71% for ^{Nt}2F5, 88% for ^{Nt}2F5ELP and 90% for ^{Nt}2F5LELP. The relative abundance of the CT *N*-glycans was 33% for ^{Nt}2F5HELP, 29% for ^{Nt}2F5, 12% for ^{Nt}2F5ELP and 10% for ^{Nt}2F5LELP. The most prominent CT *N*-glycan was GnGnX, and small amounts of AGnX carrying terminal β(1,3)-galactose were also detected. The other CT *N*-glycans (GnM, GnGn, GnMX and, in particular, GnGnXF) were only found in trace amounts. Antibody variants containing the light chain–ELP fusion (^{Nt}2F5LELP and ^{Nt}2F5ELP) contained more Man7 glycans than did the ^{Nt}2F5 and ^{Nt}2F5HELP derivatives. The higher relative abundance of Man7 corresponded with a lower relative abundance of total CT *N*-glycans, predominantly through the loss of GnGnX.

These results clearly demonstrate that the fusion of the antibody light chain, heavy chain or both chains to 100 × ELP has no impact on the interaction of the oligosaccharides attached to Asn297 with the *N*-glycan processing machinery of the plant host cell.

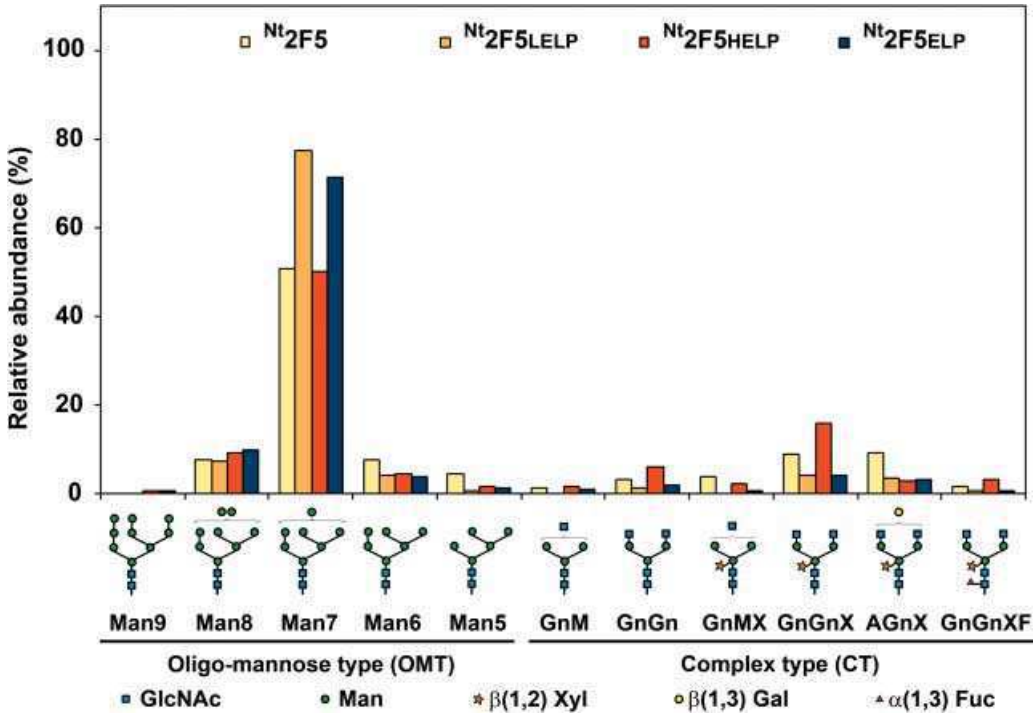

Figure 4 Relative abundance of *N*-glycoforms detected on 2F5 heavy chains by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). The relative abundances (percentages) were calculated from the summed and deconvoluted mass spectra of the *N*-glycosylated Fc peptides EEQYN297STYR and TKPR-EEQYN297STYR derived from the corresponding 2F5 variants. The *N*-glycan names and structures are based on the nomenclature proposed by Proglycan (http://www.proglycan.com).

Functional characterization of plant-produced 2F5 antibody and 2F5–ELP fusion proteins

Surface plasmon resonance spectroscopy was used to quantify the antigen binding properties of the antibody preparations by measuring the binding kinetics to Fuzeon®, a trimeric synthetic peptide containing the 2F5 epitope. The CHO-derived 2F5 (^{CHO}2F5) was used as standard. Antibodies were captured to protein A to avoid loss of activity after coupling and regeneration, thereby allowing the determination of unaltered binding kinetics and absolute single-antigen binding site activities. Individual paratopes can only be studied independently when the antibody is used as the ligand, i.e. immobilized or captured on to the surface. Although Fuzeon® is a trimer, only monovalent binding was observed because the surface densities of the captured antibodies were sufficiently low. The double-referenced binding curves were well fitted by the 1 : 1 model with mass transport limitation (Figure 5a) and with residuals scattering uniformly (Figure 5b). Comparison of the binding parameters (k_{on}, k_{off}, K_D; Table 3) showed that all antibody preparations had very similar, if not identical, binding kinetics. This is also visible from the perfect overlay (superimposition) of the ^{CHO}2F5 and ^{Nt}2F5 binding curves (Figure 5a).

Table 3 Kinetic parameters of the interaction between 2F5 antibody derivatives and Fuzeon®

Antibody	R_{mAb} (RU)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (pM)	R_{max} (RU)
^{CHO}2F5	331.5	5.4×10^6	1.9×10^{-3}	366	54.1
^{Nt}2F5	326.5	6.3×10^6	1.8×10^{-3}	294	52.8
^{Nt}2F5LELP	390.5	8.2×10^6	2.5×10^{-3}	305	56.7
^{Nt}2F5HELP	325.5	7.6×10^6	2.1×10^{-3}	279	45.1
^{Nt}2F5ELP	315.0	7.0×10^6	2.7×10^{-3}	384	38.1

RU, resonance unit.

Paratopes were saturated using a high concentration (66.7 nM) of Fuzeon® to calculate the absolute activities of the antibody preparations (Equation 1). In contrast with the binding kinetics, the Fuzeon® saturation signal revealed differences between the antibody variants (Table 4). ^{CHO}2F5 and ^{Nt}2F5 exhibited only minor differences in absolute activities. As expected, ^{Nt}2F5 displayed a high absolute binding activity of 97.8%. The ^{CHO}2F5 activity was reduced slightly to 94.1% compared with a previous study (Sack *et al.*, 2007). Surprisingly, the ELP fusions generated saturation signals corresponding to absolute activities higher than 100% (Table 4). Because the absolute activity cannot exceed 100%, other reasons must be considered. One possible explanation could be a lower than expected molecular mass. The calculated (Equation 2)

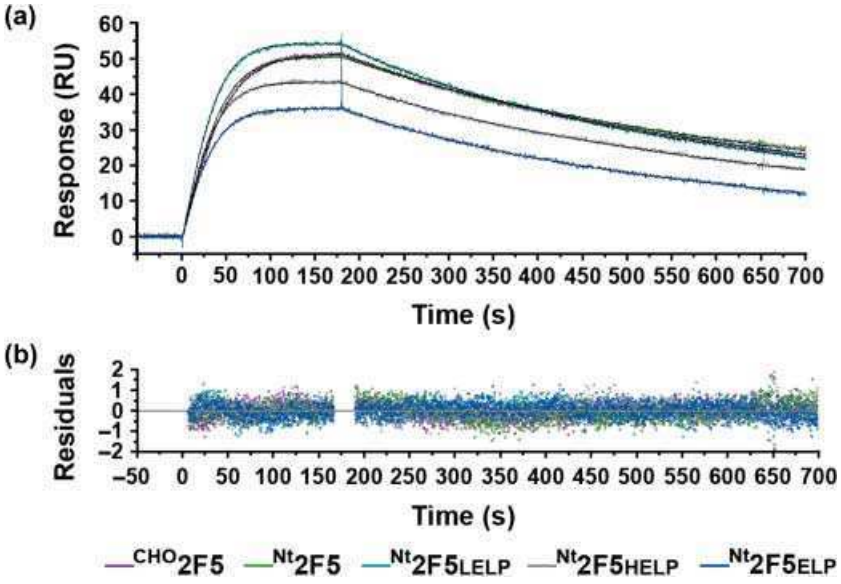

Figure 5 Biacore analysis of Fuzeon® binding to 2F5 variants. 2F5 variants were captured on a protein A surface and the binding of 6.67 nM Fuzeon® trimer was recorded. ^{CHO}2F5 was included as a reference. The blank-subtracted binding curves were well fitted by a 1 : 1 model with mass transfer; the residuals showed a uniform non-systematic distribution. The results of the fits are given in Table 3.

Table 4 Calculation of absolute antigen binding activity and putative mass loss

Antibody	R_{mAb} (RU)	R_{Sat} (RU)	Mr_{mAb} (Da)	Absolute activity (%)	ΔMr_{mAb} (Da)
^{CHO}2F5	331.5	55.75	150 814	94.1	–9448
^{Nt}2F5	326.5	54.00	159 383	97.8	–3576
^{Nt}2F5LELP	390.5	58.95	241 492	135.3	62 955
^{Nt}2F5HELP	325.5	48.45	241 492	133.4	60 421
^{Nt}2F5ELP	315.0	39.95	323 601	152.3	111 088

RU, resonance unit.

putative mass differences were 62.9 kDa for ^{Nt}2F5LELP, 60.4 kDa for ^{Nt}2F5HELP and 111.1 kDa for ^{Nt}2F5ELP. Mass differences of this magnitude can only be explained by the absence of entire antibody chains, i.e. the presence of partially assembled molecules and/or the presence of large amounts of degradation products where, for example, the entire ELP is lacking. However, all partially assembled antibody molecules would yield lower absolute antigen binding activities, which could not explain the observed results. Moreover, results from the non-reducing SDS-PAGE experiments (Figure 2c) do not support the presence of significant amounts of partially assembled or excessively degraded molecules. However, it is possible that very small amounts of degradation products present in the antibody preparations may preferentially bind to the protein A surface. To investigate this, antibodies bound to the protein A surface were eluted from the chip with 30 mM HCl, immediately neutralized, concentrated and analysed by reducing SDS-PAGE and Western blot. The comparison of the recovered samples (R) with the antibody preparations (P) showed no difference at all, and clearly confirms unbiased capture to the protein A surface (Figure S1, see 'Supplementary material'). Thus, the observed result must reflect an alternative phenomenon, two of which (the orientation of captured ELP fusions and the optical properties of the ELPs) are discussed in the 'Supplementary material'.

Discussion

ELP fusion technology provides many advantages in the large-scale production of biologicals, including increased yields (Scheller *et al*., 2004, 2006; Patel *et al*., 2007), simple downstream processing by inverse transition cycling (Meyer and Chilkoti, 1999; Scheller *et al*., 2004; Lin *et al*., 2006) and biocompatibility (Urry *et al*., 1991). However, these benefits will only fulfil their promise if ELP fusions do not interfere with the normal functions of the host cell. The aim of the present work was therefore to investigate whether or not ELP fusion technology is compatible with the production of a complex heteromultimeric pharmaceutical protein – the broadly neutralizing HIV-1-specific antibody 2F5 – which places high demands on the host cell in terms of folding, disulphide bridge formation and assembly. Antibody production in plants has been well characterized (reviewed in Ko and Koprowski, 2005; Ma *et al*., 2005; Stoger *et al*., 2005b), and our goal was to study the effects of ELP fusion on quality control and post-translational modification, particularly the *N*-glycans attached to the heavy chain Asn297 residue. The rationale was that ELP may interfere with cellular processes, such as sorting and post-translational modifications, either through passive shielding or actively through direct interaction with host cell factors.

To ensure that our observations were unbiased by position effects, approximately 100 independent primary integration events were analysed for each antibody chain variant (Table 1). Consistent with previous reports (Scheller *et al*., 2004, 2006; Patel *et al*., 2007), the accumulation of individually expressed antibody light and heavy chains was strongly enhanced by fusion to ELP (Figure 1b), and the light chain variants accumulated to higher levels than the heavy chain variants (Law *et al*., 2006). After crossing selected lines, the enhancement brought about by ELP fusion was maintained in tobacco plants co-expressing antibody heavy and light chains (Figure 2b). Three methods – sandwich ELISA of tobacco extracts (Figure 2a), non-reducing SDS-PAGE and Western blotting of antibodies purified by protein A affinity chromatography (Figure 2c) – confirmed antibody assembly *in planta*. As the light chain variants accumulated in excess, inefficient antibody assembly would result in the presence of free heavy chains. These were not detected in any of the four combinations, i.e. ^{Nt}2F5, ^{Nt}2F5LELP, ^{Nt}2F5HELP and ^{Nt}2F5ELP (Figure 2c), clearly demonstrating that *in planta* antibody assembly was efficient and unaffected by ELP fusion to the light chain, heavy chain or even to both chains. Interestingly, fusion of ELP to the light chain resulted in increased accumulation of the heavy chain, i.e. the assembled antibody is stabilized in the same manner as the individual chains. Moreover, this clearly demonstrates that the main effect of ELP fusion is to reduce protein degradation, because the transcript stability, translational and folding efficiency of the heavy chain cannot be affected by the light chain–ELP fusion. Consequently, it is concluded that the ELP fusion stabilizes the antibody heavy chain *in trans* post-translationally and after assembly, and hence confers enhanced stability on the entire assembled heteromultimeric antibody.

The purified antibodies were subjected to a detailed binding analysis using surface plasmon resonance spectroscopy. Fuzeon®, a synthetic trimeric 36-amino-acid HIV-1 fusion inhibitor containing the 2F5 core epitope ELDKWA, was used as the analyte. The binding kinetics of all 2F5 derivatives were indistinguishable from those of the CHO-derived counterpart (Figure 5 and Table 3), demonstrating that the paratope was not directly affected by ELP fusion and confirming the fidelity of protein synthesis, folding and assembly *in planta*. Quantitative determination of the absolute binding activity has been described previously for 2F5 produced in tobacco BY2 suspension cells (Sack *et al*., 2007). This method was also applied here, but absolute activities higher than 100% were recorded (Table 4). As this is not possible by definition, it was investigated whether the results could be explained by a loss of mass, i.e. overestimating the size of the captured antibody derivative. However, this was ruled out by the demonstration that the antibody eluted from the protein A surface was identical to the purified antibody (Figure S1, see 'Supplementary material'). The orientation of the captured 2F5–ELP fusions, or different optical properties of the ELP moiety, may explain these results (see 'Supplementary material'). As it is not possible to quantify these effects precisely at the present time, the absolute single-site antigen binding activities can only be determined for ^{Nt}2F5, but not for any of the ELP fusion derivatives.

The effect of ELP fusion on post-translational modification was investigated by analysing the profile of *N*-glycans attached to the heavy chain Asn297 residue of all four antibody variants. *N*-Glycosylation plays a crucial role during folding, and is also a reporter for tracking the antibody molecule along the secretory pathway (Walsh and Jefferis, 2006). Only minor differences were detected in the relative amounts of OMT and CT *N*-glycans, mainly reflecting the prominence of Man7 and GnGnX, respectively. It is believed that these slight differences are caused by the more efficient ER retrieval of ^{Nt}2F5LELP and ^{Nt}2F5ELP, relative to ^{Nt}2F5 and ^{Nt}2F5HELP, as a result of the improved accessibility of the KDEL tags attached to 2F5LCELP. The KDEL tag fused to the heavy chain is readily accessible and causes the majority of the antibody to be retrieved from the *cis*-Golgi to the ER. Although the ELP fusion to the heavy chain does not improve accessibility further, ELP fusion to the light chain may facilitate recognition by the KDEL receptor, resulting in a greater proportion of OMT glycans. The efficacy of KDEL-mediated ER retrieval is still controversial (reviewed in Stoger *et al*., 2005b). Some KDEL-tagged antibodies expressed in plants carry predominantly OMT glycans, but a significant proportion of CT glycans, indicating some leakage in the retrieval system (Ko *et al*., 2003; Tekoah *et al*., 2004; Triguero *et al*., 2005). Others have been shown to carry 100% OMT glycans, suggesting that ER retrieval could be used as a strategy to prevent the synthesis of antibodies containing core α(1,3)-fucose and core xylose (Sriraman *et al*., 2004; Petruccelli *et al*., 2006). *N*-Glycan analysis clearly showed that ELP fusion had no effect on intracellular trafficking and did not inhibit access to glycan-processing enzymes, many of which are integral membrane proteins.

In conclusion, our results demonstrate clearly that the fusion of ELP to the light chain, heavy chain or both chains of a complex, multimeric antibody produced in plants has no adverse affect on the quality of the protein, but has a positive impact on the yield. Careful comparison of antigen binding kinetics and *N*-glycan profiles showed no significant structural or functional difference between the four antibody variants. This shows that the ELP fusion does not interfere with folding,

assembly, trafficking in the secretory pathway or post-translational modification, but does enhance the stability whilst at the same time simplifying recovery. There appears to be no shielding or direct interaction between ELP and critical host factors, but the fusion appears to enhance the presentation of the light chain's KDEL tag to its cellular receptor.

ELP fusion technology offers many benefits for the development of plant-derived antibodies as microbicide components, and our data show that the addition of ELP has no adverse effects in the plant production host or on the structural and functional properties of the active pharmaceutical ingredient. The next step will be to investigate the behaviour of the 2F5–ELP fusions on mucosal surfaces, first to ensure safety, but also with particular emphasis on the possibility of mucosal surface coating by temperature-induced phase transition. Generic biological tests for materials in contact with tissues, tissue fluids and blood have shown that VPGVG polymers are extraordinarily biocompatible (Urry *et al*., 1991) and suitable for biomedical applications (reviewed in Chilkoti *et al*., 2006; Simnick *et al*., 2007).

Plant-based systems are ideal for the large-scale, economic production of pharmaceutical proteins in low-technology settings, although the costs of extraction and downstream processing obviate many of the economic benefits upstream. By combining plant-based production and ELP technology, the downstream steps of production will be simplified and the costs reduced. This may be the best way forward for the efficient, inexpensive and safe production of pharmaceutical proteins in developing countries, where the demand for microbicides is greatest, but where the financial resources to pay for them are the most limited.

Experimental procedures

Plant transformation vectors

The genes encoding the light and heavy chains of the 2F5 antibody were amplified by polymerase chain reaction (PCR) using pTRAk-2F5L and pTRAk-2F5H (T. Rademacher, unpubl. data) as templates. The light chain product was cloned as an *Nco*I-*Not*I fragment in plasmid pRTRA15 (Artsaenko *et al*., 1995) and as an *Nco*I-*Bam*HI fragment in plasmid pRTRA-100 × ELP (J. Scheller, unpubl. data) to produce the constructs pRTRA-2F5LC and pRTRA-2F5LC-100 × ELP, respectively. The 100 × ELP fusion protein has been described previously by Scheller *et al*. (2004). The heavy chain PCR product was inserted as an *Nco*I-*Not*I fragment into the pRTRA15 plasmid to produce pRTRA-2F5HC. A two-step cloning strategy was used to create pRTRA-2F5HC-100 × ELP. First, a 170-bp *Nco*I-*Bam*HI fragment was inserted into the pRTRA-100 × ELP vector, and then the missing 1273-bp fragment was introduced into the *Bam*HI site of the above-mentioned plasmid to produce pRTRA-2F5HC-100 × ELP. All expression cassettes (Figure 1a) were excised by *Hin*dIII and individually inserted into the binary vector pCB301-Kan (J. Scheller, unpubl. data), which is based on the binary vector pCB301 (Xiang *et al*., 1999).

Transgenic plants

The binary vectors described above were introduced into *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere *et al*., 1985), and used to produce transgenic tobacco (*Nicotiana tabacum* cv. Samsun NN) plants using the leaf disc transformation method (Zambrinski *et al*., 1983). T_0 plants were grown on Murashige–Skoog medium containing 50 mg/L kanamycin prior to transfer to soil in the glasshouse, and then selfed to produce the T_1 generation. T_1 lines showing Mendelian segregation consistent with a single locus insertion were used for crosses to obtain the full-length 2F5 antibody and the corresponding antibody–ELP fusions in the T_2 generation.

SDS-PAGE and Western blot

Leaf discs from transgenic plants were homogenized in SDS sample buffer (72 mM Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.25 μM bromophenol blue, pH 6.8), incubated for 10 min at 95 °C and centrifuged. The concentration of TSP was determined by the Bradford assay (Bio-Rad, Munich, Germany). Plant extracts were separated by reducing SDS-PAGE on 10% or 12% polyacrylamide gels. The assembly of affinity-purified antibodies was analysed by non-reducing SDS-PAGE on 6% polyacrylamide gels. For immunoblotting, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using 25 mM Tris, 0.1% SDS, 192 mM glycine and 20% methanol. Nitrocellulose membranes were blocked for 3 h at room temperature in 5% fat-free dried skimmed milk dissolved in 180 mM NaCl, 20 mM Tris (pH 7.8). The immunological detection of antibody chains was performed with anti-c-myc (9E10) supernatant, followed by horseradish peroxidase (HRP)-conjugated sheep anti-mouse immunoglobulin G (IgG) (Amersham Biosciences, Piscataway, NJ, USA), or with anti-human Fc-specific IgG conjugated to HRP (Sigma-Aldrich, St. Louis, MO, USA). The antibody chains were visualized using the ECL Western blotting analysis system (Amersham Biosciences).

Sandwich ELISA

Ninety-six-well plates (MaxiSorp™ Surface, Nunc A/S, Roskilde, Denmark) were coated overnight with 1 μg/mL protein L (Actigen, Oslo, Norway) in phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl, pH 7.6), and blocked with 3% bovine serum albumin (BSA) in PBS containing 0.1% Tween-20 for at least 1 h. Leaf extracts (in PBS containing 0.1% Triton X-100 or 100 mM Tris-HCl, pH 8.0) were diluted in 1% BSA in PBS and applied to the protein L-coated plates. ^{CHO}2F5 (Polymun Scientific, Vienna, Austria) was used as standard, and extract from wild-type *N. tabacum* cv. SNN as negative control. Bound antibodies were detected with HRP-conjugated protein A (Amersham Biosciences) using tetramethylbenzidine as the substrate for HRP. The 650 nm/450 nm extinction ratio was used for qualitative evaluation.

Affinity purification of antibodies

Tobacco leaf material (500 g) was homogenized with a Waring blender (Schütt Labortechnik, Göttingen, Germany) in 100 mM Tris-HCl (pH 8.0) containing 0.1% Triton X-100 to extract soluble proteins. After centrifugation (3500 *g*, 30 min, 4 °C), protein A agarose (Roche Diagnostics GmbH, Mannheim, Germany) was added to the supernatant and incubated overnight at 4 °C with gentle continuous stirring. Protein A agarose was collected in a disposable chromatography column (5 mL; Qiagen GmbH, Hilden, Germany) and extensively rinsed with 100 mM Tris-HCl and 10 mM Tris-HCl (pH 8.0), consecutively. Recombinant antibody derivatives were eluted with 100 mM glycine (pH 2.8) and immediately neutralized with 1 M Tris-HCl (pH 8.0). Fractions containing the antibody were pooled, extensively dialysed against 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 150 mM NaCl (pH 7.6) and concentrated by ultrafiltration [iCON™ Concentrator, molecular weight cut-off (MWCO) = 20 kDa; Pierce, Rockford, IL, USA]. The final retentate was centrifuged at 15 000 *g* (4 °C, 30 min) to remove any residual insoluble material.

Inverse transition cycling

Tobacco leaf material was ground in a mortar under liquid nitrogen and 100 mM Tris-HCl (pH 8.0) was added. The extract was cleared by centrifugation (15 000 *g*, 30 min, 4 °C) and NaCl was added to a final concentration of 2 M; 4 mL of the solution was incubated in a water bath for 30 min at 37, 40 and 45 °C to allow for the aggregation of the antibody–ELP fusions. The antibody–ELP variants were precipitated by centrifugation at 4500 *g* for 30 min at 37, 40 or 45 °C. The precipitate was solubilized on a shaker overnight in 100 mM Tris-HCl (pH 8.0). Insoluble material was removed by centrifugation (15 000 *g*, 30 min, 4 °C). The solubilized precipitate and the supernatant were analysed by Western blot.

Surface plasmon resonance spectroscopy

Binding assays were performed using a Biacore 2000 instrument (Biacore, GE Healthcare, Uppsala, Sweden). About 4 kilo resonance unit (kRU) of recombinant protein A (200 μg/mL in 10 mM sodium acetate, pH 4.5) was coupled to a CM5-rg sensorchip following the standard 1-ethyl-3-oliaminopropyl-carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) protocol, resulting in a surface with high binding capacity and negligible dissociation for human IgG_1. An activated/deactivated surface was used as reference for blank subtraction. Regeneration was achieved with a 30-s pulse of 30 mM HCl. Comparative binding experiments were performed using Fuzeon® (http://www.fuzeon.com), a trimeric, 36-amino-acid synthetic peptide containing the 2F5 epitope. Data evaluation was carried out using BIAevaluation software version 4.0 and Microcal Origin version 5.0 (OriginLab Corporation, Northampton, MA, USA). Absolute activities were calculated as described in Sack *et al*. (2007) using Equation (1). To obtain putative mass differences, Equation (1) was rearranged and the absolute activity was set to 100% to yield Equation (2).

$$\text{Absolute activity} = \frac{R_{Sat}}{R_{mAb}} \cdot \frac{Mr_{mAb}}{2 \cdot Mr_{Fuz}} \quad (1)$$

$$\Delta Mr_{mAb} = Mr_{mAb} - 2 \cdot Mr_{Fuz} \cdot \frac{R_{mAb}}{R_{Sat}} \cdot 100\% \quad (2)$$

R_{mAb} is the amount of antibody captured to the protein A surface, R_{Sat} is the saturation signal obtained at high Fuzeon® concentrations, Mr_{Fuz} is the molar mass of the trimer (13 476 Da) and Mr_{mAb} is the molecular mass of the various 2F5 antibody derivatives (Table 4).

N-Glycan analysis

Coomassie-stained bands were excised from polyacrylamide gels, destained, carbamidomethylated and digested with trypsin. Peptides were extracted and analysed as described previously (Kolarich and Altmann, 2000; Kolarich *et al.*, 2006) using a capillary LC-ESI-MS system consisting of an Aquasil C-18 precolumn (30 × 0.32 mm, 5 μm; Thermo Fisher Scientific, Waltham, MA, USA), a BioBasic C-18 analytical column (150 × 0.18 mm, 5 μm; Thermo Fisher Scientific), a Waters CapLC, a Rheodyne 10-port valve and a Waters Q-TOF Ultima fitted with a standard ESI source. Data analysis was carried out using MassLynx 4.0 SP4 (Waters Micromass, Milford, MA, USA).

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Supplementary material

The following supplementary material is available for this article:

Figure S1 Two hypotheses are offered to explain the results of the absolute activity determination assays. The first assumes that ELP dictates the orientation of the captured antibodies and that the antibody enters the dextran matrix head-on (Figure S2). The approximately 80 nm ELP chains probably do not diffuse entirely into the dextran matrix. As a result, the centres of mass for the antibody and the bound antigen are not the same as would be observed in the case of random orientation (Figure S2). The response signal change detected by the evanescent wave depends on the distance from the surface; molecules closer to the surface generate a stronger signal than molecules that are more distant (Jung *et al*., 1998). If the antibody is randomly captured, the average centres of mass of the antibody and the subsequently bound antigen are both located at the attachment site as a result of rotational symmetry, i.e. the centre of the two protein A binding sites. For the antibody–ELP fusions, it is assumed that the ELP portion is further away from the surface (Figure S2). As a result, the signal change is lower than expected and the amount of captured antibody–ELP is underestimated. A consideration of the dimensions of the molecules and a crude estimation of the centre of mass for a uniform orientation yield a displacement of up to 30 nm (Figure S3). This is sufficiently high to at least partially explain the observed effect.

The second hypothesis is based on the fact that the response signal depends on the index of refraction (RI) of the molecules deposited on the sensor surface. Most proteins have an RI of 1.6, but it has been noted that glycoproteins and lipoproteins have somewhat lower RIs (Stenberg *et al*., 1991; Jung *et al*., 1998). When analysing Biacore data, a general conversion factor (G = 1000 RU mm^2/ng) is used (Sjolander and Urbaniczky, 1991; Liedberg *et al*., 1993) and small differences in the RI of different proteins are usually ignored. The 100 × ELP fusion partner used in this study is a random coiled, non-structured polypeptide chain, 500 amino acids in length, and with a highly biased and non-representative amino acid composition. These characteristics can produce optical properties that are distinct from those of 'normal' proteins, which therefore should not be ignored. The dependence of the Biacore response signal on the protein conformation has also been noted in previous studies, and signal changes caused by on-chip denaturation and conformational change on analyte binding have been

described (Sota *et al*., 1998; Mannen *et al*., 2001; Zako *et al*., 2001; May and Russell, 2002; Paynter and Russell, 2002; Hsieh *et al*., 2004). In addition, for small molecules, this issue has recently been addressed and investigated in more detail (Davis and Wilson, 2000). Therefore, it seems that, as a result of the special nature of the 100 × ELP fusion partner, the absolute binding activities of the antibody–ELP fusion cannot be determined easily and reliably by Biacore.

Integrity of 2F5–elastin-like peptide (ELP) variants eluted from the protein A surface, demonstrated by Western blot. (a) Separation under reducing conditions by 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Detection with anti-human kappa-ALP and B-ZIP staining after electroblotting. (b) Separation under reducing conditions by 12% SDS-PAGE. Detection with antihuman Fc-specific immunoglobulin G-horseradish peroxidase (IgG-HRP) and ECL after electroblotting. ^{CHO}2F5, 2 ng per lane; P, protein A purified antibody used for capture to the protein A surface; R, antibody recovered from the protein A surface.

Figure S2 Schematic representation of the dimensions and orientation of different 2F5 variants within the dextran matrix. The antibody size (~14 nm), length of 100 × ELP (~80 nm) and thickness of the dextran matrix (~100 nm) are approximately to scale.

Figure S3 Schematic drawing of an ^{Nt}2F5HELP model and estimation of the different centre-of-mass positions. The dimensions of the antibody and antigen were determined using SWISS-PDBviewer 3.7.

Figure S4 Comparison of antibody expression in tobacco leaf material by Western blot. The proteins were separated under reducing conditions by 10% SDS-PAGE. The detection of the antibody heavy chains was performed by anti-human Fc-specific IgG conjugated with HRP. Different amounts of total soluble protein (TSP) of leaf extracts (2.5, 5 and 10 μg) were applied per lane and compared with the ^{CHO}2F5 heavy chains. (a) Analysis of ^{Nt}2F5 and ^{Nt}2F5LELP. (b) Analysis of ^{Nt}2F5HELP and ^{Nt}2F5ELP. ^{CHO}2F5, 2F5 standard antibody; Wt, wild-type *Nicotiana tabacum* cv. SNN.

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