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Plant Biotechnology Journal

Plant Biotechnology Journal (2009) **7**, pp. 442–455 doi: 10.1111/j.1467-7652.2009.00414.x

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Received 11 September 2008; revised 15 January 2009/30 January 2009; accepted 31 January 2009.
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Keywords: antibody, glycoengineering, *N*-glycosylation, plant, transient expression, yield.

Summary

Plant-based transient expression is potentially the most rapid and cost-efficient system for the production of recombinant pharmaceutical proteins, but safety concerns associated with plant-specific *N*-glycosylation have hampered its adoption as a commercial production system. In this article, we describe an approach based on the simultaneous transient co-expression of an antibody, a suppressor of silencing and a chimaeric human β1,4-galactosyltransferase targeted for optimal activity to the early secretory pathway in agroinfiltrated *Nicotiana benthamiana* leaves. This strategy allows fast and high-yield production of antibodies with human-like *N*-glycans and, more generally, provides solutions to many critical problems posed by the large-scale production of therapeutic and vaccinal proteins, specifically yield, volume and quality.

Introduction

Therapeutic monoclonal immunoglobulins (Igs) (monoclonal antibodies, mAbs) have an increasingly important position on the market of anti-inflammatory and anti-cancer drugs, and hundreds of new candidates are currently under research and clinical development for improved or novel applications. The annual market demand for mAbs ranges from a few grams (diagnostics) through a few kilograms (anti-toxin) to up to one or several hundreds of kilograms (bio-defence, anti-cancer, anti-infectious, anti-inflammatory).

Although Chinese hamster ovary (CHO) cell culture is still the preferred production host for IgGs at the commercial scale, it is generally accepted that alternative production systems must be developed, as the facilities required for these cultures are neither rapidly nor easily modulated in scale, their building and maintenance costs are extremely high and steadily increasing, and their validation under Good Manufacturing Practice still requires an average of 3 years following construction.

A steady stream of data has demonstrated that plants are suitable hosts for the production of mAbs (Ko and Koprowski, 2005; Ma *et al*., 2005; Yusibov *et al*., 2006; for recent reviews). mAbs have been produced in stable transgenic plant lines at relatively low yields, and through transient expression at yields of up to 20 mg/kg fresh weight (FW) (Vaquero *et al*., 1999; Kathuria *et al*., 2002; Hull *et al*., 2005; Negrouk *et al*., 2005; Orzaez *et al*., 2006). Recently, Giritch *et al*. (2006) reported expression levels of 200–300 mg/kg of leaf weight for an IgG, with one cited maximum of 500 mg/kg, through the use of a multi-virus-based transient expression system. Although these yields were extrapolated from samples of syringe-infiltrated leaf material, this technical accomplishment, combined with the fact that transient expression has a seemingly unlimited surge response capacity in time and volume, suggests that transient plant-based expression can become a viable alternative to CHO cell culture for the production of mAbs.

However, most pharmaceutical proteins, including mAbs, are complex proteins which require many co- and post-translational modifications for their biological activity. Although plants are able to perform most of these modifications, including *N*-glycosylation (Gomord and Faye, 2004), structural differences between plant and mammalian complex *N*-glycans currently limit the commercial production of glycosylated plant-made pharmaceuticals, especially when

destined for parenteral administration (Gomord *et al*., 2005; Walsh and Jefferis, 2006). Indeed, plant complex *N*-glycans contain α1,3-fucose and β1,2-xylose residues constitutive of glycoepitopes known for their immunogenicity in humans (Aalberse *et al*., 1981; Faye and Chrispeels, 1988; van Ree and Aalberse, 1995; Garcia-Casado *et al*., 1996; van Ree *et al*., 2000; Bardor *et al*., 2003a). The presence of these glycan-specific antibodies in human serum may, at the very least, induce a rapid immune clearance of glycosylated plant-made pharmaceutical proteins (PMPs) from the circulation, which may greatly compromise their effectiveness as *in vivo* therapeutic agents. In addition to this accelerated clearance, the presence of IgE antibodies against carbohydrate epitopes of plant glycoproteins suggests that a percentage of allergic patients could experience hypersensitivity reactions after intravenous injection. Such adverse hypersensitivity reactions were observed recently when a glycosylated variant of cetuximab (a chimaeric mouse–human IgG1 mAb produced in SP2/0 mice) was injected into patients, producing IgE antibodies against an α1,3-galactose glycoepitope not present in human *N*-glycans (Chung *et al*., 2008). Although topical application of PMPs with complex *N*-glycans proved not to cause adverse effects in patients (Ma *et al*., 1998; Zeitlin *et al*., 1998), modulating plant-specific maturation of complex *N*-glycans remains a key objective in the development of plant-based protein manufacturing systems.

Different strategies have been developed to reduce the immunogenicity of plant *N*-glycans (for a recent review, see Saint-Jore-Dupas *et al*., 2007). For instance, the addition of an H/KDEL endoplasmic reticulum (ER) retention signal at the C terminus of the light and/or heavy chains of mAbs has been used by different groups (Ko *et al*., 2003; Sriraman *et al*., 2004; Triguero *et al*., 2005; Petruccelli *et al*., 2006; Floss *et al*., 2008) to produce antibodies with low relative abundance of fucosylated and xylosylated *N*-glycans. Control of α1,3-fucose and β1,2-xylose addition has also been attained by knocking out the α1,3-fucosyltransferase and β1,2-xylosyltransferase genes in different plant expression systems (Koprivova *et al*., 2004; Strasser *et al*., 2004; Cox *et al*., 2006; Kang *et al*., 2008; Sourrouille *et al*., 2008; Strasser *et al*., 2008). Knock-in strategies have also been used to humanize plant *N*-glycan structures, the main example of this approach being the stable expression of human β1,4-galactosyltransferase (GalT) in transgenic plants used as production platforms (for a review, see Saint-Jore-Dupas *et al*., 2007). Recently, targeting of GalT expression to the medial Golgi through the use of a fusion with the transmembrane domain of a xylosyltransferase from *Arabidopsis thaliana* has resulted in an increased efficiency of this knock-in strategy for both humanization and competition with specific maturations of plant *N*-glycans (Bakker *et al*., 2006).

The objective of this study was to develop an IgG transient expression system that would provide yields similar to or even higher than those obtained with the recently described multi-virus systems (Giritch *et al*., 2006), but that would lend itself easily to the use of knock-in strategies; that is, a system that would use expression vectors based on the simple transcription kinetics of a non-viral promoter.

Using this strategy, together with co-transient expression of a chimaeric form of GalT, we obtained a high-yield production of antibodies with human-like *N*-glycans only 6 days after agroinfiltration of wild-type *Nicotiana benthamiana* leaves.

Results

High-yield production of IgG using co-transient expression in agroinfiltrated *N. benthamiana* leaves

As transient expression in leaves is initiated by the agroinfection of palisade and spongy parenchyma cells, which are the most active in photosynthesis, it was postulated that some of the promoters driving the expression of photosynthesis-related genes would provide the high transcription rates required for expression. Two such promoters were tested and compared with the double 35S cauliflower mosaic virus (CaMV) promoter: a ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) promoter (Khoudi *et al*., 1997) and a plastocyanin promoter (Ferrara *et al*., 2006), both from *Medicago sativa* sp. As the plastocyanin promoter gave significantly higher expression levels for reporter proteins than the Rubisco promoter in preliminary studies (data not shown), it was decided to pursue the development of the co-transient expression system for IgGs with the plastocyanin promoter.

The coding sequences of the light and heavy chains of C5-1 (a murine anti-human IgG; Khoudi *et al*., 1999) were assembled in tandem constructs downstream of the plastocyanin promoter and 5′ untranslated sequences, and flanked with the plastocyanin 3′ untranslated and transcription termination sequences on the same T-DNA segment of a pCAMBIA binary plasmid, as presented in Figure 1. In both the R612 and R610 expression cassettes, the light and heavy chain coding sequences contained the native signal peptide of C5-1 (Khoudi *et al*., 1999), but, in R610, the coding sequence of a KDEL peptide was added at the C terminus of the heavy chain to accumulate the assembled IgG in the ER. Leaves of *N. benthamiana* plants were syringe-infiltrated with *Agrobacterium* strains transformed with plasmids R612

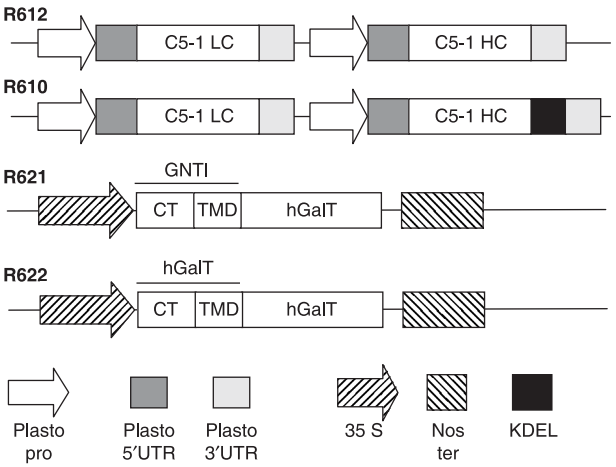

Figure 1 Schematic representation of fusion protein constructs analysed in this study. R612 and R610, plastocyanin-based cassettes assembled for C5-1 expression: C5-1 LC, C5-1 light chain coding sequence; C5-1 HC, C5-1 heavy chain coding sequence. R621 and R622, cassettes assembled for native and hybrid versions of galactosyltransferase expression: GNTI, cytosolic tail and transmembrane domain of *N*-acetylglucosaminyltransferase I; GalT, human β1,4-galactosyltransferase.

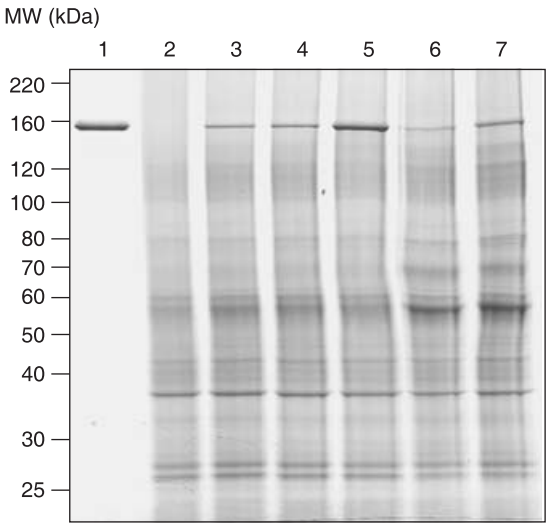

Figure 2 C5-1 accumulation in leaves of *Nicotiana benthamiana*. Leaf protein extracts were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–12%) under non-reducing conditions and stained with Coomassie blue. Lane 1, commercial murine IgG1 (Sigma; M9269) was loaded as a control of electrophoretic mobility; lane 2, 12 μg of total protein extracted from mock-infiltrated biomass (empty vector) was used as negative control, and 12 μg of the same extract, spiked with 250 ng (lane 3), 500 ng (lane 4) and 1 μg (lane 5) of commercial IgG1, was used as reference; lanes 6 and 7, 12 μg of total proteins extracted from leaves infiltrated with R612 + HcPro and R610 + HcPro, respectively.

and R610 (three plants each). Six days after infiltration, the leaves of each plant (approximately 20 g of biomass) were frozen, ground, and the frozen powder was mixed to produce a homogeneous sample from which two sub-samples of 1.5 g each were taken for extraction (from each plant). Coomassie blue staining of sodium dodecylsulphate-polyacrylamide gels, run under non-reducing conditions, was used to provide the first estimates of C5-1 accumulation in leaf protein extracts from R612- and R10-infiltrated plants (Figure 2). By comparing the intensity of the band corresponding in molecular weight to that of the control murine IgG1 (lane 1), and by using calibrated dilutions of murine IgG1 loaded in control plant extracts, it was possible to estimate that the accumulation level of C5-1 was in the region of 4% total soluble protein (TSP), or 500 ng in 12 μg of total protein.

C5-1 was quantified in total protein extracts from each sample by an enzyme-linked immunosorbent assay (ELISA). As shown in Figure 3, agroinfiltration of R612 led to the accumulation of 106 mg of antibody/kg FW, whereas the ER-retained form of the antibody (R610) reached 211 mg/kg FW.

As post-transcriptional gene silencing (PTGS) has been shown to limit the expression of transgenes in agroinfiltrated *N. benthamiana* plants, transient co-expression of a suppressor of silencing from the potato virus Y (HcPro; Brigneti *et al.*, 1998) was tested for its efficiency at increasing the expression of C5-1. The co-expression of R612 and R610 with HcPro

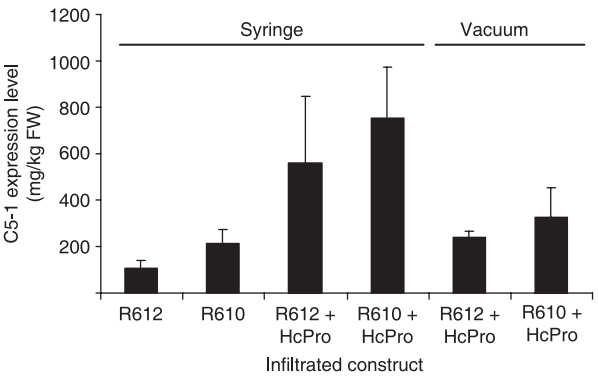

Figure 3 Accumulation of the C5-1 antibody in leaves of *Nicotiana benthamiana* agroinfiltrated with R610 and R612 (plastocyanin-based expression cassettes) with or without co-expression of the suppressor of silencing HcPro. The values presented correspond to the mean accumulation level and standard deviation obtained from six measurements on three plants (syringe), or six measurements on individual infiltration batches of approximately 12 plants (250 g). FW, fresh weight.

increased antibody accumulation levels by 5.3-fold and 3.6-fold, respectively. In the presence of HcPro, plastocyanin-controlled C5-1 expression reached average values of 558 mg/kg FW with R612 and 757 mg/kg FW with R610 (Figure 3).

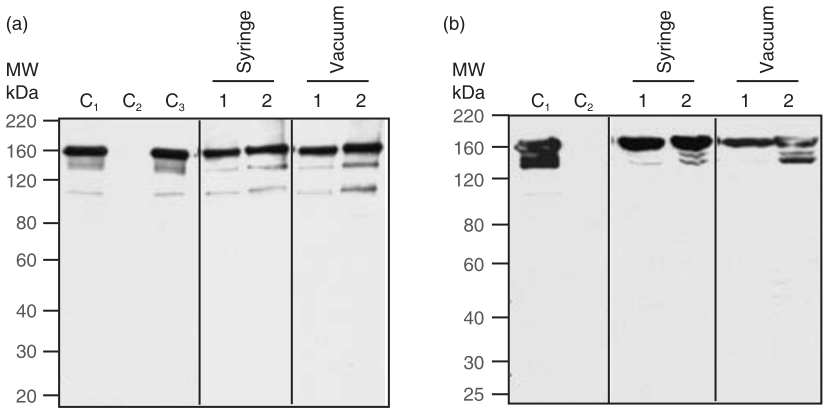

Figure 4 Protein blot analysis of C5-1 accumulation in crude extracts of syringe- and vacuum-infiltrated plants. (a) Immunoblotting with a peroxidase-conjugated goat anti-mouse IgG (H + L) on extracts from plants infiltrated with R612 (for secretion, lanes 1) or R610 [for endoplasmic reticulum (ER) retention, lanes 2]. C_1, 100 ng of commercial murine IgG1 (Sigma; M9269), loaded as a control for electrophoretic mobility; C_2, 12 μg of total proteins extracted from mock-infiltrated biomass (empty vector); C_3, 100 ng of commercial murine IgG1 (Sigma, M9269) spiked in 12 μg of total protein extracted from mock-infiltrated biomass (empty vector). (b) Activity immunoblotting with a peroxidase-conjugated human IgG1 on extracts from plants infiltrated with R612 (for secretion, lanes 1) or R610 (for ER retention, lanes 2). C_1, 2 μg of control C5-1 purified from hybridoma (Khoudi *et al*., 1999); C_2, 75 μg of total proteins extracted from mock-infiltrated biomass (empty vector).

Maximum C5-1 expression levels exceeded 1.5 g/kg FW (25% of TSPs) in some extracts from both R612- and R610-infiltrated leaves.

In order to assess the scalability of the agroinfiltration expression system, the accumulation of C5-1 was quantified following a vacuum infiltration procedure adapted from Kapila *et al*. (1997). In this series of experiments, the aerial parts of whole plants were vacuum infiltrated in repeated batches of 1 kg (16 plants) with R612 + HcPro or R610 + HcPro, and returned to the glasshouse for 6 days before harvest. In an effort to provide data which are representative of a large-scale production system, 250-g sublots of leaves/petioles were frozen, ground to homogeneity, and three subsamples of 7.5 g of material per batch were collected for analysis. As shown by ELISA quantification, average C5-1 accumulation levels reached 238 and 328 mg/kg FW for R612 and R610 infiltrations, respectively (Figure 3), which corresponds to the yield that could be obtained per 0.5 m^2 glasshouse surface using this strategy.

Characterization of the C5-1 antibody produced through transient expression

Immunodetection was used to show the assembly and fragmentation of the C5-1 IgG in crude leaf extracts of *N. benthamiana* producing either the secreted (R612) or ER-retained (R610) forms of mAb, following both syringe and vacuum infiltration. A Western blot probed with a peroxidase-conjugated goat anti-mouse IgG (H + L) was first used to highlight the presence of C5-1 antibody fragments independent of their origin on the C5-1 molecule. As shown in Figure 4a, all protein extracts contained fragments of similar molecular sizes and in similar relative abundance, irrespective of the subcellular targeting strategy or infiltration method used. In each case, a major band (≥ 85%) corresponding to the complete antibody was present at approximately 150 kDa, with two minor bands at approximately 135 and 100 kDa, showing that the antibody accumulated mainly as its fully assembled form (H_2L_2). Fragments of similar electrophoretic mobility were also present in the control IgG1 purified from a murine tumour cell line (MOPC-21) (Sigma, Oakville, Ontario, CA, #M9269), suggesting that the fragmentations occurring in plant and mammalian cells were similar and probably resulted from weak nonspecific proteolytic activity. Identical results were obtained when an anti-mouse heavy chain-specific antibody was used for detection (data not shown).

To test the antigen-binding capacity of the antibody produced through transient expression, we complemented these initial analyses by probing the blotted proteins with a peroxidase-conjugated human IgG1, the antigen of C5-1 (Figure 4b; see Khoudi *et al*., 1997). In addition to confirming unequivocally the identity and biological activity of the fully assembled antibody (≈ 150-kDa band), this analysis also confirmed the fragmentation pattern observed in Figure 4a, except for a 100-kDa band, which reacted with the anti-mouse IgG (H + L) (Figure 4a), but not with the C5-1 antigen. This result suggests that the 100-kDa fragment does not contain the Fab regions of the C5-1 antibody, which are essential for antigen binding, and, more probably, corresponds to dimers of heavy chains, an intermediate of antibody assembly.

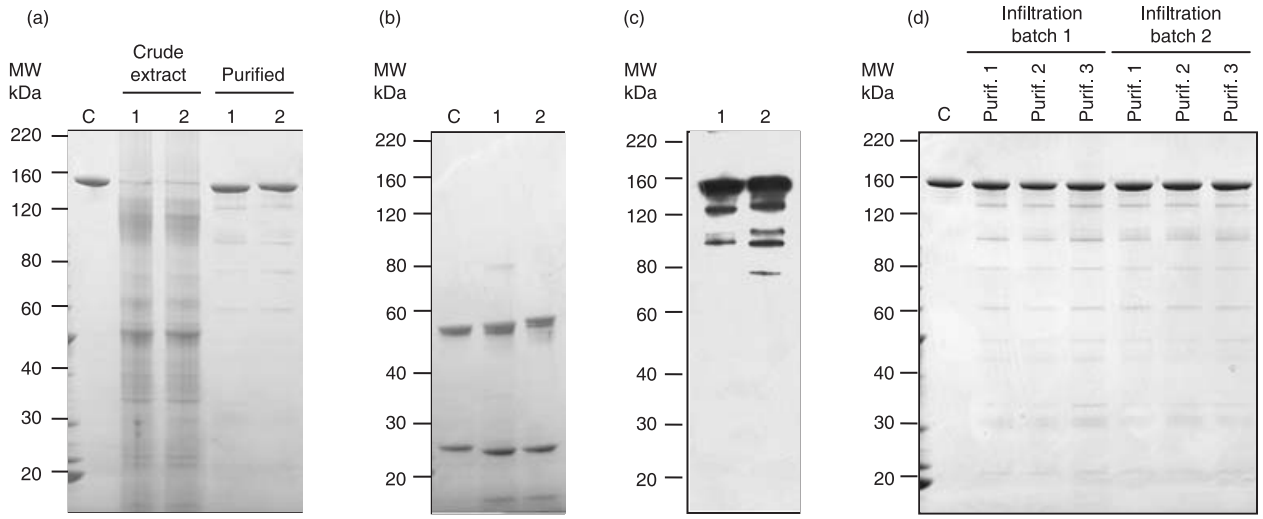

Figure 5 Analysis of antibodies purified from plants infiltrated with either R612 (for secretion, lanes 1) or R610 [for endoplasmic reticulum (ER) retention, lanes 2]. (a) Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of crude extracts and purified antibodies was performed in non-reducing conditions. Lane C, 2.5 μg of commercial murine IgG1 (Sigma; M9269), loaded as a control for electrophoretic mobility. Lanes 1 and 2, 12 μg of proteins. Lanes 3 and 4, 5 μg of proteins. (b) SDS-PAGE of purified antibodies was performed under reducing conditions. Lane C, 2.5 μg of commercial murine IgG1 (Sigma; M9269), loaded as a control for electrophoretic mobility. Lanes 1 and 2, 2 μg of antibody purified from R612- and R610-infiltrated plants, respectively. (c) Activity immunoblotting of purified antibodies was performed with a peroxidase-conjugated human IgG1. (d) Comparison of six lots of C5-1 purified from two different infiltration batches. Lane C, 2.5 μg of commercial murine IgG1 (Sigma; M9269), loaded as a control for electrophoretic mobility.

Antibody purification and characterization of the purified product

The antibody was purified from crude protein extracts using a single Protein G affinity chromatographic step, and the product obtained was analysed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The Coomassie blue-stained gel presented in Figure 5a shows a major band at 150 kDa in the eluate fraction from the Protein G column. This band represents more than 85% of the purified product in both the secreted and ER-retained forms, and the contents in contaminants are identical for both forms (Figure 5a). A Western blot analysis, probed with a polyclonal anti-mouse IgG, showed that the major contaminants in the purified C5-1 fractions are of IgG origin (data not shown). Under reducing conditions, two major products were detected at approximately 26 and 55 kDa, which correspond to the molecular weights of the light and heavy chains, respectively (Figure 5b). The heavy chain of the ER-retained antibody (Figure 5b, lane 2) showed a lower electrophoretic mobility than the heavy chain of the apoplastic antibody (Figure 5b, lane 1), which is interpreted as the combined result of additional KDEL amino acids being present at the C terminus and of differences in *N*-glycosylation as a result of the retention in the ER. Figure 5c shows that the purified antibodies (150 kDa) are bound to human IgG1, as are contaminating C5-1 fragments of 75, 90, 100 and 120 kDa, highlighting the presence of at least one Fab segment in these fragments. The presence of Fab in the 100-kDa fragment contrasts with the result obtained from crude extract analysis, where the 100-kDa band does not bind to human IgG. It is hypothesized that either the amount of Fab-containing fragments migrating at 100 kDa in the crude extract is too low for detection with the antigen-binding procedure, or that the fragment migrating at 100 kDa consists of two different molecules, one being heavy chain dimers (without Fab) and the other containing antigen-binding regions.

The reproducibility of this system for antibody production was assessed by a side-by-side comparison of purified products from two different infiltration batches and three distinct purification lots from each batch. The Coomassie blue-stained SDS-PAGE analysis of the purification lots showed the presence of identical bands in all lots, and in very similar relative abundances (Figure 5d).

High-yield production of an antibody with human-like *N*-glycans by co-transient expression of a chimaeric human GalT

To investigate whether transient co-expression could also be used to produce antibodies with human-compatible *N*-glycosylation in plants, a chimaeric construct containing the

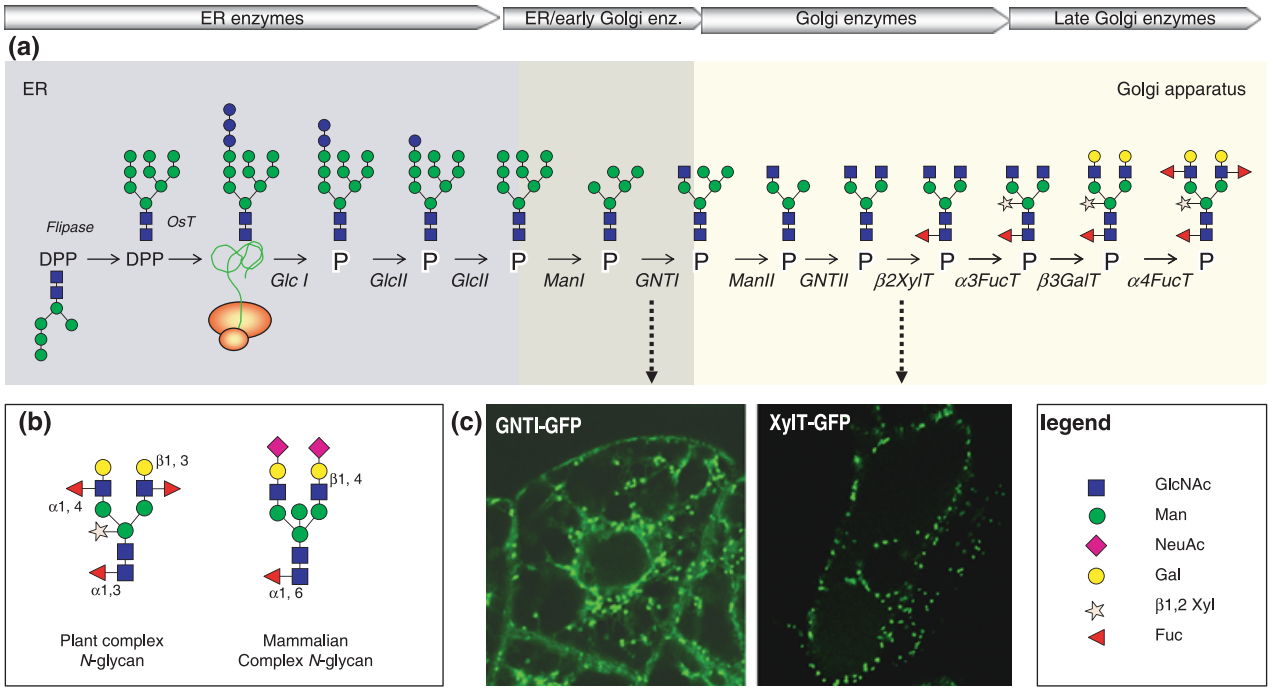

Figure 6 Protein *N*-glycosylation pathway in plants. (a) Illustration of the *N*-glycan maturation pathway in plants with the distribution of *N*-glycan-modifying enzymes from the endoplasmic reticulum (ER) to the late Golgi apparatus. P, protein backbone; DPP, dolichol pyrophosphate; *OsT*, oligosaccharyltransferase; *GlcI*, glucosidase I; *GlcII*, glucosidase II; *ManI*, mannosidase I; *GNTI*, *N*-acetylglucosaminyltransferase I; *ManII*, mannosidase II; *GNTII*, *N*-acetylglucosaminyltransferase II; *β2XylT*, β1,2-xylosyltransferase; *α3FucT*, α1,3-fucosyltransferase; *β3GalT*, β1,3-galactosyltransferase; *α4FucT*, α1,4-fucosyltransferase. (b) Structural differences between plant and mammalian complex *N*-glycans. (c) GNTI–green fluorescent protein (GFP) protein fusion detected by confocal laser scanning microscopy showing localization in both the ER reticulated network and the plant Golgi apparatus. In contrast, the XylT–GFP fusion is exclusively accumulated in the Golgi apparatus. Here, both GFP fusions are expressed in suspension-cultured tobacco cells (from Saint-Jore-Dupas *et al.*, 2006 and Pagny *et al.*, 2003).

catalytic domain of human GalT fused to the N-terminal part (cytosolic tail and transmembrane domain) of *A. thaliana* *N*-acetylglucosaminyltransferase I (GNTI) was assembled, and cloned under the control of the 35S promoter expression cassettes (Figure 1). More precisely, the first 123 N-terminal amino acids of GalT were removed and replaced by the first 77 N-terminal amino acids of GNTI to give the GNTI/GalT construct. This construct was transiently co-expressed with C5-1 light and heavy chains and HcPro in agroinfiltrated leaves of *N. benthamiana*. As illustrated in Figure 6c for green fluorescent protein (GFP), the cytosolic and transmembrane domains of GNTI are sufficient for targeting of a protein in the ER and the *cis*-Golgi apparatus membranes of tobacco cells (Saint-Jore-Dupas *et al.*, 2006; Gomord *et al.*, 2007). Our working hypothesis was that the sequestering of GalT activity in the early compartments of the secretory pathway would result in a more efficient addition of β1,4-galactose on plant *N*-glycans, together with an increased efficiency in the inhibition of α1,3-fucosylation and β1,2-xylosylation of the core.

Nicotiana benthamiana plants were simultaneously co-infiltrated for expression of C5-1 and HcPro (R612) with the GNTI/GalT chimaeric glycosyltransferase (R621). Effects of co-expression with GNTI/GalT were investigated using analysis by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) of the *N*-glycan profile of the tryptic glycopeptide EEQFNSTFR of C5-1. As illustrated in Figure 7a, when C5-1 was expressed in the absence of GNTI/GalT, its *N*-glycan population was represented by complex *N*-glycans, including an important proportion of ions consisting of α1,3-fucosylated and β1,2-xylosylated oligosaccharides and high-mannose-type *N*-glycans, as observed previously for stable expression (Bardor *et al.*, 2003b). The presence of non-mature ER-specific glycans, such as Man-8 and Man-9, could be associated, at least in part, with a fraction of proteins 'en-route', as reported previously for other plant-derived antibodies produced by transient expression (Sriraman *et al.*, 2004). The co-expression of C5-1 with GNTI/GalT yielded a purified C5-1 preparation in which the *N*-glycan population was significantly different from that of

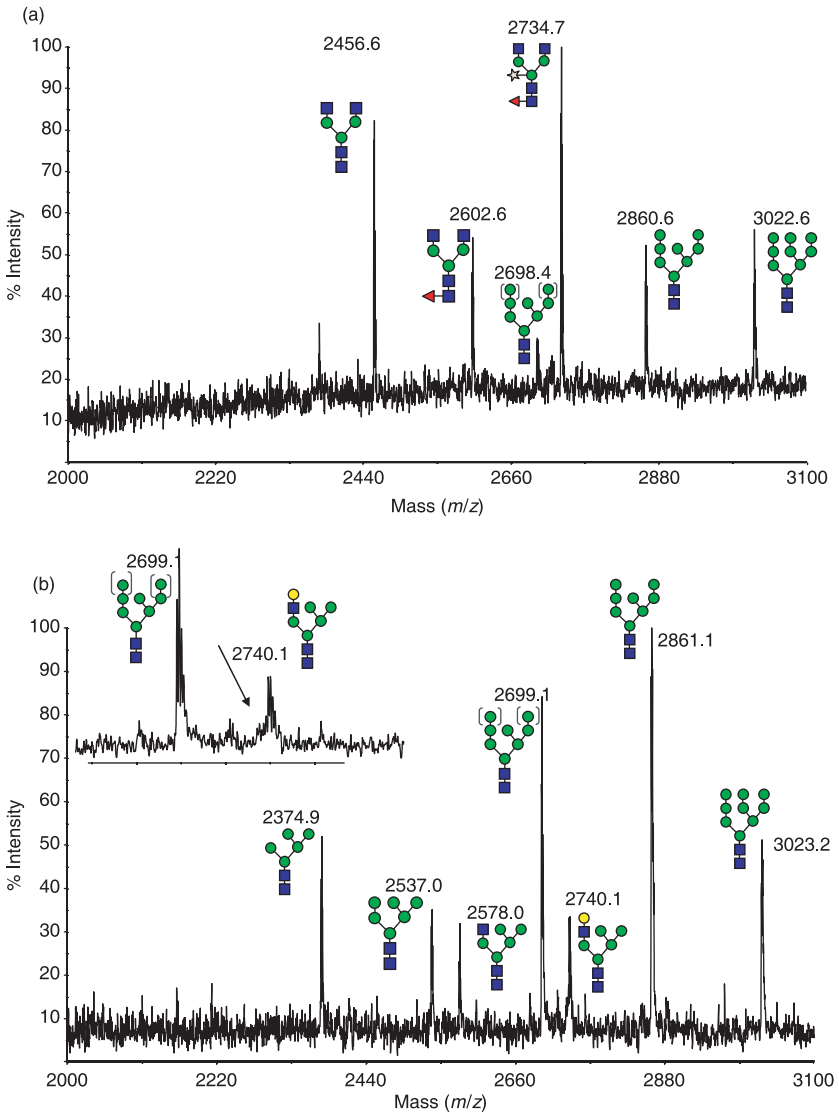

Figure 7 Matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) of the tryptic glycopeptides EEQFNSTFR from purified C5-1 expressed alone (a) or co-expressed with *N*-acetylglucosaminyltransferase I (GNTI)/ β1,4-galactosyltransferase (GalT) (b). Inset presents the enlargement of *m/z* 2650–2800 to illustrate the absence of the main complex *N*-glycan (*m/z* 2735 indicated by the arrow) form detected on the antibody expressed alone. *N*-glycan structure is shown in Figure 6.

C5-1 expressed alone. As shown in Figure 7b, galactosylated and non-galactosylated hybrids ($GalGlcNAcMan_5GlcNAc_2$ and $GlcNAcMan_5GlcNAc_2$) were present, together with immature oligomannose *N*-glycans. $GlcNAcMan_5GlcNAc_2$ and $Man_5GlcNAc_2$ oligosaccharide side-chains are most probably fragments derived from the degradation of the hybrid $GalGlcNAcMan_5GlcNAc_2$ by endogenous glycosidases, rather than intermediates of mature *N*-glycan formation. The effect of the GNTI membrane anchorage was striking, as C5-1 purified from plants in which the synthetic enzyme GNTI/GalT was co-expressed transiently with C5-1 contained no traces (≤ 1%) of glycans harbouring the plant-specific α1,3-fucose or β1,2-xylose residues (Figure 7b, inset), thus demonstrating that complete control of α1,3-fucosylation and β1,2-xylosylation was achieved during transient co-expression. As illustrated in Figure 5a, such a result can be explained by the action of the hybrid GalT at the earliest stage of complex *N*-glycan synthesis, allowing the human enzyme to act on the intermediate $GlcNAcMan_5GlcNAc_2$, thus inhibiting further transfer of plant-specific glycoepitopes on the core oligosaccharide.

Discussion

This study demonstrates that the combined effect of the strong alfalfa plastocyanin promoter (with its 5′ and 3′ regulatory elements) and the enhancing activity of the HcPro suppressor of silencing is capable of driving the expression of an antibody in agroinfiltrated *N. benthamiana* plants to levels reaching 1.5 g/kg FW (approximately 25% TSP), with average levels of 558 and 757 mg/kg FW for the secreted and ER-retained forms, respectively. This antibody expression

level is threefold higher than the antibody expression level obtained using the multi-virus transient expression system recently described by Giritch *et al*. (2006).

This vacuum-based agroinfiltration system adapted from Kapila *et al*. (1997) was easily brought to a scale in which more than 1 g of IgG could be produced per day with a small pilot unit. The transient expression system recently proposed by Giritch *et al*. (2006) relies on the expression of the light and heavy chains of antibodies on two non-competing viral vectors. The system proposed requires the co-infiltration of six different *Agrobacterium* cultures for the expression of provector modules, a recombinase and two viral replicases. In comparison, the system proposed here requires the co-infiltration of only two *Agrobacterium* cultures, and even this has now been reduced to a single culture by cloning the HcPro expression cassette in the same plasmid as the antibody expression cassette (data not shown).

In conclusion, the plastocyanin-based transient expression system presented here, together with the enhancing activity of the HcPro suppressor of silencing, yields expression levels reaching 1.5 g of antibody per kilogram of leaf FW, exceeding the accumulation level reported for any antibody in plants with other expression systems, including multi-virus-based systems and transgenic plants.

The high efficacy of the pea plastocyanin promoter has been demonstrated previously for transgenic expression in tobacco (Pwee and Gray, 1993), and this efficacy has been attributed to the specific characteristics of the upstream regulatory regions. Although a detailed functional analysis of the alfalfa plastocyanin promoter has not been performed, the high sequence homology of its specific regulatory domains to those of the pea plastocyanin promoter suggests that the alfalfa promoter contains regions which function as a general enhancer by associating with the nuclear matrix and by triggering acetylation of histones in the vicinity of the promoter region (Sandhu *et al*., 1998; Chua *et al*., 2003). Hibino *et al*. (2000) have shown that the addition of matrix/scaffold attachment regions (MAR/SAR) of various origins (including a plant MAR) augmented the SV40-regulated expression of a reporter gene in different types of transiently transformed mammalian cells.

The beneficial effect of HcPro co-expression in agroinfiltration has previously been demonstrated for viral-based expression systems (Chiba *et al*., 2006; Sainsbury *et al*., 2008) and transcription-based expression strategies using viral- and plant-derived promoters (Voinnet *et al*., 2003). It is worth noting here that, although the plastocyanin regulatory elements lead to high expression of the antibody, the co-expression of the viral suppressor of silencing HcPro significantly increases the accumulation level of the antibody to reach unprecedented levels in agroinfiltrated leaves.

As antibodies are complex multimeric proteins, their assembly, maturation and secretion are complex processes. At high levels of accumulation, it is thought that the cellular machinery involved in IgG synthesis and processing could be overwhelmed, eventually resulting in inadequate maturation and assembly of IgGs. The production system must therefore be tested for product quality in addition to product quantity. Here, we estimated the productivity of our system in crude extracts by a combination of procedures: spiked crude leaf protein extracts were used to calibrate the Coomassie blue-stained gels for direct visual estimates of fully assembled IgG, and Western and activity blots were performed on the same extracts. Combined together, these estimates confirmed that this antibody expression system reached yields of up to 1.5 g/kg FW, with over 85% of the product consisting of full-size tetrameric IgG of approximately 150 kDa. The contaminating immunoreactive compounds of crude preparations migrated at 135 and 100 kDa. Fragments of similar sizes have been described previously for different IgGs produced in plant expression systems (Sharp and Doran, 2001a,b; Giritch *et al*., 2006; Peterson *et al*., 2006), including C5-1 preparations extracted from transgenic alfalfa. Immunological and antigen-binding analysis in the current study indicated that the 135-kDa polypeptide contains at least one Fab region, and may therefore be either an H2L form of the antibody or a proteolytic fragment.

The addition of a KDEL peptide at the C terminus of the heavy chain has been used previously to increase antibody accumulation (2–10 times) by mediating the retrieval of the antibody from the Golgi back to the ER (Pagny *et al*., 2003; Schillberg *et al*., 2003). We have shown here that the addition of a KDEL peptide to the heavy chain of C5-1 doubled the yield when the HcPro suppressor of silencing was not used, but that this difference was much reduced when HcPro was used to reduce silencing. ER retention did not influence the product quality, as the fragments observed in the crude extracts from plants producing the ER-retained and secreted forms of the antibody were identical in size and relative abundance. These results differ significantly from those published by Sharp and Doran (2001a,b), where it was found that blocking the secretion of antibodies from transgenic tobacco cell culture with Brefeldin A resulted in an increase in the accumulation of fully assembled antibodies and a decrease in fragmentation.

Together with the concerns about production yields, concerns related to the characteristics of plant *N*-glycosylation

patterns have also limited the broad acceptance of plant production platforms by the pharmaceutical industry. The first and foremost concern about glycosylation of plant-made pharmaceuticals is safety. Plants produce *N*-glycans with typical core β1,2-xylose and α1,3-fucose, which are not found in human glycoproteins, and from a regulatory perspective, the ability to produce therapeutic glycoproteins with human-compatible glycosylation patterns is a requirement of any production platform (Gomord *et al*., 2005; Walsh and Jefferis, 2006). The second concern is efficacy, based on an increasing amount of data now demonstrating that glycans play a key role in the potency and stability of most therapeutic glycoproteins. As a result, the trend is now to develop glycoproteins with modified glycan profiles which are more efficient as biological effectors, rather than eliminating *N*-glycans from glycoproteins (Ferrara *et al*., 2006; Saint-Jore-Dupas *et al*., 2007). A major goal of plant glycoengineering is to improve the plant-based platforms to meet these two key industrial requirements.

To date, only a few plant-based production platforms have been successful at modelling the *N*-glycans of recombinant protein into a human-compatible form (for a recent review, see Saint-Jore-Dupas *et al*., 2007). In each case, expression of the recombinant proteins has been performed in modified (transgenic or mutants) host plants obtained through lengthy genetic transformation, selection and breeding procedures. In this study, our objective was to adapt transient expression technology to the production of human-compatible glycoproteins without affecting the features of speed and capacity. Our hypothesis was that we would achieve more stringent control of glycosylation through transient modulation, rather than through permanent transformation of the glycophenotype. The challenge was to achieve strong and synchronous co-expression of the target and modulator within the short time frame of transient expression.

In this case, the genes encoding for the light and heavy subunits of a murine IgG1 (C5-1) were transiently and simultaneously co-expressed with HcPro to increase antibody yield, and with a construct containing the chimaeric form of a GalT gene to produce C5-1 with human-compatible *N*-glycans.

The modification of the native GalT in this study was based on the recent availability of a large panel of signals, allowing targeted expression of heterologous glycosyltransferases in the plant secretory pathway (Gomord *et al*., 2007). In a previous study, it was shown that positioning GalT activity in the medial Golgi by a fusion with the *Arabidopsis* XylT N-terminal sequence (Bakker *et al*., 2006) strongly increased its ability to perform β1,4-galactosylation on nascent glycoproteins, but only partially inhibited plant-specific modifications of complex *N*-glycans. Considering both these results and recent data published by Saint-Jore-Dupas *et al*. (2006), we hypothesized that coupling GalT to the membrane anchorage domain of a tobacco GNTI to allocate GalT activity further upstream in the plant secretory pathway than with the XylT N-terminal sequence (as illustrated in Figure 5 where these targeting signals are fused to the reporter protein GFP) would result in a more efficient addition of terminal galactose and a more complete competition with β1,2-xylosyltransferase and α1,3-fucosyltransferases.

The effect of the GNTI membrane anchorage was striking, as C5-1 purified from plants in which the chimaeric GNTI/GalT was transiently co-expressed contained glycans with no detectable (< 1%) α1,3-fucose or β1,2-xylose residues. This demonstrates that complete inhibition of fucosylation and xylosylation was achieved during co-expression. This can be explained as a consequence of the action of GalT at the earliest stage of complex *N*-glycan biosynthesis, allowing the human enzyme to act on intermediate $GlcNAcMan_5GlcNAc_2$, thus inhibiting further transfer of plant-specific glycoepitopes to the core oligosaccharide. In contrast, when C5-1 was co-infiltrated with native GalT, it contained a significant amount of glycans bearing β1,2-xylose residues (data not shown).

The results shown here demonstrate that the current transient co-expression based on agroinfiltration has the capacity to produce mAbs at yields and quality similar or even higher than those published recently by Giritch *et al*. (2006), but with less complex molecular tools and procedures. Together with speed and simplicity, this method allows the high-yield production and modulation of post-translational glycomodifications during transient expression, i.e. in this case, the concomitant terminal β1,4-galactosylation and complete inhibition of α1,3-fucosylation and β1,2-xylosylation of a plant-made mAb. This approach avoids the lengthy production and selection of optimal mutant or glycomodulator transgenic lines and their subsequent use as parental lines, as described previously for plant-based production platforms developed for the production of human-compatible therapeutic proteins. As the impact of the modulator on the plant is restricted to the 6 days of incubation, it is hypothesized that this method will be amenable to the control of a broad range of post-translational modifications and not limited to *in vivo* protein design through glycoengineering. To our knowledge, this is the first demonstration of a transient system which combines simplicity, speed, high yields, product quality and *in vivo* modulation of post-translational modifications.

Table 1 Oligonucleotide primers used in this study

Name	Sequence
*Xma*I-pPlas.c	5′-AGTTCCCCGGGCTGGTATATTTATATGTTGTC-3′
*Sac*I-ATG-pPlas.r	5′-AATAGAGCTCCATTTTCTCTCAAGATGATTAATTAATTAATTAGTC-3′
*Sac*I-PlasTer.c	5′-AATAGAGCTCGTTAAAATGCTTCTTCGTCTCCTATTTATAATATGG-3′
EcoRI-PlasTer.r	5′-TTACGAATTCTCCTTCCTAATTGGTGTACTATCATTTATCAAAGGGGA-3′
Plasto-443c	5′-GTATTAGTAATTAGAATTTGGTGTC-3′
Plas + *LC-C51*.r	5′-ATCTGAGGTGTGAAAACCATTTTCTCTCAAGATG-3′
LC-C51.c	5′-ATGGTTTTCACACCTCAGATACTTGG-3′
LC-C51XhoSac.r	5′-ATATGAGCTCCTCGAGCTAACACTCATTCCTGTTGAAGC-3′
HC-C51.c	5′-ATGGCTTGGGTGTGGACCTTGC-3′
Plas + *HC-C51*.r	5′-CAAGGTCCACACCCAAGCCATTTTCTCTCAAGATG-3′
HC-C51XhoSac.r	5′-ATAAGAGCTCCTCGAGTCATTTACCAGGAGAGTGGG-3′
HC-C51*KDEL*(*Sac*I).r	5′-ATAAGAGCTCTCA*AAGTTCATCCTT*TTTACCAGGAGAGTGGG-3′
RGalTFlagStuI	5′-AAGGCCTACGCTACTTGTCATCGTCATCTTTGTAGTCGCACGGTGTCCCGAAGTCCAC-3′
FGalTSpe	5′-GGACTAGTGCACTGTCGCTGCCCGCCTGC-3′
RGNTspe	5′-CGGGATCCACTAGTCTGACGCTTCATTTGTTCTTC-3′
FGNT	5′-ATCGAAATCGCACGATGAGAGGGTACAAGTTTTGC-3′
FGalT	5′-GACTCTAGAGCGGGAAGATGAGGCTTCGGGAGCCGCTC-3′

Experimental procedures

Assembly of expression cassettes

All manipulations were performed using the general molecular biology protocols of Sambrook and Russell (2001). The oligonucleotide primers used are presented in Table 1. The first cloning step consisted of assembling a receptor plasmid containing upstream and downstream regulatory elements of the alfalfa plastocyanin gene (Vézina and D'Aoust, 2006). The plastocyanin promoter and 5′ untranslated region (UTR) sequences were amplified from alfalfa genomic DNA using oligonucleotide primers *Xma*I-pPlas.c and *Sac*I-ATG-pPlas.r. The resulting amplification product was digested with *Xma*I and *Sac*I and ligated into pCAMBIA2300, previously digested with the same enzymes, to create pCAMBIA-PromoPlasto. Similarly, the 3′ UTR sequences and terminator of the plastocyanin gene were amplified from alfalfa genomic DNA using the primers *Sac*I-PlasTer.c and *Eco*RI-PlasTer.r, and the product was digested with *Sac*I and *Eco*RI before being inserted into the same sites of pCAMBIA-PromoPlasto to create pCAMBIAPlasto.

Plasmids R610 and R612 were prepared so as to contain C5-1 light and heavy chain coding sequences under the plastocyanin promoter of alfalfa as tandem cassettes; R610 was designed to allow retention in the ER, whereas R612 was designed to allow secretion of the assembled IgG. The assembly of C5-1 expression cassettes was performed using the polymerase chain reaction (PCR)-mediated ligation method described by Darveau *et al*. (1995). To assemble the light chain coding sequences downstream of the plastocyanin promoter, a first step consisted of amplifying the first 443 base pairs (bp) of the alfalfa plastocyanin promoter upstream of the initial ATG by PCR with primers Plasto-443c and Plas+*LC-C51*.r, with pCAMBIAPlasto as template. In parallel, the light chain coding sequence was PCR amplified from plasmid pGA643-kappa (Khoudi *et al*., 1999) with primers LC-C51.c and LC-C51XhoSac.r. The two amplification products obtained were mixed together and used as template in a third PCR using primers Plasto-443c and LC-C51XhoSac.r. The overlap between primers Plas+*LC-C51*.r and LC-C51.c used in the first reactions leads to the assembly of the amplification products during the third reaction. The assembled product, resulting from the third PCR, was digested with *Dra*III and *Sac*I and ligated into pCAMBIAPlasto, previously digested with *Dra*III and *Sac*I, to generate plasmid R540.

The heavy chain coding sequence was fused to the plastocyanin upstream regulatory element as follows: the 443 bp upstream of the initial ATG of plastocyanin was amplified by PCR with primers Plasto-443c and Plas+*HC-C51*.r, with pCAMBIAPlasto as template. In parallel, the heavy chain coding sequence was amplified using primers HC-C51.c and HC-C51XhoSac.r on plasmid pGA643-gamma (Khoudi *et al*., 1999). The products of these reactions were mixed and assembled in a third PCR using primers Plasto-443c and HC-C51XhoSac.r. The resulting fragment was digested with *Dra*III and *Sac*I and ligated into pCAMBIAPlasto between the *Dra*III and *Sac*I sites. The resulting plasmid was named R541. The KDEL tag was added to the C terminus of the heavy chain coding sequence by PCR amplification with primers Plasto-443c and HC-C51*KDEL*(*Sac*I).r on plasmid R541. The resulting fragment was digested with *Dra*III and *Sac*I cloned into the same sites of pCAMBIAPlasto, creating plasmid R550. The assembly of light and heavy chain expression cassettes on the same binary plasmid was performed as follows: R541 and R550 were digested with *Eco*RI, blunted, digested with *Hin*dIII and ligated into the *Hin*dIII and *Sma*I sites of R540 to create R610 (with KDEL) and R612 (without KDEL).

Plasmids for GalT and GNTI/GalT expression were assembled from pBLTI121 (Pagny *et al*., 2003). The human GalT (hGalT) gene (UDP galactose:β-*N*-acetylglucosaminide:β(1,4)-galactosyltransferase; EC 2.4.1.22) was isolated from pUC19-hGalT (Watzele *et al*., 1991) with *Eco*RI digestion. After Klenow treatment, the 1.2-kb hGalT fragment was cloned into pBLTI221 at *Sma*I sites. A flag tag was then fused to the C-terminal end of the coding region by PCR (FGal-T-RGalTFlagStu). R622 was then produced by cloning this *Xba*I-*Stu*I fragment into the binary vector pBLTI121. The cDNA encoding the first 77 amino acids from GNTI – corresponding to the cytosolic tail and the transmembrane domain – was amplified by PCR using

primers FGNT and RGNTspe, and cloned first into the pGEM-T vector, and then by *Apa*I/*Bam*HI into pBLTI221. For the fusion between GNTI and hGalT, PCR amplification was performed from pUC19-hGalT to eliminate its own transmembrane domain and create *Spe*I and *Stu*I sites (FgalTspe-RgalTFlagStu). The *Spe*I/*Stu*I hGalT fragment was then cloned into pBLTI221-GNTI. Finally, digestion by the surrounding sites *Xba*I/*Stu*I enabled a 1030-bp fragment to be isolated, and R622 was then produced by cloning this 1030 stretch into the binary vector pBLTI121-Plasto.

All clones were sequenced to confirm the integrity of the constructs. The different constructs were used to transform *Agrobacterium tumefaciens* (AGL7) according to Höfgen and Willmitzer (1988).

Preparation of plant biomass, inoculum and agroinfiltration

Nicotiana benthamiana plants were grown from seeds in flats filled with a commercial peat moss substrate. The plants were allowed to grow in the glasshouse under a 16-h/8-h photoperiod and a temperature regime of 25 °C day/20 °C night. Three weeks after seeding, individual plantlets were picked out, transplanted into pots and left to grow in the glasshouse for three additional weeks under the same environmental conditions. *Agrobacterium* strains R612, R610, R621, R622 or 35SHcPro were grown in a yeast extract broth (YEB) medium supplemented with 10 mM 2-[*N*-morpholino]ethanesulphonic acid (MES, pH 5.6), 20 μM acetosyringone, 50 μg/mL kanamycin and 25 μg/mL carbenicillin, until they reached an optical density at 600 nm (OD_{600}) between 0.6 and 1.6. *Agrobacterium* suspensions were centrifuged before use and resuspended in the infiltration medium [10 mM $MgCl_2$ and 10 mM MES (pH 5.6)]. For vacuum infiltration, *Ag. tumefaciens* suspensions were centrifuged, resuspended in the infiltration medium and stored overnight at 4 °C. On the day of infiltration, culture batches were diluted 1 : 2.5 in infiltration medium and allowed to warm before use. Whole plants of *N. benthamiana* were placed upside down in the bacterial suspension in an air-tight stainless steel tank under a vacuum of 20–40 Torr for 2 min. Following vacuum infiltration, plants were returned to the glasshouse for a 4–6-day incubation period until harvest.

Leaf sampling and total protein extraction

Following incubation, the aerial parts of the plants were harvested, frozen at –80 °C, crushed into pieces and separated into 1.5- or 7.5-g subsamples. TSPs were extracted by homogenizing (Polytron) each subsample of frozen-crushed plant material in three volumes of cold 50 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.4, 0.15 M NaCl, 0.1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride and 10 μM chymostatin. After homogenization, the slurries were centrifuged at 20 000 ***g*** for 20 min at 4 °C, and these clarified crude extracts (supernatant) were kept for analyses. The total protein content of clarified crude extracts was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as the reference standard.

Protein analysis and immunoblotting

As C5-1 is an anti-human murine IgG, detection and quantification can be performed through either its characteristic affinity to human IgGs (activity blots) or by its immunoreactivity to anti-mouse IgGs. Proteins from total crude extracts or purified antibody were separated by SDS-PAGE and either stained with Coomassie blue R-250 or G-250 or electrotransferred on to polyvinylidene difluoride membranes (Roche Diagnostics Corporation, Indianapolis, IN, USA) for immunodetection. Prior to immunoblotting, the membranes were blocked with 5% skimmed milk and 0.1% Tween-20 in Tris-buffered saline (TBS-T) for 16–18 h at 4 °C. Immunoblotting was performed by incubation with the following antibodies: a peroxidase-conjugated goat anti-mouse IgG (H + L) antibody (Jackson ImmunoResearch, West Grove, PA, USA; Cat# 115-035-146) (0.04 μg/mL in 2% skimmed milk in TBS-T); a peroxidase-conjugated human IgG antibody (Gamunex® Bayer Corp., Elkhart, IN, USA) (0.2 μg/mL in 2% skimmed milk in TBS-T); or a polyclonal goat anti-mouse IgG antibody (heavy chain specific) (Sigma-Aldrich, St Louis, MO, USA) (0.25 μg/mL in 2% skimmed milk in TBS-T). A peroxidase-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch) (0.04 μg/mL in 2% skimmed milk in TBS-T) was used as a secondary antibody for membranes treated with the heavy chain-specific antibody. Immunoreactive complexes were detected by chemiluminescence using luminol as the substrate (Roche Diagnostics Corporation). Horseradish peroxidase–enzyme conjugation of human IgG antibody was carried out using the EZ-Link Plus® Activated Peroxidase conjugation kit (Pierce, Rockford, IL, USA).

Quantitative ELISA

Multiwell plates (Immulon 2HB, ThermoLab System, Franklin, MA, USA) were coated with 2.5 μg/mL of goat anti-mouse antibody specific to IgG1 heavy chain (Sigma; M8770) in 50 mM carbonate buffer (pH 9.0) at 4 °C for 16–18 h. Multiwell plates were then blocked through a 1-h incubation in 1% casein in phosphate-buffered saline (PBS) (Pierce Biotechnology, Rockford, IL, USA) at 37 °C. For each plate used, a standard curve was generated with 0, 8 16, 32, 48, 64 and 80 ng/mL of purified mouse IgG1 control (Sigma; M9269). When performing the immunoassays, all dilutions (control and samples) were performed in a plant extract obtained from plant tissue infiltrated and incubated with a mock inoculum so that any matrix effect be eliminated. Plates were incubated with protein samples and standard curve dilutions for 1 h at 37 °C. After three washes with 0.1% Tween-20 in PBS (PBS-T), the plates were incubated with a peroxidase-conjugated goat anti-mouse IgG (H + L) antibody (0.04 μg/mL in blocking solution) (Jackson ImmunoResearch; 115-035-146) for 1 h at 37 °C. The washes with PBS-T were repeated and the plates were incubated with a 3,3′,5,5′-tetramethylbenzidine (TMB) Sure Blue peroxidase substrate (KPL, Gaithersburg, MD, USA). The reaction was stopped by the addition of 1 N HCl (1 N = 1 M) and the absorbance was read at 450 nm. Each sample was assayed in triplicate and the concentrations were interpolated in the linear portion of the standard curve. To be considered valid, the values (read and calculated) of the standard curve had to meet the following criteria: a calculated linear coefficient (R^2) of the standard curve of over 95% and a calculated coefficient of variation (CV) between the triplicates of the standard curve not exceeding 15%. Only the plates in which the standard curve met these acceptability criteria were used. Samples were analysed in triplicate. For each replicate, four one-third serial dilutions were distributed randomly on plates. For each replicate, the values outside of the highest and lowest points of the standard curve were not used. In addition, samples for which the coefficient of variation exceeded 15% were not considered.

IgG purification

All purification batches of C5-1 from leaf material were performed with the following standardized procedure. Frozen leaves of *N. benthamiana* (100–150 g) were extracted in 20 mM sodium phosphate, 150 mM NaCl and 2 mM sodium metabisulphite at pH 5.8–6.0 using a commercial blender for 2–3 min at room temperature. Insoluble fibres were removed by a coarse filtration on Miracloth™ (Calbiochem, San Diego, CA, USA) and 10 mM phenylmethanesulphonyl fluoride (PMSF) was added to the filtrate. The extract was adjusted to pH 4.8 ± 0.1 with 1 M HCl and clarified by centrifugation at 18 000 ***g*** for 15 min at 2–8 °C. The clarified supernatant was adjusted to pH 8.0 ± 0.1 with 2 M Tris, clarified again by centrifugation at 18 000 ***g*** for 15 min at 2–8 °C, and filtered on sequential 0.8 and 0.2 μm membranes (Pall Corporation, Mississauga, Ontario, Canada). The filtered material was concentrated by tangential flow filtration using a 100-kDa molecular weight cut-off ultrafiltration membrane of 0.2 ft^2 of effective area (GE Healthcare, Chicago, IL, USA) to reduce the volume of the clarified material by 5–10-fold. The concentrated sample was then applied to a 5-mm × 5-cm column (1 mL column volume) of recombinant protein G-Sepharose Fast Flow (Sigma-Aldrich; Cat.# P4691). The column was washed with five column volumes of 20 mM Tris-HCl, 150 mM NaCl, pH 7.5. The antibody was eluted with 100 mM glycine pH 2.9–3.0, and immediately brought to neutral pH by collection in tubes containing calculated volumes of 1 M Tris-HCl pH 7.5. The pooled fractions of eluted antibody were centrifuged at 21 000 ***g*** for 15 min at 2–8 °C and stored at –80 °C until analysis. After purification, the affinity column was cleaned and stored according to the manufacturer's instructions. The same chromatographic media could be reused for several purifications without significant modification of purification performances (up to 10 cycles tested).

N-Glycosylation analysis

Fifty micrograms of purified C5-1 were run on 15% SDS-PAGE. Heavy and light chains were revealed with Coomassie blue and the gel band containing the heavy chain was excised and crushed into small pieces. Fragments were washed three times with 600 μL of a solution of 0.1 M NH_4HCO_3/CH_3CN (1 : 1) for 15 min each time and dried. Reduction of disulphide bridges occurred by incubation of the gel fragments in 600 μL of a solution of 0.1 M dithiothreitol (DTT) in 0.1 M NH_4HCO_3 at 56 °C for 45 min. Then, alkylation was carried out by adding 600 μL of a solution of iodoacetamide 55 mM in 0.1 M NH_4HCO_3 at room temperature for 30 min. Supernatants were discarded and polyacrylamide fragments were washed once again in NH_4HCO_3 0.1 M/CH_3CN (1 : 1). Proteins were then digested with 7.5 μg of trypsin (Promega, Charbonnières-les-Bains, France) in 600 μL of 0.05 M NH_4HCO_3 at 37 °C for 16 h. Two hundred microlitres of CH_3CN were added and the supernatant was collected. Gel fragments were then washed with 200 μL of 0.1 M NH_4HCO_3, and then with 200 μL CH_3CN again, and finally with 200 μL formic acid 5%. All supernatants were pooled and lyophilized. Peptide separation by high-performance liquid chromatography (HPLC) was carried out on a C_{18} reverse-phase column (4.5 × 250 mm) with a linear gradient of CH_3CN in trifluoroacetic acid 0.1%. Fractions were collected and lyophilized, and analysed by MALDI-TOF-MS on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems, Cortaboeuf, France) equipped with a 337-nm nitrogen laser. Mass spectra were performed in the reflector delayed extraction mode using α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich) as matrix.

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (CNRS), the French Ministère de la Recherche and Medicago Europa SAS.

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