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High-yield production of a human monoclonal IgG by rhizosecretion in hydroponic tobacco cultures

Luisa M. Madeira[1,†], Tim H. Szeto[1], Maurice Henquet[2], Nicole Raven[3], John Runions[4], Jon Huddleston[5], Ian Garrard[5], Pascal M.W. Drake[1,*,‡] and Julian K-C. Ma[1,‡]

[1]*The Hotung Molecular Immunology Unit, Institute for Infection and Immunity, St. George's University of London, London, UK*

[2]*Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands*

[3]*Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Aachen, Germany*

[4]*Department of Biological and Medical Sciences – Faculty of Health and Life Sciences, Oxford Brookes University, Oxford, UK*

[5]*Brunel Institute for Bioengineering, Brunel University, London, UK*

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**Correspondence* (Tel 0044 2087255691;
fax 0044 2087253487;
email pdrake@sgul.ac.uk)
[†]Present address: Department of Plant Sciences, University of Oxford, Oxford, UK.
[‡]These authors contributed equally to this work.

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Summary

Rhizosecretion of recombinant pharmaceuticals from *in vitro* hydroponic transgenic plant cultures is a simple, low cost, reproducible and controllable production method. Here, we demonstrate the application and adaptation of this manufacturing platform to a human antivitronectin IgG_1 monoclonal antibody (mAb) called M12. The rationale for specific growth medium additives was established by phenotypic analysis of root structure and by LC-ESI-MS/MS profiling of the total protein content profile of the hydroponic medium. Through a combination of optimization approaches, mAb yields in hydroponic medium reached 46 μg/mL in 1 week, the highest figure reported for a recombinant mAb in a plant secretion-based system to date. The rhizosecretome was determined to contain 104 proteins, with the mAb heavy and light chains the most abundant. This enabled evaluation of a simple, scalable extraction and purification protocol and demonstration that only minimal processing was necessary prior to protein A affinity chromatography. MALDI-TOF MS revealed that purified mAb contained predominantly complex-type plant *N*-glycans, in three major glycoforms. The binding of M12 purified from hydroponic medium to vitronectin was comparable to its Chinese hamster ovary (CHO)-derived counterpart. This study demonstrates that *in vitro* hydroponic cultivation coupled with recombinant protein rhizosecretion can be a practical, low-cost production platform for monoclonal antibodies.

Introduction

Monoclonal antibodies (mAbs) are the most commercially important products in the field of biologics (Aggarwal, 2009). Therapeutic mAbs often serve a large patient population, involving long-term therapy with high doses, for example the chimeric monoclonal antibody Rituximab, used for the treatment of haematological cancers and autoimmune diseases such as rheumatoid arthritis (Edwards *et al.*, 2004; Saini *et al.*, 2011). Consequently, extremely high amounts of mAbs are needed to meet demand. The main challenge for mAb manufacturing is the development of scalable, consistent and reproducible systems for their production at moderate costs (Shukla and Thömmes, 2010). To date, mammalian cell culture has been the method of choice. However, in spite of the high production capacity achieved after over a decade of optimization, costs associated with bioreactor establishment/maintenance, cell culture medium and purification are still very high.

Transgenic plants offer a low cost, readily scalable alternative to mammalian cell production systems. Since mAbs were first produced in transgenic plants in 1989 (Hiatt *et al.*, 1989), the feasibility of the plant expression system has been demonstrated in numerous reports (reviewed in De Muynck *et al.*, 2010; Yusibov *et al.*, 2011). Initial scepticism regarding the practicalities of establishing a regulatory compliant production process that could match the mammalian cell culture production system has eased, following regulatory approval for Phase I safety trials of plant-derived mAbs in both the USA and UK (www.icongenetics.com/html/5954.htm; www.pharma-planta.org). Importantly, Protalix's Elelyso™ (taliglucerase alfa) recently became the first plant-derived pharmaceutical to be approved for human use by the FDA, followed by several other countries' regulatory agencies (http://www.protalix.com/procellex-platform/overview-procellex-platform.asp). This milestone established plant systems as manufacturing platforms for pharmaceutical proteins, paving the way for other plant-produced recombinant pharmaceuticals.

Rhizosecretion has been investigated as a process which could be exploited to harvest recombinant protein when genetically modified plants were grown hydroponically, as a way to simplify downstream processing (Borisjuk *et al.*, 1999). Other advantages were also proposed: the possibility of recombinant product extraction throughout the life of the plant, exposure to fewer proteolytic enzymes, the fact that the final product is fully secreted and therefore potentially more homogeneous than a product extracted from plant tissue and the potential for continuous in-line extraction process (Drake *et al.*, 2009).

Drake *et al.* (2009) used plant-growth regulators to improve rhizosecretion yields of two model mAbs and an HIV microbicide candidate, cyanovirin-N (CV-N) (Drake *et al.*, 2009). They demonstrated that the auxin α-naphthaleneacetic acid (NAA), added to hydroponic medium at 1 mg/L, increased antibody yields 50-fold and the yield of CV-N sixfold over a 7-day period. The

maximum rhizosecretion rates achieved were 0.95 μg/mL/week for Guy's 13 and 6.25 μg/mL/week for CV-N.

Here, we applied these findings to the rhizosecretion of a recombinant human IgG antibody, M12. This mAb was derived from a single-chain variable fragment (scFv) selected from a naïve human phage display library using a breast carcinoma cell line (Raven *et al.*, 2011). M12 binds to vitronectin, a plasma glycoprotein which may influence tumour haemostasis and malignancy (Bloemendal *et al.*, 2004; Kirchhoff *et al.*, 2012). Transgenic tobacco plants which expressed M12 at high levels in leaf tissue were used, and studies were undertaken to determine whether this was reflected in the yields produced by rhizosecretion. The aim of this work was to optimize hydroponic cultivation and rhizosecretion of the antibody, to understand the biological limitations of the production system and to identify the downstream processing and purification challenges resulting from the manipulation of the plant cultivation process. Following on from our previous findings, we included NAA in our culture media and compared mAb yields in plants cultured either in MS medium (Murashige and Skoog, 1962) or MS supplemented with additional nitrate (MSN), a formulation that has previously boosted mAb production in tobacco BY-2 cells (Holland *et al.*, 2010). The rhizosecretion system was further characterized with microscopy analysis of roots, a proteomic profile of hydroponic medium, the rhizosecretome, and glycan structure and functionality analysis of the M12 mAb. Finally a simple, scalable standard operating procedure was developed for purification of rhizosecreted mAbs from hydroponic culture.

Results

M12 antibody is consistently rhizosecreted at high yields in nitrate-enriched hydroponic medium

Tobacco plants expressing a secreted version of M12 antibody were cultivated inside glass jars in either MS medium or MSN medium, containing NAA and gelatin. Samples were collected weekly during the harvesting phase (weeks 11–18) and quantified by ELISA. M12 yields in MSN medium were significantly higher than in MS medium at all time points (Student's *t*-test, $P < 0.0001$). As shown in Figure 1a, at week 11, the average M12 level accumulated in hydroponic medium of plants grown in MS medium was ~1 μg/mL and decreased to undetectable levels after week 13. However, the mean yield for the MSN group was 35 μg/mL at week 11. At week 12, the mean yield increased to 46 μg/mL in the MSN plants (with a maximum of 75 μg/mL measured in the highest yielding individual plant). The levels decreased slightly thereafter, but were maintained between 13 and 25 μg/mL until week 18. Over the eight weeks of the harvesting phase, an average of 23 μg/mL per week of M12 antibody was produced by the plants in the MSN group.

Rhizosecreted M12 from MS and MSN groups was also visualized on a Western blot probed with antiheavy chain or antilight chain antiserum (Figure 1b,c). With antiheavy chain antiserum, under nonreducing conditions, there were three major high molecular weight bands, representing the full-length antibody and two assembly intermediate or degradation products, in the leaf-derived positive control and in M12 harvested from plants cultured in MS or MSN. The intensity of all three bands was much stronger for the MSN sample including the 150 kDa full-length antibody (arrow). Under reducing conditions, a 50 kDa strong band was observed in the MSN sample (arrow), which was not observed in the MS sample. The same band was observed in

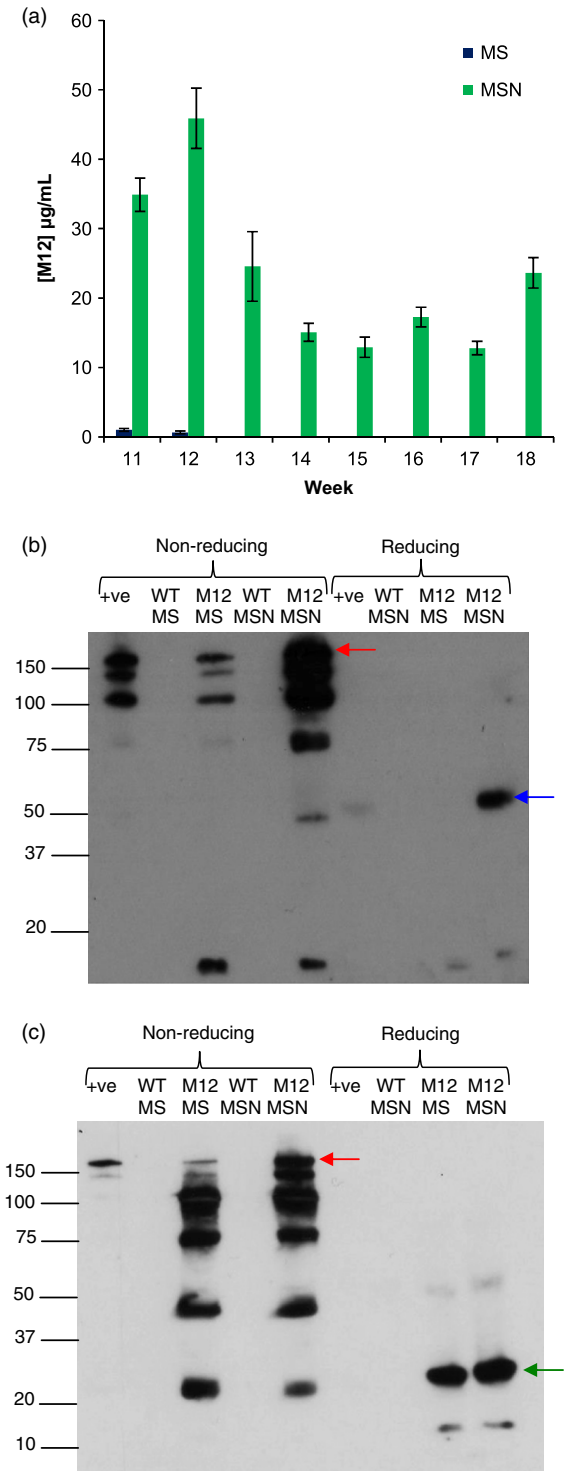

Figure 1 M12 production in MS or MSN hydroponic medium. (a) M12 yields in hydroponic medium measured by ELISA of plants cultivated in MS or MSN. Values are mean ± SE of a minimum of seven plants. (b) Western blot of pooled samples from week 11 detected with anti-gamma chain antiserum, at reducing and nonreducing conditions. (c) Western blot of pooled samples from week 11 detected with anti-lambda chain antiserum, under reducing and nonreducing conditions. M = molecular weight marker. +ve = tobacco leaf-derived purified M12 antibody control (40 ng). *Mr* (kDa) is indicated on the left. Red arrow indicates fully assembled antibody, blue arrow indicates heavy chain, and green arrow indicates light chain.

the purified tobacco leaf-derived M12 positive control. A further 10–15 kDa band was detected in both MS and MSN samples, suggesting degradation of the heavy chain. When detected with antilight chain antiserum under nonreducing conditions, the same banding pattern was observed for both MS and MSN samples, but the 150 kDa full-length antibody (arrow) was much more intense in the MSN sample, confirming the result observed with antiheavy chain antiserum. Under reducing conditions, a 25 kDa band of comparable intensity was detected in both samples suggesting that the effect induced by the high nitrate medium is exclusive to the heavy chain. No bands were observed for the WT samples under any conditions.

The root phenotype in the rhizosecretion is consistent with auxin treatment

The rhizosecretion-based production system used here involved treatment of plants with NAA over a prolonged period. This was associated with an altered root phenotype that was evident to the naked eye. Whilst wild type and M12 control root tips appeared similar and normal, those treated with NAA displayed root hair proliferation and elongation, and initiation of nonelongating lateral root primordia along the entire length of the vascular cylinder right to the root tip. This phenotype was more pronounced in M12 3NAA roots compared to M12 1NAA roots. Microscopy of roots from plants treated with NAA illustrates a proliferation of elongated root hairs (Figure 2, top panel, M12 1NAA and M12 3NAA), compared to plants not treated with NAA (Figure 2, top panel, WT and M12). Root hairs were remarkably long in the group of plants receiving three doses of NAA, so much so that they formed a mat that had to be pulled apart with forceps to allow for imaging of individual root tips.

The nodular structures (Figure 2, bottom panel, M12 1NAA and M12 3NAA) were identified under magnification as nonelongating lateral root primordia. Their proliferation was more remarkable in the group of plants receiving three doses of NAA, whilst they could not be observed in the plants not treated with NAA (Figure 2, bottom panel). These lateral root primordia initiated but failed to elongate and thus did not result in disruption to enclosing root cortical or epidermal cell layers.

The rhizosecretome is a complex mix of proteins; the recombinant antibody chains are the most abundant proteins

In order to further characterize the rhizosecretion platform and to address the challenge of developing downstream processing and

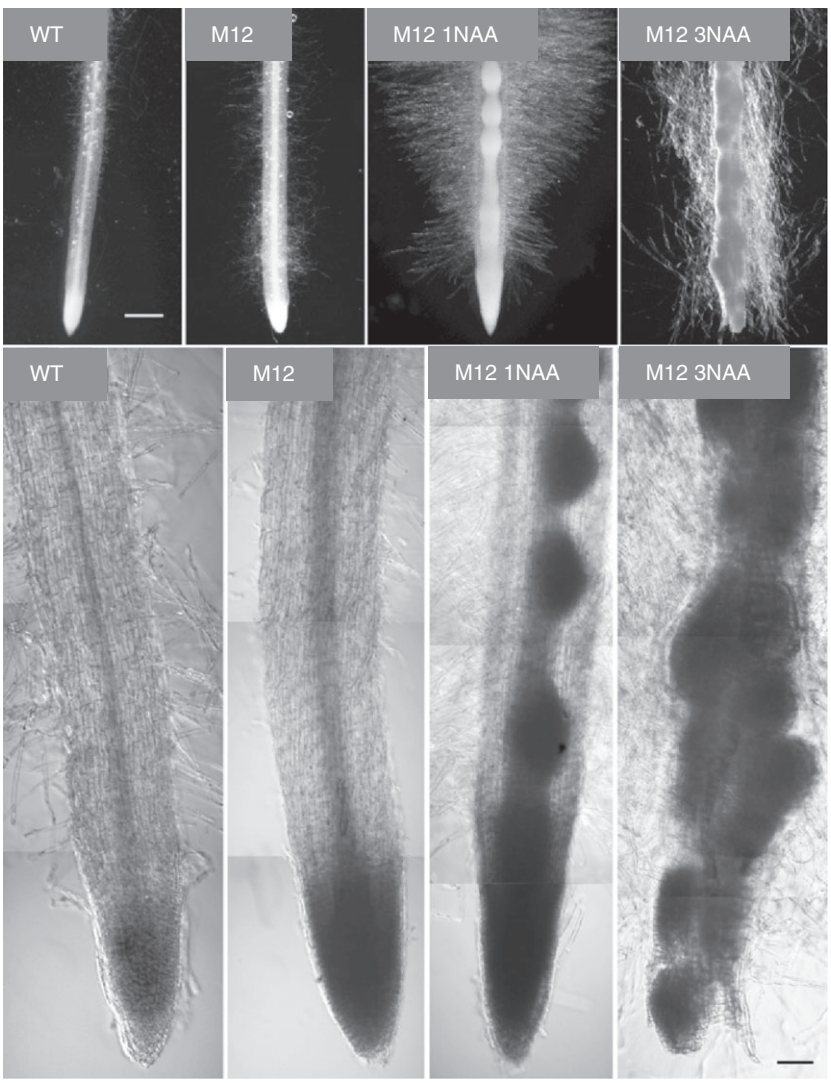

Figure 2 Root phenotypes after different NAA treatments. Root tips from 8-week-old wild-type and M12 tobacco plants grown in hydroponic medium (M12) or hydroponic medium supplemented with NAA. M12 1NAA plants received one 1 mg/L dose of NAA, and M12 3NAA plants received three 1 mg/L doses of NAA (1 per week) over a period of 3 weeks. Scale bars: top row, 100 μm; bottom row 30 μm. NAA, α-naphthaleneacetic acid.

purification strategies, a proteomic analysis was performed to identify the range and relative abundance of rhizosecreted proteins in the optimized hydroponic medium of tobacco plants expressing M12. The protein content of hydroponic medium (week 11), which was shown by Western blot to contain M12 heavy and light chains (Figure 1b,c), was initially concentrated and then resolved by SDS-PAGE (Figure 3a). A range of protein bands between 10 and 150 kDa was observed, and these were subsequently identified by LC/MS/MS.

A total of 104 proteins were identified (Table S1). The proteins identified in each sample were ranked according to the exponentially modified protein abundance index (emPAI). M12 heavy and light chains were the two most abundant individual proteins, accounting for 13% and 14% of the total protein content, respectively (Figure 3b). The other groups of plant proteins identified in hydroponic medium were chitinases, glucosidases, pathogenesis-related proteins and peroxidases. Two proteases were identified in the medium—a subtilisin-like protease and CND41, a chloroplast nucleoid DNA-binding protein, which contains an aspartic protease domain (Murakami *et al.*, 2000)—comprising only 3% of the total protein content.

M12 mAb is readily and efficiently purified from hydroponic medium

A simple, scalable standard operating procedure for purification of rhizosecreted mAbs from hydroponic culture was developed, to identify the potential efficiency of purification.

At 1 L scale, coarse filtration followed by 0.45 μM microfiltration resulted in permeate 98% of total volume and 2% retentate remaining at the end of the filtration. The overall recovery of the mAb at this stage was 99%. During the course of the UF, mAb concentration increased from 40 to 153 μg/mL which was in line with the degree of dewatering achieved. Following Protein A chromatography, overall recovery of the mAb was 84% accounting for all mAb found in all fractions, with 83% recovery of the product present in the two principal product elution fractions.

At 100 mL scale, hydroponic medium was passed directly through a 0.22 μm filter before purification by protein A affinity chromatography. Samples were visualized on a Coomassie-stained SDS-PAGE gel (Figure 4a). The extraction and concentration of the antibody, calculated to be ~85-fold, was achieved at a very high level of purity (Figure 4a, lane 5). Western blot of purified M12 under nonreducing conditions revealed the presence of the fully-assembled antibody (Figure 4b, arrow) and two additional bands at ~125 and ~100 kDa (Figure 4b). This indicates that antibody degradation or assembly intermediate products were co-purified with the whole antibody. The final purified antibody yield from 100 mL medium was 480 μg. The recovery of mAb was 80%.

Rhizosecreted M12 mAb consists mainly of complex glycoforms

The glycosylation pattern of purified rhizosecreted M12 mAb at the first, fourth and seventh week of the harvesting period (weeks 11, 14 and 17) was investigated. The relative results are shown in Table 1. At the first week, M12 purified from hydroponic medium contained mainly complex-type plant *N*-glycans, with three major glycoforms: $M_3Gn_2X_1F_1$, $M_3Gn_3X_1F_1$ and $M_3Gn_4X_1F_1$. This was unchanged at the third week, but the sample harvested at the seventh week did contain small percentages of two additional glycoforms, including a high-mannose type (M_7Gn_2). In comparison, M12 antibody extracted and purified from root tissue at the seventh week (Table 1, final column) contained a similar heterogeneity of glycoforms to the hydroponic medium, but with a greater proportion of M_7Gn_2.

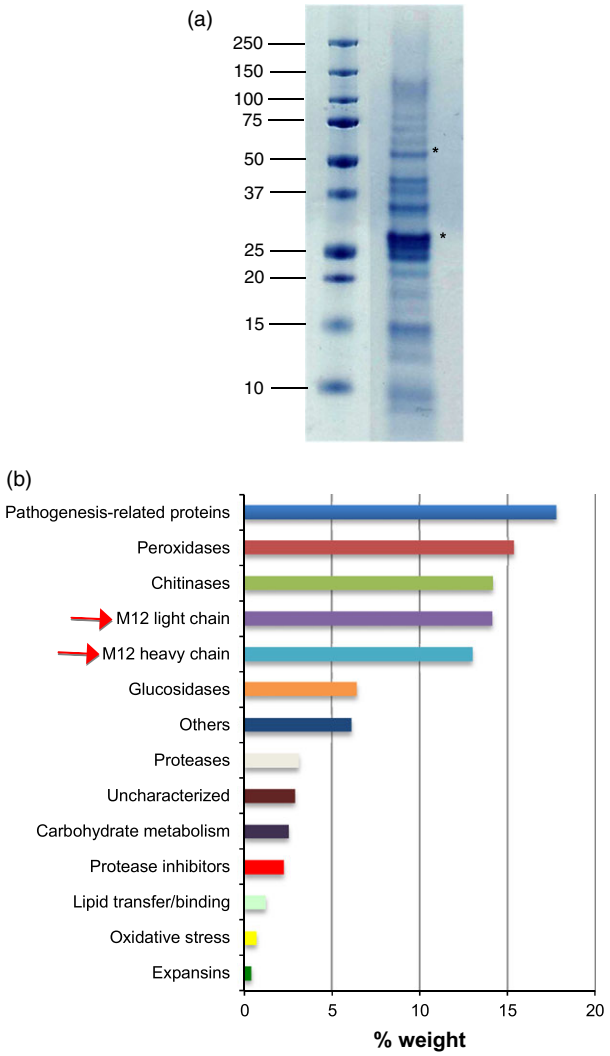

Figure 3 The rhizosecretome. (a) SDS-PAGE separation of hydroponic medium proteins and Coomassie brilliant blue staining for the identification of proteins by LC-ESI-MS/MS. Samples were concentrated 20 times prior to loading. Lane 1—molecular marker (*Mr* is indicated on the left in kDa) and lane 2—hydroponic medium sample (MSN + NAA). (*) bands correspond to M12 heavy and light chain. (b) Categories of rhizosecreted proteins identified by LC/MS. Categories are displayed in percentages calculated according to the emPAI. The PAI was calculated by dividing the number of observed peptides by the number of observable peptides per protein, which was then exponentially modified to make it directly proportional to protein content. NAA, α-naphthaleneacetic acid; emPAI, exponentially modified protein abundance index.

Rhizosecreted M12 binds to vitronectin

In order to verify the M12 binding activity to vitronectin, an ELISA was undertaken with antibody purified from hydroponic medium in comparison with a purified Chinese hamster ovary (CHO)-derived M12 control. Both antibody samples bound the cognate antigen, and no differences were seen between the binding curves (Figure 5).

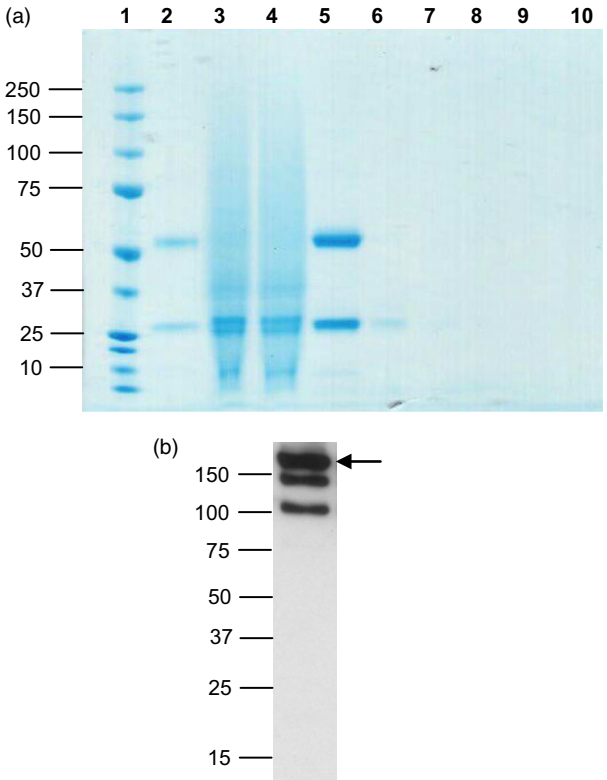

Figure 4 M12 purification from hydroponic medium using protein A chromatography. (a) Coomassie-stained SDS-PAGE under reducing conditions with samples from M12 purification from hydroponic medium. Lane 1—protein marker; lane 2—M12 positive control (1 μg); lane 3—0.22 μm filtered hydroponic medium; lane 4—Protein A column flow through, 5 to 10—elution fractions 1 to 6. (b) Western blot of elution fraction 1 under nonreducing conditions; 1 μL of a 1 : 10 dilution was loaded onto the gel. Arrow indicates fully assembled antibody. *M*r is indicated on the left in kDa.

Table 1 Glycosylation analysis of rhizosecreted M12 mAb at the first, fourth and seventh week of the harvesting phase (weeks 11, 14 and 17 from culture initiation, respectively) and of M12 extracted from root tissue after the final collection. Major glycoforms and their masses are shown; their relative abundance is expressed as a percentage of total glycoforms

Glycoform	Mass (Da)	Medium 1st Rel. abundance (%)	Medium 4th	Medium 7th	Root 7th
High mannose					
M_3Gn_4	2487.99	Not detected	Not detected	3	Not detected
M_7Gn_2	2730.04	Not detected	Not detected	2.2	5.6
Complex					
$M_3Gn_3X_1$	2416.95	4.3	5.4	3	6.2
$M_3Gn_4X_1$	2620.03	9	10.2	5.8	8.2
$M_3Gn_2X_1F_1$	2359.93	15.5	16.4	24.3	25.4
$M_3Gn_3X_1F_1$	2563.01	21.6	24	29.4	18.1
$M_3Gn_4X_1F_1$	2766.09	49.6	44	32.3	36.5

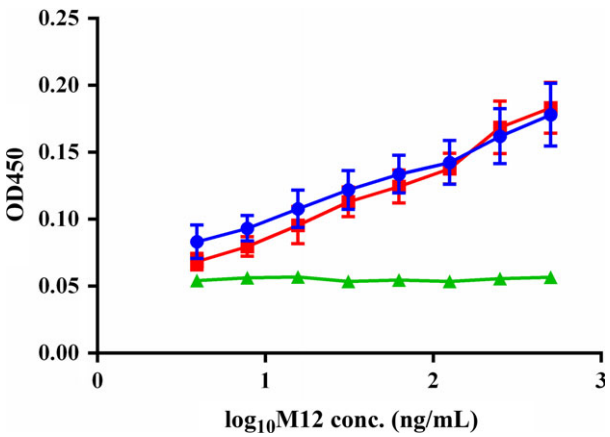

Figure 5 M12 binding activity to vitronectin measured by ELISA. Purified M12 from hydroponic medium (■) was incubated alongside a Chinese hamster ovary-derived purified M12 (●) and PBS (▼) on ELISA plates coated with human vitronectin. Bound M12 was detected with HRP-labelled anti-lambda light chain antiserum. The data represent an average from three independent experiments with three replicates each with standard deviations.

Discussion

Monoclonal antibodies are valuable therapeutic biomolecules often required in large quantities for the treatment of a range of conditions and diseases. To date, mAbs have been produced commercially in mammalian cell culture, but the costs associated with this manufacturing system are high. Plants are an inexpensive alternative to mammalian systems, offering additional advantages such as low risk of contamination with human pathogens. Here, the production of the antivitronectin M12 mAb was explored using a relatively low-tech, very low-cost plant-based expression platform.

An initial assessment of M12 antibody transgenic plants demonstrated a higher level of recombinant antibody rhizosecreted in the hydroponic medium, compared to previous published reports for mAbs. The concentration of the murine Guy's 13 mAb following rhizosecretion into MS medium was originally reported as 0.05 μg/mL (Drake *et al.*, 2009), compared to 0.3 μg/mL for M12 here (data not shown). Guy's 13 yields were improved nearly 50-fold when the auxin NAA was included into the hydroponic medium for 4 weeks prior to harvesting. In the present study, a significant increase in M12 yield was also obtained in MS medium when NAA was used, reaching 7.5 μg/mL at week 8 (data not shown). However, the initial high yields were not maintained and decreased during harvesting phase (week 11 onwards) to ~1 μg/mL, probably due to plant ageing and production of proteases.

Utilization of nitrate-enriched medium (MSN) greatly enhanced M12 rhizosecretion levels well beyond those observed in MS medium, and this enhancement was maintained throughout the harvesting phase. A nitrate enrichment strategy was previously reported in BY-2 cell culture, where the yield of a human IgG_1, 2G12, was improved 10- to 20-fold in medium with increased concentration of nitrate (Holland *et al.*, 2010). A similar effect was observed in hairy root cultures for the same M12 antibody, reaching a maximum secreted concentration of 5.9 μg/mL (Hakkinen *et al.*, 2014). In the present study, a peak M12 concentration of over 45 μg/mL was observed. This figure is higher than any previously reported either for a rhizosecreted antibody or for antibody produced in extracellular medium of BY-

2 tobacco cells or hairy root cultures (De Guzman *et al.*, 2011; Drake *et al.*, 2009; Hakkinen *et al.*, 2014; Xu *et al.*, 2011).

Western blot analysis under nonreducing conditions confirmed the increase of the fully assembled M12 mAb yields obtained when nitrate-enriched medium was used. Under reducing conditions, however, it was shown that the medium boosted yield of mAb heavy chain but not light chain, suggesting that the nitrate effect may not be purely due to an increase in protein synthesis. In our future studies, we shall seek to elucidate the mechanism by which this enhancement of mAb yield in medium with elevated nitrate occurs.

We have previously demonstrated that root hairs can secrete mAb (Drake *et al.*, 2003). In an unpublished study, we also previously observed that NAA increased root hair formation along the length of the root in hydroponic cultures of tobacco (Figure S1). In the present study, this was also observed and characterization of the root tips in the rhizosecretion system by microscopy was undertaken. Here, a clear increase in the amount and length of root hairs on root tips, following application of NAA, was demonstrated. Formation of root hairs is likely to have provided increased surface area for the production and secretion of recombinant antibodies. Another observation was the striking development of lateral root primordia. An important event in the formation of lateral roots is the reorganization of endodermal, cortical and epidermal layers of cells to allow the emergence of lateral root primordia. It has been suggested that these tissues actively participate in the emergence process by expressing cell wall remodelling enzymes to loosen the tissues (Laskowski *et al.*, 2006; Neuteboom *et al.*, 1999; Roberts *et al.*, 2002). Analysis of root transcripts induced by auxin in the tissues surrounding lateral root primordia has identified mRNAs encoding several cell wall remodelling genes, including a subtilisin-like protease and expansins (Swarup *et al.*, 2008), and both were identified in our study, in the rhizosecretome.

LC-ESI-MS/MS analysis was used for determination of the rhizosecretome, to the best of our knowledge the first of its kind for transgenic tobacco plants in hydroponic culture. After identification, the abundance of each protein was calculated using the emPAI (Ishihama *et al.*, 2005), a well-established index which has been used for proteomic studies in diverse organisms (Degnan *et al.*, 2009; Eubel *et al.*, 2008; Morris *et al.*, 2007; Ohta *et al.*, 2010; Rice *et al.*, 2010; Taylor *et al.*, 2011). A total of 104 proteins were identified, and the light and heavy antibody chains were the most abundant proteins. The other main groups of proteins identified—chitinases, endoglucanases, pathogenesis-related proteins and peroxidases—have all been described as stress-related or secreted proteins with defence activity (Bais *et al.*, 2004). They are consistent with proteins identified in an extracellular proteomic analysis of medium in which BY-2 cells (Basu *et al.*, 2006; Okushima *et al.*, 2000) *Arabidopsis thaliana* and *Brassica napus* roots had been cultured (Basu *et al.*, 2006).

Less abundant groups included proteases, protease inhibitors, carbohydrate metabolism, oxidative stress and expansins. One of the proteases identified was a subtilisin-like protease (GenBank accession # GI:148299083—Table S1), first identified in the extracellular medium of *Nicotiana tabacum* BY-2 culture (Navarre *et al.*, 2012). The presence of proteases in the medium is not attractive for the production of antibodies by rhizosecretion; however, we have previously demonstrated that hydroponic culture medium contained significantly lower proteolytic activity than tobacco leaf intercellular fluid (Drake *et al.*, 2009). In addition, the Guy's 13 mAb produced by rhizosecretion in this report was less degraded than the leaf-derived counterpart. In the present study, the amount of proteases in the MSN medium accounted for only 3% of all the proteins.

An important advantage of the rhizosecretion system, in comparison with extraction from vegetative tissues, is the potential for simplified purification. BY-2 cells and hairy roots also have a similar advantage because they are also based on the secretion of the recombinant protein into a simple medium, but both systems require expensive bioreactors for cultivation. BY-2 cell culture also requires more complex purification steps, as the cells retain large volumes of medium and produce high amounts of extracellular polysaccharides resulting in the need for additional pressing and filtration steps (Hellwig *et al.*, 2004).

Protein A is the affinity ligand of choice for commercial mAb purification, and the simplification of downstream processing is likely to revolve around reduction of the preparative steps normally required prior to protein A chromatography. An efficient standard operating procedure was established for hydroponic medium at 1 L scale, that involved coarse filtration, followed by 0.45 μm microfiltration and concentration by ultrafiltration, a process that is broadly in line with standard practice. Here, we also demonstrated that purification of M12 from hydroponic medium by protein A chromatography was possible after a single microfiltration step. Eighty per cent recovery of antibody was achieved in this preliminary study. Thus, antibody purification from hydroponic medium could be achieved using established downstream processing protocols for recombinant mAbs, avoiding all the preliminary processing steps that are required for other plant cell-based or tissue-based expression systems. The quality and functionality of the purified antibody purified from medium was confirmed.

The structure of complex glycans in plants is known to differ from those in mammals (Bosch and Schots, 2010; Faye *et al.*, 2005; Whaley *et al.*, 2011), and it has been suggested that plant glycosylated mAbs may have altered Fc effector functionality (Forthal *et al.*, 2010; Jin *et al.*, 2008). The relevance of these glyco-epitopes in the context of human therapy is still a matter for study (Faye *et al.*, 2005). However, phase I clinical trials in humans with antibodies produced in plants given orally (Ma *et al.*, 1998), subcutaneously (McCormick *et al.*, 2008) or vaginally (www.pharma-planta.org) have shown no immunological responses to plant-specific glycans following antibody administration.

As the antibody chains traffic through the ER and Golgi, the glycosylation process may not proceed to completion; therefore, *N*-glycan structures can be heterogeneous (Bosch and Schots, 2010; Elbers *et al.*, 2001). It has been reported that when recombinant antibodies were purified from tobacco leaf tissue, diverse glycoforms, including high-mannose species, were detected, which was probably due to the extraction of a mixture of fully secreted apoplastic molecules and of those that were still *in transit* through the secretory pathway (Cabanes-Macheteau *et al.*, 1999). Extraction of recombinant proteins from hydroponic medium should in theory result in a more homogeneous mixture as all of the recombinant protein has been secreted. Indeed, we have shown here that rhizosecreted antibody collected at the beginning and middle of the harvesting phase (1st and 4th weeks) consisted only of complex glycoforms. At a later stage (7th week), a very small percentage of high-mannose glycans were detected, but this was less than that found following antibody purification from transgenic root tissue. Heterogeneity of antibody glycoforms has been associated with senescence and

degradation (Elbers *et al.*, 2001; Stevens *et al.*, 2000). The appearance of high-mannose glycans may have been due to cell death caused by a combination of auxin treatment and plant senescence with subsequent release of intracellular antibody.

Glyco-engineering in plants has advanced rapidly, as demonstrated in several published reports (reviewed by Bosch *et al.*, 2013). For example, antibody molecules lacking the plant-specific glycans can be produced in β-1,2-xylosyltransferase and α-1,3-fucosyltransferase RNAi-mediated knocked-down plants (Strasser *et al.*, 2008). Neuraminic acid, a glycan not found in plant proteins, has also been added to a plant-produced protein by co-expression with protein sialylation pathway genes (Jez *et al.*, 2013). In future, the rhizosecretion platform could be used to produce glycoengineered proteins using appropriately modified transgenic plants.

In this study, we have identified a cultivation protocol for transgenic tobacco that produces a high yield of glycosylated, functional monoclonal antibody. Despite the anatomical changes induced in the root system, and the consequent complex mixture of host proteins released into the hydroponic medium, it was demonstrated that antibody extraction and purification was extremely simple, involving a very small number of steps. Establishment of hydroponic plant cultures is simple and economical, using disposable or recyclable vessels, allowing controlled and contained production with inexpensive, defined medium. The system is scalable and amenable to automation, which would only require medium changes on a weekly basis. From the volume of hydroponic medium obtained in these studies and the yield achieved, an estimated 13 mg of functional, purified M12 per batch of 10 plants can be produced over a 15-week production period. Considering the amount of jars that can be stacked per m^3, 1 kg of purified antibody could be produced in a space of 320 m^3 in the same period.

In future studies, it will be interesting to compare rhizosecretion of recombinant proteins in different cultivars of tobacco and other plant species. It will also be important to make this an 'animal-free' production platform by finding an alternative to gelatin, which has been used as a stabilizer for the production of mAbs in the system to date. Removal of gelatin from the cultivation process reduces M12 concentration in medium by ~50% (data not shown). Alternatives to gelatin may include plant proteins, for example derived from soya or, conceivably, animal proteins such as albumin that have been produced in transgenic plants (He *et al.*, 2011).

In conclusion, we propose that the rhizosecretion production platform is a simple, low-tech and economical method to produce high-quality mAbs with a variety of applications.

Experimental procedures

Establishment and maintenance of plant cultures

Transgenic tobacco (*N. tabacum* cv. Petite Havana SR1) plants expressing the heavy and the light chains of the M12 antibody were previously generated by *Agrobacterium*-mediated transformation with the expression vector pTRAkc-MTAD (Raven *et al.*, 2011). Briefly, the heavy and the light chain coding sequences, including leader sequences for secretion to the apoplast, were introduced as separate expression cassettes within a single T-DNA, driven by the double-enhanced Cauliflower mosaic virus (CaMV) 35S promoter. The expression cassettes were flanked by scaffold attachment regions.

Seeds from wild-type tobacco plants (WT) or a M12 homozygous line were surface-sterilized and germinated as previously described (Drake *et al.*, 2003). Seedlings were grown to a stem length of circa 1 cm and transferred individually to glass jars containing liquid Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium under sterile conditions so that the root was immersed in liquid medium and the shoot was supported by a polystyrene platform (Drake *et al.*, 2003). After 6 weeks of establishment in liquid culture, each group of 10 plants received 30 mL per plant of either MS or MSN (MS + 100 mM HNO_3, pH adjusted to 5.8 with NaOH). Both types of media contained 1 mg/L α-naphthalene acetic acid (NAA; Sigma-Aldrich, Poole, UK) and 8 g/L gelatin (Sigma-Aldrich). Further 1 mg/L NAA doses (i.e. 300 μL of medium containing 100 μg/mL NAA) were added to the medium of each individual plant at weeks 7 and 8 (Drake *et al.*, 2009). Medium with NAA was replaced weekly by pipetting under sterile conditions from week 10 until week 18—this period was named the harvesting phase. The yield of M12 in the medium recovered during these weekly collections (~25 mL medium per plant) was determined by ELISA. The medium was not processed in any way prior to the ELISA.

Experimental design and statistical analysis

Plants were assigned randomly to each experimental group, and protein concentration was measured by ELISA. A normality test (Kolmogorov–Smirnov) was performed to judge applicability of parametric statistical analysis, and a Student's *t*-test was performed using the Graphpad Prism™ Software (Version 5.04; GraphPad Software, Inc., San Diego, CA).

ELISA

For detection of M12 antibody, ELISA plates (Nunc Maxisorp®; Nunc, Roskilde, Denmark) were coated with 50 μL/well of 5 μg/mL anti-human IgG antiserum (The Binding Site, Birmingham, UK) diluted in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_3, 1.5 mM KH_2PO_3, pH 7.5), for 2 h at 37 °C. Plates were blocked with 2.5% w/v bovine serum albumin (BSA; Sigma-Aldrich) in PBS with 0.1% v/v Tween 20 (BSA-PBST), 200 μL/well, for 16 h at 4 °C. After washing the plate with distilled water, serial dilutions of samples were added alongside a known concentration of a tobacco leaf-derived purified M12 positive control, and the plate was incubated for 2 h at 37 °C. The plate was then washed three times with distilled water containing 0.1% v/v Tween 20 (Sigma-Aldrich) and incubated for 2 h at 37 °C with BSA-PBST containing 1 μg/mL of sheep HRP-conjugated anti-human-lambda light chain antiserum (The Binding Site), 50 μL/well. Tetramethylbenzidine dihydrochloride peroxidase substrate was added (Sigma-Aldrich), and the reaction was stopped with 2 M H_2SO_4. The optical density was read at 450 nm on a plate reader (Tecan Sunrise™; Tecan, Reading, UK).

Antibody concentration was calculated by interpolation of values obtained from an IgG positive control sample titration curve. Calculation was made using the Graphpad Prism™ (Graphpad Software).

SDS-PAGE

Hydroponic medium samples and the purified leaf-derived positive control were mixed with 5 μL of 4X Tris-Bis loading buffer (Invitrogen, Life Technologies, Paisley, UK) to a final volume of 20 μL. For reducing SDS-PAGE, 10% v/v β-mercaptoethanol was added to the samples. Samples were boiled for 3 min and loaded onto a 4–12% gradient Tris-Bis gel (Invitrogen).

Samples were electrophoresed at 20 mA/gel, and visualization of the separated proteins was performed by Coomassie brilliant blue staining with Instant Blue™ (Expedeon, Harston, UK) or by Western blotting.

Western blot

The gels were blotted onto nitrocellulose membrane using a semi-dry transfer system (Hoefer; GE Healthcare, Little Chalfont, UK) and blocked with 5% w/v nonfat dried milk in TBS/0.1% w/v Tween 20 (milk-TBST) overnight at 4 °C. Detection was after incubation with milk-TBST containing 1 μg/mL of sheep-derived HRP-conjugated anti-human-lambda light chain antiserum (The Binding Site) for 2 h at 37 °C. The blot was developed using the ECL Plus Western blotting Detection System (GE Healthcare).

Analysis of roots by microscopy

M12 and WT plants were cultivated as described above. After 4 weeks of establishment in liquid culture, WT plants and a group of M12 plants received 30 mL of MSN medium with 8 g/L gelatin. Another two group of M12 plants, named M12 1NAA and M12 3NAA, received MSN medium containing 1 mg/L of NAA and 8 g/L gelatin. Further 1 mg/L NAA doses were added to individual plants in the M12 3NAA group at weeks 5 and 6. Medium was replaced at week 7, without NAA for the WT and M12 plants, and with 1 mg/L NAA for the M12 1NAA and 3NAA plants. Roots were excised from plants at week 8 and fixed at 4 °C in a 50% v/v methanol and 10% v/v acetic acid solution. Roots were stored in fixing solution at room temperature.

For light microscopy, fixed roots were cleared using the following procedure. Roots were rinsed in water to remove fixative and then put into 1% sodium dodecyl sulphate with 0.2N NaOH overnight at room temperature. Roots were then rinsed with water and placed into a 25% bleach solution for 5 min. Bleached roots were rinsed in water and placed into a chloral hydrate solution (80 g chloral hydrate in 30 mL water) overnight at room temperature. Chloral hydrate-treated roots were transferred onto microscope slides in a polyvinyl alcohol solution containing 75 g chloral hydrate, 2.5 g polyvinyl alcohol (average molecular weight 85 000–124 000), 7 g glycerol and 25 mL water. Coverslips were added, and mountant was allowed to set for 24 h before observation by darkfield stereomicroscopy, or brightfield compound microscopy.

Determination of total protein profile of hydroponic medium (the rhizosecretome)

Protein separation and digestion

Hydroponic medium from M12 plants grown in MSN with NAA was collected at week 11 and concentrated 20-fold using a Vivaspin 2 column (Sartorius Stedim, Epsom, UK). The concentrated mixture was resolved by reducing SDS-PAGE and the gel stained with Instant Blue™ (Expedeon). Stained protein bands and seemingly blank gel regions in each lane were excised and placed in a microtiter tray. A digesting robotic system (Projester; Genomic Solutions, Huntingdon, UK) was set to first wash and shrink the gel plugs using 100 mM ammonium bicarbonate (Ambic, Witney, UK) and acetonitrile (Sigma-Aldrich). The protein was then reduced with DTT and alkylated using iodoacetic acid. Digestion was performed using a 3% w/v trypsin solution (proteomics grade; Sigma) in 2 mM HCl and 10% v/v acetonitrile. Digested peptides were extracted with 10% v/v formic acid, frozen at −80 °C for 16 h and then freeze dried. The resulting powder was resuspended with 30 μL of a 5% v/v acetonitrile and 0.1% v/v formic acid solution for MS analysis. Gel plugs containing BSA were also digested alongside the experimental samples as a system control.

Liquid chromatography—electrospray ionization—tandem mass spectrometry (LC-ESI/MS/MS) analysis

Peptides were analysed by LC-ESI-MS/MS using a Surveyor LC system and LCQ Deca XP Plus (Thermo Scientific, Northumberland, UK). Peptides were resolved by reverse phase liquid chromatography (180 μM × 15 mm Biobasic column; Thermo Scientific) over a 30 min acetonitrile gradient at a flow rate of 2 μL/min. Peptides were ionized by electrospray ionization, and MS/MS was acquired on ions dependant on their charge state and intensity. Mass accuracy and sensitivity of the MS was confirmed with the direct infusion of glufibrinopeptide (2.5 pmol/μL), and LC/MS/MS performance was assessed with a digest of BSA.

Data processing

The obtained data files (.raw) were converted into mascot generic files using the MassMatrix File Conversion Tool (Version 2.0; http://www.massmatrix.net) for input into the Mascot searching algorithm (Matrix Science). The data files (all merged in one search) were searched against NCBInr (v. 20080527) with plant taxonomy parameter using the following search criteria: tryptic peptides with up to one missed cleavage and carbamidomethylation of cysteines and oxidation of methionines, which were set as variable modifications. In a separate search, individual bands were searched against an in-house database containing sequences of the recombinant proteins. Results were exported to Microsoft Office Excel® software with a score threshold set at 34; scores above this indicate identity or extensive homology.

The proteins identified in each sample were ranked with the protein with the highest mascot score listed first. The emPAI was also calculated as previously described (Ishihama *et al.*, 2005). The percentage of the total protein weight contributed by each individual protein was calculated.

Protein A affinity chromatography

Two approaches were adopted to investigate downstream processing and purification. To process a volume of ~1 L of hydroponic medium, coarse filtration through a 100 μM nylon mesh filter was first used to remove particulates. The hydroponic solution was then applied under vacuum with a flow rate of 100 mL/min. A microfiltration step was then performed using a 0.45 μM pore filter (Millipore Pelicon XL, Millipore, Watford, UK). Ultrafiltration (UF) through a 30 kDa molecular weight cut-off membrane (Millipore Biomax 30) was employed to reduce the volume of the feed prior to protein A affinity chromatography. The concentrated sample was adjusted to pH 7.4 and applied to a packed bed protein A column (GE Healthcare). Elution was with 20 mM potassium citrate buffer (pH 3.6).

To assess the possibility of simplifying the initial preparative steps, the pH of hydroponic medium was adjusted to 7.5 and simply passed through a 0.22 μm filter prior to loading onto a Protein A agarose (Sigma) packed column (BioRad, Hemel Hempstead, UK) with a final bed volume of 500 μL. Samples (100 mL) were loaded using a constant flow rate of 1 mL/min. The column was washed with ≥20 column volumes of PBS, and antibody was eluted with five column volumes of 0.1 M glycine (pH 2.5), in 1 mL fractions. Each fraction was immediately

neutralized by the addition of 50 μL of 1 M Tris–HCl pH 9. For regeneration, the column was washed extensively with 0.1 M glycine (pH 2.5) and then with ≥10 column volumes of PBS.

MALDI analysis of *N*-linked glycosylation

M12 heavy chain *N*-linked glycosylation was purified and sequenced by MALDI-TOF as described previously (Hakkinen *et al.*, 2014).

Vitronectin-binding assay

The ability of rhizosecreted M12 mAb to bind to its cognate antigen was assessed by a vitronectin-binding ELISA. 96-well microtiter plates were coated with human vitronectin (R&D Systems, Abingdon, UK) to a final concentration of 500 ng/mL in PBS, 50 μL/well and incubated at 37 °C for 2 h. Plates were blocked with 2.5% w/v BSA in PBST, 200 μL/well, for 2 h at 37 °C and washed with distilled water containing 0.1% v/v Tween 20. Samples were diluted to the desired concentration in PBS, loaded on the plate in PBS, 50 μL/well and incubated for 2 h at 37 °C. M12 produced in CHO cells (kindly provided by Fraunhofer IME) was used as a reference. Plates were washed and then incubated for 2 h at 37 °C with BSA-PBST containing 1 μg/mL of sheep-derived HRP-conjugated anti-human-lambda light chain antiserum (The Binding Site), 50 μL/well. After washing and drying, assay was developed and the plates were read, as described for ELISA.

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References

Aggarwal, S. (2009) What's fueling the biotech engine–2008. *Nat. Biotechnol.* **27**, 987–993.

Bais, H.P., Park, S.-W., Weir, T.L., Callaway, R.M. and Vivanco, J.M. (2004) How plants communicate using the underground information superhighway. *Trends Plant Sci.* **9**, 26–32.

Basu, U., Francis, J.L., Whittal, R.M., Stephens, J.L., Wang, Y., Zaiane, O.R., Goebel, R., Muench, D.G., Good, A.G. and Taylor, G.J. (2006) Extracellular proteomes of *Arabidopsis thaliana* and *Brassica napus* roots: analysis and comparison by MudPIT and LC-MS/MS. *Plant Soil*, **286**, 357–376.

Bloemendal, H., de Boer, H., Koop, E., van Dongen, A., Goldschmeding, R., Landman, W.M., Logtenberg, T., Gebbink, M.B.G. and Voest, E. (2004) Activated vitronectin as a target for anticancer therapy with human antibodies. *Cancer Immunol. Immunother.* **53**, 799–808.

Borisjuk, N.V., Borisjuk, L.G., Logendra, S., Petersen, F., Gleba, Y. and Raskin, I. (1999) Production of recombinant proteins in plant root exudates. *Nat. Biotechnol.* **17**, 466–469.

Bosch, D., Castilho, A., Loos, A., Schots, A. and Steinkellner, H. (2013) N-glycosylation of plant-produced recombinant proteins. *Curr. Pharm. Des.* **19**, 5503–5512.

Bosch, D. and Schots, A. (2010) Plant glycans: friend or foe in vaccine development? *Expert Rev. Vaccines*, **9**, 835–842.

Cabanes-Macheteau, M., Fitchette-Laine, A.C., Loutelier-Bourhis, C., Lange, C., Vine, N.D., Ma, J.K., Lerouge, P. and Faye, L. (1999) N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. *Glycobiology*, **9**, 365–372.

De Guzman, G., Walmsley, A., Webster, D. and Hamill, J. (2011) Hairy roots cultures from different Solanaceous species have varying capacities to produce *E. coli* B-subunit heat-labile toxin antigen. *Biotechnol. Lett.* **33**, 2495–2502.

De Muynck, B., Navarre, C. and Boutry, M. (2010) Production of antibodies in plants: status after twenty years. *Plant Biotechnol. J.* **8**, 529–563.

Degnan, P.H., Yu, Y., Sisneros, N., Wing, R.A. and Moran, N.A. (2009) *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. *Proc. Natl Acad. Sci. USA*, **106**, 9063–9068.

Drake, P.M.W., Barbi, T., Sexton, A., McGowan, E., Stadlmann, J., Navarre, C., Paul, M.J. and Ma, J.K.C. (2009) Development of rhizosecretion as a production system for recombinant proteins from hydroponic cultivated tobacco. *FASEB J.* **23**, 3581–3589.

Drake, P.M.W., Chargelegue, D.M., Vine, N.D., van Dolleweerd, C.J., Obregon, P. and Ma, J.K.C. (2003) Rhizosecretion of a monoclonal antibody protein complex from transgenic tobacco roots. *Plant Mol. Biol.* **52**, 233–241.

Edwards, J.C.W., Szczepański, L., Szechiński, J., Filipowicz-Sosnowska, A., Emery, P., Close, D.R., Stevens, R.M. and Shaw, T. (2004) Efficacy of B-cell–targeted therapy with rituximab in patients with rheumatoid arthritis. *N. Engl. J. Med.* **350**, 2572–2581.

Elbers, I.J., Stoopen, G.M., Bakker, H., Stevens, L.H., Bardor, M., Molthoff, J.W., Jordi, W.J., Bosch, D. and Lommen, A. (2001) Influence of growth conditions and developmental stage on N-glycan heterogeneity of transgenic immunoglobulin G and endogenous proteins in tobacco leaves. *Plant Physiol.* **126**, 1314–1322.

Eubel, H., Meyer, E.H., Taylor, N.L., Bussell, J.D., O'Toole, N., Heazlewood, J.L., Castleden, I., Small, I.D., Smith, S.M. and Millar, A.H. (2008) Novel proteins, putative membrane transporters, and an integrated metabolic network are revealed by quantitative proteomic analysis of Arabidopsis cell culture peroxisomes. *Plant Physiol.* **148**, 1809–1829.

Faye, L., Boulaflous, A., Benchabane, M., Gomord, V. and Michaud, D. (2005) Protein modifications in the plant secretory pathway: current status and practical implications in molecular pharming. *Vaccine*, **23**, 1770–1778.

Forthal, D.N., Gach, J.S., Landucci, G., Jez, J., Strasser, R., Kunert, R. and Steinkellner, H. (2010) Fc-glycosylation influences Fcgamma receptor binding and cell-mediated anti-HIV activity of monoclonal antibody 2G12. *J. Immunol.* **185**, 6876–6882.

Hakkinen, S.T., Raven, N., Henquet, M., Laukkanen, M.L., Anderlei, T., Pitkanen, J.P., Twyman, R.M., Bosch, D., Oksman-Caldentey, K.M., Schillberg, S. and Ritala, A. (2014) Molecular farming in tobacco hairy roots by triggering the secretion of a pharmaceutical antibody. *Biotechnol. Bioeng.* **111**, 336–346.

He, Y., Ning, T., Xie, T., Qiu, Q., Zhang, L., Sun, Y., Jiang, D., Fu, K., Yin, F., Zhang, W., Shen, L., Wang, H., Li, J., Lin, Q., Sun, Y., Li, H., Zhu, Y. and Yang, D. (2011) Large-scale production of functional human serum albumin from transgenic rice seeds. *Proc. Natl Acad. Sci. USA*, **108**, 19078–19083.

Hellwig, S., Drossard, J., Twyman, R.M. and Fischer, R. (2004) Plant cell cultures for the production of recombinant proteins. *Nat. Biotechnol.* **22**, 1415–1422.

Hiatt, A., Cafferkey, R. and Bowdish, K. (1989) Production of antibodies in transgenic plants. *Nature*, **342**, 76–78.

Holland, T., Sack, M., Rademacher, T., Schmale, K., Altmann, F., Stadlmann, J., Fischer, R. and Hellwig, S. (2010) Optimal nitrogen supply as a key to increased and sustained production of a monoclonal full-size antibody in BY-2 suspension culture. *Biotechnol. Bioeng.* **107**, 278–289.

Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J. and Mann, M. (2005) Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell Proteomics*, **4**, 1265–1272.

Jez, J., Castilho, A., Grass, J., Vorauer-Uhl, K., Sterovsky, T., Altmann, F. and Steinkellner, H. (2013) Expression of functionally active sialylated human erythropoietin in plants. *Biotechnol. J.* **8**, 371–382.

Jin, C., Altmann, F., Strasser, R., Mach, L., Schahs, M., Kunert, R., Rademacher, T., Glossl, J. and Steinkellner, H. (2008) A plant-derived human monoclonal antibody induces an anti-carbohydrate immune response in rabbits. *Glycobiology*, **18**, 235–241.

Kirchhoff, J., Raven, N., Boes, A., Roberts, J.L., Russell, S., Treffenfeldt, W., Fischer, R., Schinkel, H., Schiermeyer, A. and Schillberg, S. (2012) Monoclonal

tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. *Plant Biotechnol. J.* **10**, 936–944.

Laskowski, M., Biller, S., Stanley, K., Kajstura, T. and Prusty, R. (2006) Expression profiling of auxin-treated Arabidopsis roots: toward a molecular analysis of lateral root emergence. *Plant Cell Physiol.* **47**, 788–792.

Ma, J.K., Hikmat, B.Y., Wycoff, K., Vine, N.D., Chargelegue, D., Yu, L., Hein, M.B. and Lehner, T. (1998) Characterization of a recombinant plant monoclonal secretory antibody and preventive immunotherapy in humans. *Nat. Med.* **4**, 601–606.

McCormick, A.A., Reddy, S., Reinl, S.J., Cameron, T.I., Czerwinkski, D.K., Vojdani, F., Hanley, K.M., Garger, S.J., White, E.L., Novak, J., Barrett, J., Holtz, R.B., Tuse, D. and Levy, R. (2008) Plant-produced idiotype vaccines for the treatment of non-Hodgkin's lymphoma: safety and immunogenicity in a phase I clinical study. *Proc. Natl Acad. Sci. USA*, **105**, 10131–10136.

Morris, R.M., Fung, J.M., Rahm, B.G., Zhang, S., Freedman, D.L., Zinder, S.H. and Richardson, R.E. (2007) Comparative proteomics of Dehalococcoides spp. reveals strain-specific peptides associated with activity. *Appl. Environ. Microbiol.* **73**, 320–326.

Murakami, S., Kondo, Y., Nakano, T. and Sato, F. (2000) Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells. *FEBS Lett.* **468**, 15–18.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Navarre, C., De Muynck, B., Alves, G., Vertommen, D., Magy, B. and Boutry, M. (2012) Identification, gene cloning and expression of serine proteases in the extracellular medium of *Nicotiana tabacum* cells. *Plant Cell Rep.* **31**, 1959–1968.

Neuteboom, L.W., Veth-Tello, L.M., Clijdesdale, O.R., Hooykaas, P.J.J. and van der Zaal, B.J. (1999) A novel subtilisin-like protease gene from *Arabidopsis thaliana* is expressed at sites of lateral root emergence. *DNA Res.* **6**, 13–19.

Ohta, S., Bukowski-Wills, J.C., Sanchez-Pulido, L., Alves, F.D., Wood, L., Chen, Z.A., Platani, M., Fischer, L., Hudson, D.F., Ponting, C.P., Fukagawa, T., Earnshaw, W.C. and Rappsilber, J. (2010) The protein composition of mitotic chromosomes determined using multiclassifier combinatorial proteomics. *Cell*, **142**, 810–821.

Okushima, Y., Koizumi, N., Kusano, T. and Sano, H. (2000) Secreted proteins of tobacco cultured BY2 cells: identification of a new member of pathogenesis-related proteins. *Plant Mol. Biol.* **42**, 479–488.

Raven, N., Schillberg, S., Kirchhoff, J., Brändl, I.J., Imseng, N. and Eibl, R. (2011) Growth of BY-2 suspension cells and plantibody production in single-use bioreactors. In *Single-Use Technology in Biopharmaceutical Manufacture* (Eibl, R. and Eibl, D., eds), pp. 251–261. Hoboken, New Jersey, USA: John Wiley & Sons.

Rice, R.H., Xia, Y.J., Alvarado, R.J. and Phinney, B.S. (2010) Proteomic analysis of human nail plate. *J. Proteome Res.* **9**, 6752–6758.

Roberts, J.A., Elliott, K.A. and Gonzalez-Carranza, Z.H. (2002) Abscission, dehiscence, and other cell separation processes. *Annu. Rev. Plant Biol.* **53**, 131–158.

Saini, K.S., Azim, H.A. Jr, Cocorocchio, E., Vanazzi, A., Saini, M.L., Raviele, P.R., Pruneri, G. and Peccatori, F.A. (2011) Rituximab in Hodgkin lymphoma: is the target always a hit? *Cancer Treat. Rev.* **37**, 385–390.

Shukla, A.A. and Thömmes, J. (2010) Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends Biotechnol.* **28**, 253–261.

Stevens, L.H., Stoopen, G.M., Elbers, I.J., Molthoff, J.W., Bakker, H.A., Lommen, A., Bosch, D. and Jordi, W. (2000) Effect of climate conditions and plant developmental stage on the stability of antibodies expressed in transgenic tobacco. *Plant Physiol.* **124**, 173–182.

Strasser, R., Stadlmann, J., Schahs, M., Stiegler, G., Quendler, H., Mach, L., Glossl, J., Weterings, K., Pabst, M. and Steinkellner, H. (2008) Generation of glyco-engineered *Nicotiana benthamiana* for the production of monoclonal antibodies with a homogeneous human-like N-glycan structure. *Plant Biotechnol. J.* **6**, 392–402.

Swarup, K., Benkova, E., Swarup, R., Casimiro, I., Peret, B., Yang, Y., Parry, G., Nielsen, E., De Smet, I., Vanneste, S., Levesque, M.P., Carrier, D., James, N., Calvo, V., Ljung, K., Kramer, E., Roberts, R., Graham, N., Marillonnet, S., Patel, K., Jones, J.D.G., Taylor, C.G., Schachtman, D.P., May, S., Sandberg, G., Benfey, P., Friml, J., Kerr, I., Beeckman, T., Laplaze, L. and Bennett, M.J. (2008) The auxin influx carrier LAX3 promotes lateral root emergence. *Nat. Cell Biol.* **10**, 946–954.

Taylor, N.L., Heazlewood, J.L. and Millar, A.H. (2011) The *Arabidopsis thaliana* 2-D gel mitochondrial proteome: refining the value of reference maps for assessing protein abundance, contaminants and post-translational modifications. *Proteomics*, **11**, 1720–1733.

Whaley, K.J., Hiatt, A. and Zeitlin, L. (2011) Emerging antibody products and Nicotiana manufacturing. *Hum. Vaccin.* **7**, 349–356.

Xu, J., Ge, X. and Dolan, M.C. (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol. Adv.* **29**, 278–299.

Yusibov, V., Streatfield, S.J. and Kushnir, N. (2011) Clinical development of plant-produced recombinant pharmaceuticals: vaccines, antibodies and beyond. *Hum. Vaccin.* **7**, 313–321.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Root phenotypes of wild-type tobacco plants cultivated in hydroponic MS medium without NAA (No NAA) and after receiving three doses of NAA (3NAA).

Table S1 Proteins identified by LC/MS/MS in hydroponic medium in which M12 expressing tobacco plants had been cultivated.