Production of a tumour-targeting antibody with a human compatible glycosylation profile in *N. benthamiana* hairy root cultures

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Dear Editor,
attached please find the revised version of the manuscript entitled: 'Production of a tumour-targeting antibody with a human-type compatible glycosylation profile in N. benthamiana hairy root cultures'.

We appreciate precious comments provided. Following reviewers' suggestions, we have carefully revised the manuscript by adding the requested major changes and experiments (New Figure 2; Figure 3E and Figure 5).
A detailed response to all points raised by the Reviewers is given below.

Review Editor
The manuscript has been reviewed by three experts in the field, and several concerns have been raised.
A serious concern refers for example to the comparison of two different plant species (benthamiana and tabacum), one of them containing the deletion ΔXTFT. This setup does not allow for meaningful conclusions.

We have revised the paper avoiding direct comparison between the two plant species. Text was changed to emphasize the main aim of this work, which is the use of multiple-gene co-transformation in glyco-engineered N. benthamiana to obtain immunotherapeutic antibodies having a human-compatible glycosylation profile.

Also, statements about efficient expression, secretion and accumulation should be well separated and carefully used to interpret the experimental data.

We have carefully revised these statements using the appropriate terms to interpret the experimental data.

An elaboration of the function and intended application of the antibody would help to demonstrate the applied relevance of the study.

We have carefully described the tumour-targeting antibody mAb H10 to highlight the relevance of this study in the production of immunotherapeutic antibodies.

Nevertheless, the study is interesting and provides valuable information. If the points raised by the reviewers can be addressed, it could make a valuable contribution to the field.

Reviewer: 1

The work described by Lonoce et al. describes the production of a tumor targeting monoclonal antibody H10 in hairy roots derived from H10 transgenic N. tabacum or ?XTFT N. benthamiana plants. The presented technical results were completely expected and thus little if any new findings are presented. The work is technical and does not address any novel aspect. Parts of the experimental data are not of good quality and the authors do not critically discuss their results. Prior publications e.g. on the co-transformation efficiency via hairy root induction have been ignored. Scientific terms are used sloppy, inaccurately or with inappropriate units (e.g. growth rate), and there are cases where ill-defined terms have been introduced (e.g. expression efficiency). The figures and the text descriptions are not always in agreement (e.g. apparent sizes of antibody bands). While the manuscript contains several redundant (i.e. with no or little additional information) figures of western blots, whereas the primary data for the analysis of the antibody N-glycans is not presented. In light of the little information and the many shortcomings of the manuscript a publication in the Biotechnology Journal is not warranted.

Author response 1:

We appreciated the Reviewer's criticisms that helped us to improve the manuscript with the aim of emphasizing the novelty of this study, i.e. the use of a rapid multiple-gene co-transformation in glyco-engineered N. benthamiana to obtain immunotherapeutic antibodies having a human compatible glycosylation profile. In this context, we do not believe that this work is technical and does not address any novel aspect; it simply describes the development/application of various technological approaches in engineered plants to generate novel pharmaceutical products useful for human health and having improved characteristics. To emphasize this point, we have revised the whole text eliminating all inappropriate terms and we have introduced a discussion on previous communications regarding the co-transformation efficiency via hairy root induction. As requested, novel experiments have been performed to improve the quality of the data and to eliminate redundant figures. In particular, novel results on Western blot analysis using gradient SDS-PAGE gels (amended Figure 2) and on quantification of intracellular antibodies (amended Figure 3E) have been presented in this revised study to address the comments of Reviewer 2 and 3, respectively. Similarly, antibody purification from root biomass has been conducted; results are now reported in amended Figure 4C. The primary data (MS spectra) for N-glycan analysis are shown in Figure 5A.

There are several issues that raise additional questions and concerns:

• The hairy root fresh weights are not expressed as gram fresh weight per liter of culture volume.

Author response 2:
Along with the experimental result obtained in 40 ml of culture volume, now we have also added the root fresh weight parameter (expressed as FW per liter of culture volume) in the Results and Discussion section.
'Starting from an inoculum size of ~0.2 g in a 40 ml culture, the FW biomass value of ΔXTFT N. benthamiana HRs was 11 g (275 g/L) after 25 days, while in the case of N. tabacum it was lower (100 g/L) but comparable to that already described by other groups [11].'

• Hairy root cultures have been claimed to be an advantageous biotechnological manufacturing platform with several advantages, including fast biomass accumulation and manufacturing scalability. This directly calls for a comparison of the antibody accumulation and productivity obtained in the hairy root suspension cultures to other systems (transgenic plants, infiltrated plants/leaves, suspension cell cultures). This was not done by the authors, possibly due to the unfavourable outcome.

Author response 3:
At the end of the discussion section, we already highlighted the main disadvantage of using HRs in terms of antibody accumulation and productivity, with respect to other systems such as transient expression strategies based on agroinfiltration. In this context, we also proposed possible future improvements to enhance protein production and secretion.

These concepts have now been strengthened in the Introduction section:
'Despite recent improvements, antibody yields obtained in HRs do not meet those reported for other plant expression systems, such as transgenic plants and agrobacterium-based transient expression systems. In fact, by agroinfiltration of N. benthamiana leaves using plant virus derived expression vectors, up to 500mg/Kg FW of the human tumour-specific mAb A5 were obtained [12].'

and in the Discussion section, also highlighting the advantages of the use of HRs for heterologous protein production:

'As expected, mAb H10 purification yields in HRs are sensibly lower compared to those obtained by using transient expression systems based on N. benthamiana agroinfiltration [27]; the latter yielded up to 75 mg/Kg FW of the antibody.

Nevertheless, HR cultures still represent and advantageous system in terms of manufacturing and downstream processing for the contained root growth in sterile conditions and the secretion of the recombinant protein in the culture medium.'

• The hairy-root suspension cultures exhibit a slow growth and a pronounced lag phase of two weeks. The MS medium was supplemented with 10% sucrose, which raises concerns about the osmolarity of the growth medium.

Author response 4:
We apologize for the typing error that occurred during manuscript preparation. The medium used was supplemented with 3% sucrose, as described in the Materials and methods section (MS medium with 30 g/L sucrose). We have now modified the text accordingly. Under our culture conditions, we have always observed this slow growth lag phase in all clones in the first weeks, when an initial inoculum of 0.2 g in 40 ml was used. It must be also noted that the initial inoculum was taken from roots growing on a solid medium.

• The yields are far from convincing, and the rationale for selection of the mab H10 has not been presented. In fact, little if any information on this antibody is present and hence one must conclude that mAb H10 represents a model protein at best. If so, why didn't the authors select an antibody that is expressed at higher levels and is not degraded?

Author response 5:

We have now described the rationale for the selection of mAb H10, providing information about this antibody and the importance of anti-TenascinC mAbs in the treatment of several tumours. We also highlighted the importance of producing glyco-modified immunotherapeutic antibodies with enhanced biological activity.

Introduction section:
'The goal of the present work was to produce the tumour-targeting antibody mAbH10 in HR cultures derived from glyco-engineered ΔXTFT N. benthamiana, with the aim of improving the quality of the final product. This human IgG1 (λ), which derived from a selected single-chain variable fragment (scFv), is directed against the C domain of the large isoform of the tumour-associated antigen TNC. TNC has been associated with a variety of tumours including breast, squamous cells, lung and prostate carcinomas, melanoma and malignant glioma [12, 13]. Anti-TNC antibodies have been recently used with success for the therapy of patients with acute myeloid leukemia and metastatic breast cancer [14,15].'

Discussion section:
'Plant-derived mAbs differ from their mammalian counterpart in their Fc N-glycan composition. Non-human glycosylation may represent a limitation in their therapeutic application since plant typical complex glycan structures moieties may cause immunogenic reactions [35,36] and may also interfere with Fcγ receptors binding [19, 37]. This has a negative impact on immunotherapeutic mAbs, since their effector functions, such as antibody dependent cell cytotoxicity (ADCC), rely on the interaction with Fcγ receptors [39]. Recent studies demonstrated that antibodies lacking plant specific xylose and fucose have enhanced binding to Fcγ receptors and improved biological activity [40].'

• The comparison between N.tabacum and N.benthamiana is of little use only and provides no additional insight. The data is too limited to allow for a thorough comparison of the two different approaches and the experimental design is severely limited by the choice of a bad transgenic line in the first place. Notably, efficient co-transformation has been reported previously by Huang et al. (Appl Microbiol Biotechnol. 2013 Oct;97(19):8637-47), neither was this reference included, nor were the results discussed in light of these previous findings.

Author response 6:
We have now modified the text in the 'Discussion' section as reported below, pointing out that: 'This result is merely indicative, due to the low number of HR clones screened, and does not allow for a thorough comparison of the two different approaches …'

Moreover, we discussed the results obtained by Huang and colleagues.

'We showed that the percentage of clones expressing detectable amounts of recombinant protein was higher in N. tabacum (60% of the clones were positive in Western blot analysis) than in co-transformed ΔXTFT N. benthamiana (20% of the clones were positive). This result is merely indicative, due to the low number of HR clones screened, and does not allow for a thorough comparison of the two different approaches. It simply demonstrates that co-infection of leaf discs with a mix of A. rhizogenes carrying antibody HC and LC coding sequences, although at a lower efficiency, can be used for the rapid establishment (approx. 2 month) of stable HR clones expressing functional mAbs. A previous work based on the same multiple gene co-trasformation strategy, i.e. transgenes located in different plasmids and transformed in different batches of A. rhizogenes, was used to co-express GUS and GFP in N. tabacum hairy roots. In this case a co-transformation efficiency of 65% was obtained, while a higher value (82%) was achieved when the reporter genes were inserted in two different T-DNAs within the same binary vector [4].'

• Huge variations in transgene expression (due to positional effects) but also in growth and morphology of hairy-root suspensions have been reported previously. A comparison based on a low number of cell lines is thus meaningless. Conclusions regarding the "antibody secretory efficiency" cannot be made.

Author response 7:
In the light of what has been described in literature we have now commented on the variations in both transgene expression and growth observed among the different hairy root clones. Based on these evidences, we avoided drawing any conclusion from the observed different growth and antibody yields in the two HR clones expressing mAb H10.

Discussion section:
'It has been previously demonstrated that transgene expression in HRs is strongly influenced by positional effects (genes are randomly inserted in different regions of the genomic DNA), affecting not only protein expression but also hairy root growth and morphology [4,28]. This accounts for the strong variation in protein expression and growth that we and other groups observed among individual transgenic hairy roots expressing monoclonal antibodies in different plant species [8,11].'

For this reason the comparison in terms of root growth between the two clones does not allow to draw any conclusion.

We have also removed the sentence on "antibody secretory efficiency" and performed new experiments, as requested by the 3rd Reviewer, for evaluating the amount of intracellular antibody in the root biomass (Figure 3E). This allowed us to calculate the 'Total antibody' in the HR cultures as the total amount of antibody (secreted antibody + intracellular antibody) in the system as already described in a previous work by Wongsamuth and Doran (1996) Biotechnology and Bioengineering, 54. 401-415, taking into account the amount of biomass present and the residual volume of each HR culture (initial medium volume of 40 mL).

• There is a profound difference in an agent that stabilizes a protein and an agent that improves/stabilizes the accumulation of a protein.

Author response 8:
We have rephrased the sentence correctly.

'An optimised protocol was recently developed leading to high yield accumulation of a mAb in N. tabacum HRs, which is based on KNO3 and NAA to induce rhizosecretion and PVP as protein stabilizing agent [11].'

• Instead of speculating about the effect of NAA and whether the "cellular modifications could be significantly involved in the increased" protein accumulation in the culture supernatant the authors could have easily tested this by culturing the hairy roots in media supplemented with a single additive only. Note that the authors have no data or rationale to make conclusions from the protein accumulation in the culture supernatant to "protein synthesis".

Author response 9:
We agree, and removed this sentence.
Deleted: 'On this basis, it is conceivable to speculate that these cellular modifications could be significantly involved in the increased protein synthesis and secretion in roots.'
The development of an optimized protocol for the induction of antibody secretion in HRs was already reported from other groups (Hakkinen et al. 2014; Sharp et al. 2001). In the present manuscript, we were not interested in characterizing the effect of different inducers.

• Several statements are not supported by experimental data and the declaration of "data not shown" has not been provided. Additional conclusions drawn from such statements should not be drawn.

Author response 10:
We have removed statements not supported by experimental data.

Reviewer: 2

The results presented are interesting and of value, but essential improvements have to be done according experimental data as well as according the presentation of the work.

To "Introduction"
In the introduction, any description of the monoclonal antibody H10 is completely lacking. Here, the basic paper about the expression of this antibody (Villani et al., reference 17) should be cited more at the beginning of the introduction. Moreover, essential information about this antibody (designed from a scFv; IgG1, kappa; antigen human C domain of TN-C etc.) should be given. The construct used should be explained.

Author response 1:
In the revised version, we have described the antibody mAb H10 at the beginning of the introduction section, providing the essential information requested.

'The goal of the present work was to produce the tumour-targeting antibody mAb H10 in HR cultures derived from glyco-engineered □XTFT N. benthamiana with the aim of improving the quality of the final product. This human IgG1 (□), which derived from a selected single-chain variable fragment (scFv) is directed against the C domain of the large isoform of the tumour-associated antigen. TNC has been associated with a variety of tumours including breast, squamous cells, lung and prostate carcinomas, melanoma and malignant glioma [12, 13]. Anti-TNC antibodies have been recently used with success for the therapy of patients with acute myeloid leukemia and metastatic breast cancer [14, 15]'

We have also added information on the construct used:
'In this study, we have monitored different approaches for the generation of HRs producing human mAb H10. To this purpose, we used two plant binary expression

vectors bearing heavy chain (HC) and light chain (LC) coding sequences under the control of the cauliflower mosaic virus promoter (35S) and the tobacco mosaic virus (TMV) translational enhancer (Ω) with the nopaline synthase terminator sequence at the 3′ end. A signal peptide sequence deriving from an embryonic mouse immunoglobulin HC coding gene was used to direct the recombinant antibody to the secretory pathway [13].'

To "Materials and Methods":
The description of the ELISA is done in the results section and should be shown in Materials and Methods.

Author response 2:
Detailed description of the ELISA protocol has been moved in the 'Materials and Methods' section 2.5 ELISA assays, as requested.

To "Results":
According the N. benthamiana plants, co-infection with LC and HC constructs in relevant Agrobacterium strains was described. Was it done in the same way with N. tabacum?

Author response 3:
The transgenic N. tabacum line expressing mAbH10 (described in Villani et al 2009) was infected with wild type Agrobacterium rhizogenes to obtain HRs. This is described in detail in subsection '2.1 Hairy roots generation' of the Materials and Methods section:
'N. tabacum cv. Petite Havana SR1 HRs were prepared from a T4 generation transgenic plant line (line 7.6) expressing mAb H10 HC and LC, which was previously obtained by Villani and colleagues [13]. Briefly, wild-type A. rhizogenes A4 was grown ……'

The Western blots presented in figures 2 and S3 are not of sufficient quality for publication. The separation of complete antibodies at non-reducing conditions should be done i.e. by use of a 5-15% gradient gel. Then, clear separation of both heavy chains at reducing conditions and complete antibodies at non-reducing conditions could be achieved on the same gel. Even in the light of the discussion of glycosylation analysis results, the bands at high molecular weight are of interest.

Author response 4:
We have repeated the analysis of the two relevant clones (N. tabacum Clone 6 and N. benthamiana clone 15) using 4-15% (Mini-PROTEAN® TGX™ Precast Gels, BIORAD, USA) to better separate the antibody bands (Figure 2). The results show a much better separation of antibody bands, which permits a better interpretation of the results. In particular, both anti HC and anti LC Western blots show the presence of a band at about 150 kDa corresponding to the complete antibody in both clones, thus confirming the observed homogeneous glycosylation.

The corresponding results have now been described as follows:
'Two clones were selected (clone 15 for N. benthamiana and clone 6 for N. tabacum) and analysed; they were resolved on a gradient gel under reducing and non-reducing conditions and then subjected to Western blot analysis using both anti-HC and LC antibodies (Fig. 2). In the anti-HC blotting under non-reducing conditions, both N tabacum and ΔXTFT N. benthamiana clones showed a similar band pattern, with the expected band migrating at high molecular mass (□150 kDa) corresponding to the assembled mAb. Additional lower bands were also observed: an intense band (at about 100 kDa) and very faint ones (at about 135, 75 and 50 kDa) that probably relate to antibody assembly intermediates (HC dimers/monomers) or putative degradation fragments. In the corresponding analysis under reducing conditions, a 50 kDa band associated with intact HC was visible as well as a faint band at about 25 kDa indicating specific degradation of the heavy chain (Fig. 2A). The anti-LC blot under non-reducing conditions (Fig. 2B) showed a 150 kDa band in both clones, together with additional lower

faint bands at about 135, 100 and 75 kDa, which were already observed in the anti-HC blot. A very strong band at about 45 kDa was visible, which also occurred in the control. This may correspond to the homodimeric form of free LC. Under reducing conditions, the 25 kDa band corresponding to the LC was detected in both clones.'

In figure 1, the HC-band at reducing conditions is obviously at 60 kDa, and not at 50 kDa as described in the text. In figure 3 of Villani et al. 2009, it is at 50 kDa. The purified antibody's HC are then at 55 kDa (Figure S4). There is obviously a problem with the markers. This should be clarified.

Author response 5:
The reviewer refers here to Figure 2. The novel analysis (performed on gradient gel) showed the expected molecular mass value for the HC (about 50 kDa). For this analysis, we used a new broad range molecular mass marker that allows a better evaluation of the molecular size values, especially at high molecular masses. In the previous figure, the HC-band at apparently 60 kDa derived from the fact that a non-optimal separation of lower molecular mass bands occurred in the 12% SDS-PAGE gel. The same applies for the Figure S4, where the 45 and 55 kDa bands of the marker are very close to each other.

The FW data presented in Figure 3A do not significantly show a loss in FW (mentioned at Page 12), if one looks to the standard deviations.

Author response 6:
We agree that there is no significant loss of FW; accordingly, we removed the sentence
'A net loss of FW was ……'

The ca. 45 kDa band at non-reducing conditions seems to me a kind of Bence-Jones protein, a dimer formed by 2 kappa chains, because it is obviously cleaved at reducing conditions. Dimers formed by expression of nanobody-C-Kappa fusion proteins in plants are described i.e. by Giersberg et al.

Author response 7:
We have now discussed this point in the 'Discussion' section, also in the light of the comments by Reviewer 3. We have also added the suggested reference.

'Identical band patterns for the two HR clones were also observed in Western blot analysis of root extracts under non-reducing conditions using an anti-LC antibody (Fig. 2). A strong signal at about 45 kDa was obtained suggestive for the formation of LC homodimers. The occurance of LC dimers in N. benthamiana had been already described in a recent work in which dimerized monoclonal camelidae variable heavy chain domain antibodies (VHH) ……..'

In the discussion, I miss a few sentences according the planned use of this antibody. Glycosylation, i.e. is of high importance for antibody dependend cellular cytotoxicity. If this antibody is planned to use exclusively for tumor labelling/detection, then this is not of importance.

Author response 8:
We have better clarified the intended use of mAb H10 in immunotherapy and the importance of glyco-modification in enhancing antibody effector functions such as ADCC.
We added in the discussion section the following sentences:
'Plant-derived mAbs differ from their mammalian counterpart in their Fc N-glycan composition. Non-human glycosylation may represent a limitation in their therapeutic application since plant typical complex glycan structures moieties may cause immunogenic reactions [35,36] and may also interfere with Fcγ receptors binding [19, 37]. This has a negative impact on immunotherapeutic mAbs, since their effector functions, such as antibody dependent cell cytotoxicity (ADCC), rely on the interaction with Fcγ receptors [39]. Recent

studies demonstrated that antibodies lacking plant specific xylose and fucose have enhanced binding to Fcγ receptors and improved biological activity [40].'

Reviewer: 3

In their Manuscript biot.201500628 "Production of a tumour-targeting antibody with a human-type glycosylation profile in N. benthamiana hairy root cultures", the authors have analyzed the production of the tumor-targeting mAb H10 by secretion from transgenic hairy root (HR) cultures.

mAb H10 transgenic HR cultures were obtained either by infection of transgenic N. tabacum expressing mAb H10 with A. rhizogenes or from the N-glyco-engineered N. benthamiana line ΔXTFT, which lacks xylosyl and fucosyl transferases, with a mixture of recombinant A. rhizogenes bacteria transformed with the H10 heavy or light chain.

N. tabacum mAb H10 and N. benthamiana ΔXTFT HR cultures were compared in terms of growth rate and antibody production.

The structural differences between plant and mammalian N-linked glycans are major limitations in using plant-derived pharmaceuticals for human health. N. benthamiana ΔXTFT line minimizes plant-specific N-glycosylation and is successfully used for the production of mAbs. The development of hairy root cultures starting from the ΔXTFT line by direct infection with the A. rhizogenes mixture is the novel strategy proposed in this work for rapid production of mAbs and other pharmaceutical proteins without the immunogenic xylose and fucose sugars.

The paper is well written and the aim is clear; however, there are some points that need to be clarified and discussed.

General comment:

The differences in HR growth and mAb H10 production could be due to the fact that two plant species have been compared (benthamiana and tabacum). The best comparison would have been between HRs obtained from transgenic mAb H10 N.benthamiana transgenic plants and HRs obtained from N.benthamiana ΔXTFT infected with the HC/LC A. rhizogenes mixture.

Author response 1:
As suggested by the Reviewer 1, it is not possible to draw any conclusion since huge variations in transgene expression (due to positional effects) but also in growth and morphology of hairy-root suspensions have been reported previously. We have added this statement in the discussion section of the amended manuscript:
'It has been previously demonstrated that transgene expression in HRs is strongly influenced by positional effects (genes are randomly inserted in different regions of the genomic DNA), affecting not only protein expression but also hairy root growth and morphology [4,28]. This accounts for the strong variation in protein expression and growth that we and other groups observed among individual transgenic hairy roots expressing monoclonal antibodies in different plant species [8,11].'

Also in the light of the suggestions by the other reviewers, we have revised the paper avoiding a direct comparison between the two plant species. The manuscript focus was shifted on the main aim of this work, which is the use of multiple-gene co-transformation in glyco-engineered N. benthamiana to obtain human-compatible immunotherapeutic antibodies.

1) Title:

The human-type N-glycans of secretory proteins contain α1,4 and α1,6 fucose, sialic acid, β1,4 galactose. Therefore, the definition of the "plant N-glycans that lack β1,2 xylose and α1,3 fucose" as "human-type" is incorrect. I suggest substitute "human-type glycosylation profile" with "human-compatible glycosylation profile", both in the title and the text.

Author response 2:
We changed both title and text accordingly

2) - Figure 2A, non-reducing condition:
In all the three ΔXTFT HR clones, anti-HC antibodies recognize an additional 50 kDa band (more evident in clone 11) that is secreted in addition to the assembled mAbs, and most probably corresponds to free HC.
- Figure S3A, non-reducing condition:
In ΔXTFT HR clone 15, anti-LC antibodies recognize an additional 45 kDa band that could correspond to the homodimeric form of free LC.

The presence of free HC and LC polypeptides indicates that they are not synthesized in stoichiometric ratios. This unbalance can be due to several reasons: HC and LC transgene copy number; ii) mRNA stability; iii) transcription efficiency; iii) quality control.
The authors should discuss this point and formulate some hypothesis.

Author response 3:
We have discussed this point in the Discussion section:
'Identical band patterns for the two HR clones were also observed in Western blot analysis of root extracts under non-reducing conditions using an anti-LC antibody (Fig. 2). A strong signal at about 45 kDa was obtained suggestive for the formation of LC homodimers. The occurance of LC dimers in N. benthamiana had been already described in a recent work in which dimerized monoclonal camelidae variable heavy chain domain antibodies (VHH) were obtained through the C-terminal fusion of a k light chain domain [34]. Non-reducing analysis using anti-HC antibody revealed the presence of an intense band at about 100 kDa and a fainter one at about 50 kDa possibly representing HC dimers and monomers, respectively. The presence of free LC and HC polypeptides in HR clones indicated that they are not synthesized in stoichiometric ratios. A marked difference was observed in the presence of free HC and LC homodimers between the two co-transformed XTFT N. benthamiana hairy root clones 11 and 15 (Fig. S3). Since HC and LC coding genes were located in separate plasmids and transformed in different batches of A. rhizogenes, further used for hairy roots co-transformation, it is very likely that transgenes were inserted in these two clones in different regions of genomic DNA and possibly in different copy numbers, thus accounting for the differential accumulation of monomeric HC and LC.'

3) Figure 3A
The authors have compared the growth rates of ΔXTFT N. benthamiana and N. tabacum HRs. They conclude that the former grows three times faster.

Do HRs from N. tabacum and N. benthamiana grow differently?
OR:
Is the difference in growth rates related to the ΔXTFT mutation?
Please, discuss this point.

Author response 4:
The answer to this question has been already treated in the general comments. As suggested by the first reviewer, it is not possible to draw any conclusion since huge variations in transgene expression (due to positional effects) but also in growth and morphology of hairy-root suspensions have been reported previously. We have added this statement in the discussion section as reported above. The

ΔXTFT mutation does not account for the difference in growth rates since variations are present also among ΔXTFT N. benthamiana HR clones.

4) Figure 3E and page 16, lines 17-18 "although XTFT HRs have a higher growth rate, antibody secretory efficiency per g of root FW is lower compared to N. tabacum"

What do you mean by "antibody secretory efficiency"? The amount of protein secreted is determined by the balance between the rates of protein synthesis, secretion and degradation, and degradation can be influenced by the glycan structure. The authors do not present data that favour one specific hypothesis. It would be nice to have some data on the rates of protein synthesis or measurements of stability: mAb H10 turnover could be assayed in N.benthamiana ΔXTFT and N. tabacum HRs by pulse/chase with radioactive aminoacids, analyzing both the intracellular and the secreted polypeptides. Alternatively, at least the amount of intracellular and secreted mAb should be determined by protein blot analysis, to have some indication on whether there is a difference in the trafficking ability of the different molecules

Author response 5:
As suggested by Reviewer 1, 'Huge variations in transgene expression (due to positional effects) but also in growth and morphology of hairy-root suspensions have been reported previously. A comparison based on a low number of cell lines is thus meaningless. Conclusions regarding the "antibody secretory efficiency" cannot be made.'

In the light of what described in literature, we have now commented the huge variations in both transgene expression and growth observed among the different hairy root clones in the Discussion section.

We have also removed the sentence on "antibody secretory efficiency" and performed new experiments, as requested, for evaluating the amount of functional intracellular antibody in the root biomass of both clones (Figure 3E). This allowed us to calculate the 'Total antibody' in the HR cultures as the total amount of antibody (secreted antibody + intracellular antibody) in the system as already described in a previous work by Wongsamuth and Doran (1996) Biotechnology and Bioengineering, 54. 401-415, taking into account the amount of biomass present and the residual volume of each HR culture (initial medium volume of 40 mL).
The calculated amount of secreted antibody as percentage of total antibody for both HR clones was similar, thus indicating that there is no major difference in the trafficking ability of the two molecules.

'Discussion section': 'Overall, our results showed that the amount of functional antibody secreted in the medium by N. benthamiana clone 15 represents about 20% of total antibody. This amount is slightly lower but comparable to that of N. tabacum clone 6 (35% of total antibody), indicating that there is no major difference in trafficking ability of the two mAb H10 glyco-variants. Significant variations in the amount of secreted antibody in three different N. tabacum HR clones expressing -mAb M12 were reported in a previous work with levels ranging from 25 to 57% of the total antibody yield [11].'

Minor points:

The anti-HC and anti-HC antibodies seem to better recognize their antigens in reducing than in non-reducing condition. Please, discuss this point.

Author response 6:
This difference in recognition has already been observed in previous studies and probably depends on the method used to obtain the polyclonal antibodies.

This concept has been added in the results section:

'It must be noted that both anti-HC and LC polyclonal antibodies seem to better recognize the antigens in their monomeric form, when they are separated under reducing conditions. This was already evidenced in previous works [13,26].'

Which is the maximum volume for HRs cultures?

Author response 7:

At lab scale, up to 20 L bioreactors have been tested for the growth of HR cultures from different species especially for the production of secondary metabolites (*Panax ginseng, Artemisia annua, Solanum tuberosum etc.)* (Yong-Eui Choi, Yoon-Soo Kim, Kee-Yoeup Paek. Types and designs of bioreactors for hairy root culture (2008). Plant Tissue Culture Engineering pp 161-172). Commercial scale bioreactors of 1000 L or more exist for the culture of hairy-like adventitious roots of *Panax ginseng.*

Research Article

Production of a tumour-targeting antibody with a human-~~type~~ compatible glycosylation profile in *N. benthamiana* hairy root cultures

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Abbreviations: **HR**, hairy root; **PVP**, polyvinylpyrrolidone; **NAA**, 1-naphtaleneacetic acid; **TNC**, tenascin C; **FW**, fresh weight; **HC**, heavy chain; **LC**, light chain; **PBS**, phosphate-buffered saline; **SDS-PAGE**, Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis; **MS**, Murashige and Skoog medium; **LC-ESI-MS**, liquid chromatography- electrospray ionization mass spectrometry; **GFP**, Green Fluorescent Protein; **GUS**, β-glucuronidase-

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Abstract

Hairy root (HR) cultures derived from *Agrobacterium rhizogenes* (*A. rhizogenes*) transformation of plant tissues are an advantageous biotechnological manufacturing platform due to the accumulation of recombinant proteins in an otherwise largely protein free culture medium. In this context, HRs descending from transgenic *Nicotiana tabacum* (*N. tabacum*) plants were successfully used for the production of several functional mAbs with plant-type glycans. Here, we ~~compared the expression of~~ expressed the tumour-targeting mAb H10 in HRs obtained either by infecting a transgenic *N. tabacum* line expressing H10 with *A. rhizogenes* or a glyco-engineered *N. benthamiana* line (ΔXTFT) with recombinant *A. rhizogenes* carrying mAb H10 heavy and light chain cDNAs. Selected HR clones derived from both plants accumulated mAb H10 in the culture medium with similar yields (2-3 mg/L). N-glycosylation profiles of antibodies purified from HR supernatant revealed the presence of plant-~~-~~typical complex structures ~~(i.e. GnGnXF)~~ for *N. tabacum*-derived mAbH10 and of GnGn structures lacking xylose and fucose for the ΔXTFT-~~-~~derived counterpart. Both antibody glyco-formats exhibited comparable antigen binding activities. Collectively, these data demonstrate that the co-infection of ΔXTFT *N. benthamiana* with recombinant *A. rhizogenes* is an efficient procedure for the generation of stable HR cultures expressing the tumour-targeting mAb H10~~s~~ with a ~~targeted~~ human-compatible~~type~~ glycosylation profile, thus representing an important step towards the exploitation of root cultures for the production of 'next generation' human therapeutic antibodies.

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1 Introduction

Expression of therapeutic proteins in *in vitro* plant systems under contained conditions represents an advantageous manufacturing strategy in terms of uniform cultivation conditions, product quality and downstream purification process [1]. Indeed, the first plant-derived human therapeutic protein, taliglucerase α for Gaucher's disease treatment, is currently produced in carrot cells [2]. Along with cell suspension cultures, hairy root (HR cultures represent a valid alternative thanks to their time efficiency in establishing transgenic HR lines, intrinsic genetic stability, fast biomass accumulation and manufacturing scalability [3]. HR cultures are normally generated by infecting transgenic plants expressing the recombinant protein with *A. rhizogenes* or by transforming plant tissues with *A. rhizogenes* strains bearing a plant expression vector encoding the protein of interest [4]. These cultures lead to the formation of extensive secondary roots that can be cultivated under contained sterile conditions in hormone free media. Importantly, recombinant proteins can be secreted in the culture medium facilitating the downstream purification processes [5]. Hairy roots have been successfully used to express a wide range of complex recombinant proteins, such as enzymes [6], vaccine components [7], monoclonal antibodies (mAbs) [8] and anti-HIV microbicides [9]. Antibodies produced in *N. tabacum* HRs demonstrated that accumulation in the culture medium can be improved by the addition of hormones as well as protein stabilizing agents such as polyvinylpyrrolidone (PVP) [7,9]. Recently, an interesting study showed that components such as 1-naphtaleneacetic acid (NAA) can rise ~~accumulation levels~~yields of the tumour targeting human IgG1 M12 produced in *N. tabacum* HRs by even 30-fold (~ 6 mg/L of culture medium) [11]. Despite recent improvements, antibody yields obtained in HRs do not meet those reported for other plant expression systems, such as transgenic plants and agrobacterium-based transient expression systems. In fact, by agroinfiltration of *N. benthamiana* leaves using plant virus derived expression vectors, up to 500mg/Kg FW of the human tumour-specific mAb A5 were obtained [12].

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The goal of the present work was to produce the tumour-targeting antibody mAb H10 in HR cultures derived from glyco-engineered ΔXTFT *N. benthamiana* with the aim of improving the quality of the final product. This human IgG1 (λ), which derived from a selected single-chain variable fragment (scFv) is directed against the C domain of the large isoform of the tumour-associated antigen. TNC has been associated with a variety of tumours including breast, squamous cells, lung and prostate carcinomas, melanoma and malignant glioma [12, 13]. Anti-TNC antibodies have been recently used with success for the therapy of patients with acute myeloid leukemia and metastatic breast cancer [14, 15].

Field Code Changed

An important feature for many therapeutically interesting mAbs is proper Fc-glycosylation. It is now well established that certain glycan structures may alter the function of antibodies [17]. A prominent example is the lack of core fucose, which confers increased *in vitro* and *in vivo* activities [12, 13]. Recently it was shown that a mAb produced in wild type *N. tabacum* HRs carries, as many other plant-produced glycoproteins, complex structures bearing plant typical β1,2-xylose and α1,3 fucose [11]. A series of studies demonstrated that plants are particularly amenable to glycan engineering towards human-type structures as reviewed recently [20]. A major achievement was the elimination of plant-typical fucose and xylose residues using RNA interference, as in the case of duckweed (*Lemna minor*) and ΔXTFT *N. benthamiana* transgenic line [15, 16]. However, whether mAbs with modulated N-glycan profiles can be produced in HR cultures was not investigated so far.

In this study we have monitored different approaches for the generation of HRs producing human mAb H10. To this purpose, we used two plant binary expression vectors bearing heavy chain (HC) and light chain (LC) coding sequences under the control of the cauliflower mosaic virus promoter (35S) and the tobacco mosaic virus (TMV) translational enhancer (Ω) with the nopaline synthase terminator sequence at the 3' end. A signal peptide sequence deriving from an embryonic mouse immunoglobulin HC coding gene was used to direct the recombinant antibody to the secretory pathway [13]. Hairy roots were either obtained

by infecting transgenic tobacco cv Petite Havana SR1 plants expressing mAb H10 [13] or by the co-infection of a glyco-engineered ΔXTFT *N. benthamiana* line with transformed *A. rhizogenes* strains bearing the plant expression vectors encoding antibody HC and LC sequences. Selected HR clones derived from both plants accumulated mAb H10 in the culture medium with similar yields. Purified antibodies were characterised for glycosylation and functional activity *in vitro* showing the presence of plant-typical complex structures (i.e. GnGnXF) for *N. tabacum*-derived mAbH10 and of GnGn structures lacking xylose and fucose for the ΔXTFT- derived counterpart. Both antibody glyco-formats exhibited comparable antigen binding activities.

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2 Materials and methods

2.1 Hairy roots generation

N. tabacum cv. Petite Havana SR1 HRs were prepared from a T4 generation transgenic plant line (line 7.6) expressing mAb H10 HC and LC which was previously obtained by Villani and colleagues [13]. Briefly, wild-type *A. rhizogenes* A4 was grown in LB medium containing 50 mg/L rifampicin to OD600 = 0.5, at 28 °C and 160 rpm. Bacteria were pelleted by centrifugation at 3000 x *g* for 10 min and resuspended at OD_{600}=1 in MS medium with 30 g/L sucrose and 100 µM acetosyringone, pH 5.8 [23]. Leaves were cut in segments (size: 1 cm x 1 cm), wounded with a sterile needle (10 pricks per 1 cm^2) and inoculated by immersion in the *A. rhizogenes* suspension for 30 min, swirling in the dark. Leaves were dried and transferred to culture plates containing MS agar medium without phytohormones in the dark, for 2 days. Leaves were rinsed with sterile water and transferred to fresh MS agar medium containing 30 g/L sucrose and 0.5 g/L cefotaxime. Leaves were transferred onto fresh plates every 15 days. Roots emerging from leaves (typically after 40 days) were excised and transferred to new plates. *A. rhizogenes* was eradicated by transferring roots every 15 days onto MS agar plates using decreasing cefotaxime concentrations (0.25 g/L, 0.125 g/L, 0.05 g/L) until no antibiotic was added. *A. rhizogenes* eradication was confirmed by PCR using *virC* specific primers (Supporting Information, Fig. S1). Glyco-engineered ΔXTFT *N. benthamiana* plants [22] were used to obtain HRs expressing mAbH10. Leaf segments were infected with a co-culture of *A. rhizogenes* A4 strains transformed with the plant expression vectors p35S-HC or p35S-LC (Fig. 1A). Each strain was resuspended in inoculation medium to a final $O.D._{600}$ of 1. Next steps were carried out using the same protocol described for *N. tabacum* HR generation with the exception that both solid and liquid MS culture media were supplemented with kanamycin (100 mg/L).

2.2 Selection of HRs expressing mAb H10 by Western blot analysis

The selection of HRs expressing mAb H10 was performed by Western blot analysis using anti-HC and LC antibodies. Root tissues (100 mg) were ground in liquid N_2 and homogenized in 100 µl of phosphate-buffered saline pH 7.2 (PBS) containing a protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany). After centrifugation at 20000 x *g*, 4 °C for 30 min, the supernatant was recovered and analyzed by Western blotting. Samples were separated on 12% or 4-15% (Mini-PROTEAN® TGX™ Precast Gels, BIORAD, USA) SDS-PAGE acrylamide gels, and proteins were electrotrasferred to a PVDF membrane (Millipore, Bedford, MA) using a Semi-Dry Transfer Unit (Hoefer TE70; GE Healthcare, Freiburg, Germany) for 40 min at 0.4 mA/cm2 and 30 V. The mAb H10 purified from agro-infiltrated *N. benthamiana* leaves was used as positive control and extracts from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein as negative control. Membranes were incubated in PBS with 4% (w/v) non-fat milk, for at least 2 h. Protein detection was performed with anti-human γ chain (A8419 Sigma-Aldrich) and with anti-human λ chain secondary antibody (A5175; Sigma-Aldrich, Gillingham Dorset, UK) horseradish peroxidase conjugated diluted 1:5000 in 2% (w/v) non-fat milk in PBS. The membranes were washed three times with 0.1% Tween-20 in PBS, two times in PBS and developed using ECL Plus Western blotting detection system (ECL Plus; GE Healthcare). The ImageQuant™ LAS 500 (GE Healthcare) was used for chemiluminescence signal detection. Protein molecular mass markers were used in this analysis as reference (Color burst™, C1992 Sigma and Sharp Mass VI, Euroclone, Italy).

2.3 Establishment of HR culture in liquid medium and antibody secretion

The tobacco HRs were grown in 250 mL Erlenmeyer flasks using 40 mL of MS medium supplemented with ~~10~~3% sucrose. Starting inoculum weight was typically ~0.2 g of root FW and flasks, sealed with a cotton plug and aluminum foil, were orbitally shaken at 80 rpm (∅19 mm), in the dark, at 26 °C. Hairy root growth was analysed by measuring roots FW

at different time points before and after induction. Measurement was performed by gently removing medium and weighing flask and roots; fresh root net weight was then obtained by subtracting flask weight. Measurements were performed in triplicate at day 8, 15, 20, 25, 28, 30 and 33 from the initial inoculum. For antibody production, HRs were grown for 3-4 weeks, as described before, and secretion was triggered by the addition of 10 mL of MS culture medium supplemented with 14 g/L of KNO_3, 19 mg/L of NAA and 1.5 g/L of PVP (Mw 40000). Three days after the first induction, flasks were again supplemented with 19 mg/L of NAA and 1.5 g/L of PVP, and left for 5 days before collecting the spent culture medium for antibody purification. An aliquot of 20 µl of HR culture medium at different time points (before and after induction) was collected in order to analyze the amount of secreted antibody by Western blot analysis (as described before). H10 purified from agro-infiltrated *N. benthamiana* leaves was used as positive control and culture medium from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein as negative controls. Densitometric quantification of bands was performed by ImageQuant TL 7.0 Image analysis software (GE Healthcare). Induced and non-induced HRs from both *N. benthamiana* and *N. tabacum* were observed by stereomicroscopy 33 days after inoculum. Analysis was performed using a macro-photo microscope WILD/Leica M420.

2.4 Antibody purification from roots and medium of HR cultures

Culture medium was separated from HRs by filtration using Miracloth™ (Millipore). Supernatant was centrifuged at 15000 x *g* for 15 min, and filtered through 0.45 µm syringe filters (Millipore) ready for protein-A purification. Remaining roots (typically 4 to 11 g FW) were washed with PBS, ground in liquid nitrogen and homogenized using Ultra-Turrax homogenizer T25 equipped with a S25N-18G disperser (IKA, Staufen,German) in two volumes of 1X PBS. Extract was filtered using Miracloth™, centrifuged at 15000 x *g* for 15 min, passed through 0.45 µm syringe filters and subjected to affinity chromatography. Antibody purification was performed by affinity chromatography essentially as previously described [24]. Briefly, the

clarified root extract or culture medium was loaded onto a protein-A affinity column (1 mL HiTrap™ rProtein A FF; GE Healthcare) previously equilibrated with extraction buffer (1X PBS) at a flow rate of 1 mL/min. The column was washed with 10 mL of PBS (10 column volumes) and the antibody was eluted with 0.1 M citric acid (pH 3) and buffered with 1/5 volume of 1 M Tris-HCl, pH 8. Eluted fractions were analysed by SDS-PAGE, followed by Coomassie staining. Reference molecular mass markers were used (Amersham, RPN5800). Antibody-containing fractions were dialysed using slide-A-Lyzer™ Dialysis cassettes (Thermo Scientific) against 1X PBS. Antibody concentration was determined spectrophotometrically by measuring the absorbance at 280 nm [25].

2.5 ELISA assays

For the quantification of mAb H10 secreted in HR culture medium or in the root biomass, aliquots taken 8 days after induction were analyzed by quantitative ELISA. Briefly, the purified antigen, human TNC (CC065; Merck Millipore), was coated directly onto Nunc-MaxiSorp® 96 well plates at a concentration of 2 µg/mL in PBS, and incubated at 4 °C overnight. Plates were then blocked with 2% milk (w/v) in PBS, at 37 °C, for 1 h. Hairy root culture media were centrifuged at 15000 x *g* for 15 min, at 4 °C, the supernatants were recovered and serial dilutions were added to the wells (100 µl final volume) and incubated at 37 °C, for 2 h. In the case of root biomass (for intracellular antibody quantification), 0.2 g of roots were ground in liquid nitrogen in two volumes of 1X PBS, clarified by centrifugation 15000 x *g* for 15 min, at 4 °C, and serial dilutions were added to the wells and incubated at 37 °C, for 2 h. After washing, the anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was added at 1:5000 dilution in 2% milk (w/v) PBS, and incubated at 37 °C, for 1 h. As an internal standard for quantification, we used serial dilutions of purified mAb H10 positive control starting from a concentration of 3 µg/mL. Each HR sample was assayed in triplicate and mAb H10 concentrations were interpolated in the linear portion of the standard curve. For the

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functional characterization of the HR purified antibodies, the ELISA assay was performed essentially as described before. Purified antibodies in triplicate samples were added to TNC coated plates at serial dilutions starting from a concentration of 3 μg/mL. The anti-human γ chain HRP-conjugated antibody (A8419; Sigma-Aldrich) was used as secondary antibody. After 30 min, enzymatic activity was measured at 405 nm on a microplate reader (TECAN-Sunrise, Groedig, Austria) using 2,2-azino-di-3-ethylbenz-thiazoline sulphonate as substrate (ABTS, KPL).

2.6 Glycan analysis

The N-glycosylation profile was determined by LC-ESI-MS as previously described by Stadlmann and colleagues [26]. In brief, purified IgG was separated by reducing SDS-PAGE, and bands corresponding to the HC and LC were excised from the Coomassie stained gel. Upon S-alkylation and tryptic or tryptic/GluC digestion, fragments were eluted from the gel with 50% acetonitrile and separated on a reversed-phase Column (150 × 0.32 mm BioBasic-18, Thermo Scientific) using a gradient of 1–80% acetonitrile. Glycopeptides were analyzed with a Q-TOF Ultima Global mass spectrometer (Waters). Spectra were summed and deconvoluted for the identification of glycoforms. Glycans were annotated according to the proglycan nomenclature (www.proglycan.com).

3 Results

3.1 Infection of transgenic *N. tabacum* and glyco-engineered ΔXTFT *N. benthamiana* leaves with *A. rhizogenes* and hairy roots generation

Twenty-three HR clones were obtained from *N. tabacum* cv. Petite Havana SR1 (T4 generation transgenic plant line -7.6-) expressing mAb H10 HC and LC [13] by leaf disc infection with wild-type *A. rhizogenes* A4 strain. Twenty-five HR clones were also obtained from leaf discs of ΔXTFT *N. benthamiana* plants [22] infected with a co-culture of *A. rhizogenes* A4 strains transformed with the plant expression vectors p35-HC and p35-LC [13] encoding mAb H10 HC and LC, respectively (Fig. 1A). Hairy root clones of both *N. tabacum* and ΔXTFT *N. benthamiana* were grown in plates with solid MS and using liquid MS in shake flasks (Fig. 1B and 1C).

3.2 Selection of tobacco hairy root clones expressing mAb H10

All ΔXTFT *N. benthamiana* HR clones were screened for mAb expression by non-reducing Western blot analysis using an anti-HC antibody. Only five clones showed a band at 150 kDa indicating the presence of the full size mAb H10 (about 20% of the clones) and three clones (11, 15 and 16) exhibited the highest mAb H10 levels (Supporting Information, Fig. S2). In the case of HRs derived from *N. tabacum* transgenic line, fourteen clones were positive to antibody expression (about 60% of the clones), with clones 3, 4, and 6 showing the highest antibody levels (Supporting Information, Fig. S2). Two clones were selected (clone 15 for *N. benthamiana* and clone 6 for *N. tabacum*) and analysed; they were resolved on a gradient gel under reducing and non-reducing conditions and then subjected to Western blot analysis using both anti-HC and LC antibodies (Fig. 2). In the anti-HC blotting under non-reducing conditions, both *N tabacum* and ΔXTFT *N. benthamiana* clones showed a similar band pattern, with the expected band migrating at high molecular mass (~150 kDa) corresponding to the assembled mAb. Additional lower bands were also observed; an intense band (at about 100 kDa) and very

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faint ones (at about 135, 75 and 50 kDa) that probably relate to antibody assembly intermediates (HC dimers/monomers) or putative degradation fragments. In the corresponding analysis under reducing conditions, a 50 kDa band associated with intact HC was visible as well as a faint band at about 25 kDa indicating specific degradation of the heavy chain (Fig. 2A). The anti-LC blot under non-reducing conditions (Fig. 2B) showed a 150 kDa band in both clones, together with additional lower faint bands at about 135, 100 and 75 kDa, which were already observed in the anti-HC blot. A very strong band at about 45 kDa was visible, which also occurred in the control. This may correspond to the homodimeric form of free LC. Under reducing conditions, the 25 kDa band corresponding to the LC was detected in both clones. It must be noted that both anti-HC and LC polyclonal antibodies seem to better recognize the antigens in their monomeric form, when they are separated under reducing conditions. This was already evidenced in previous works [13, 26].

3.3 Hairy root growth and antibody secretion in the culture medium

Hairy root cultures of ΔXTFT *N. benthamiana* (clone 15) and *N. tabacum* (clone 6) in liquid MS medium were established. Secretion of mAb H10 in the culture medium was induced by the addition of KNO_3 as nitrogen source, the plant growth regulator NAA and PVP as protein stabilizing agent [10]. Growth curves of HR cultures were obtained by measuring root fresh weight (FW) at different time points before and after induction (Fig. 3A). Antibody secretion was induced at day 25 and 28 post-inoculation and flasks were left for another 5 days prior collecting the culture medium for antibody purification (33 days after inoculum). The two clones showed a significant difference in their growth, with ΔXTFT *N. benthamiana* (clone 15) reaching about 11 g FW/40 mL (275 g FW/L) 25 days post-inoculation, compared to about 4 g FW/40 mL (100 g FW/L) in *N. tabacum* (clone 6). Moreover, we recorded a decrease in growth for both clones after the start of induction at day 25 (Fig. 3A). ~~A net loss of FW was recorded at day 30 and 33 probably due to root decline. In fact, root FW was measured by removing culture~~

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~~medium from the flask, and root debris were observed in the supernatant at these time points.~~ Microscopy observations at day 33 showed a general enhanced fragility of roots with swollen tips and root hair proliferation, compared to non-induced ones (Fig. 3B).

The accumulation of mAb H10 in culture medium was assayed by Western blot analysis using an anti-HC (γ chain) antibody. Samples were collected at different time points before (day 0) and after induction of antibody secretion (days 2, 4 and 8), and separated by non-reducing SDS-PAGE (Fig. 3C and D, left panels). Very faint bands were observed at day 0 (before induction) at about 150 kDa, while stronger bands were detected in the following days. Maximum mAb H10 levels were typically reached at day 8 for both *N. tabacum* (Fig. 3C, right panel) and ΔXTFT *N. benthamiana* (Fig. 3D, right panel).

The amount of active antibody in HR culture medium was quantified by ELISA. HR supernatant was recovered at day 8 after induction and serial dilutions were added to the ELISA wells coated with human TNC. Results showed a similar concentration of functional antibody in the medium of both HR clones, with 2.70 ± 0.32 mg/L in *N. tabacum* (mAb H10Nt) and 2.24 ± 0.19 mg/L in ΔXTFT *N. benthamiana* (mAb H10ΔXTFT) (Fig. 3E). Antibody quantification in the root biomass was performed at day 8 after induction by adding serially diluted root extracts to TNC-coated ELISA wells. Results showed a slightly higher concentration of functional antibody in *N. tabacum* clone 6 (48.7 ± 7.7 µg/g FW) compared to ΔXTFT *N. benthamiana* clone 15 (31.3 ± 5 µg/g FW) (Fig. 3E). Total antibody in the HR cultures was calculated as the total amount of antibody (secreted antibody + intracellular antibody) in the system, taking into account the amount of biomass present and the residual volume of each HR culture (initial medium volume of 40 mL). Results showed higher levels of mAb in ΔXTFT *N. benthamiana* clone 15 (0.47 ± 0.08 mg) compared to *N. tabacum* clone 6 (0.3± 0.03 mg) (Fig. 3E).

The amount of antibody in the medium (considering HR cultures with 40 mL of starting volume) corresponded to about 20% of the total antibody (secreted + intracellular) in ΔXTFT *N. benthamiana* and about 35% in *N. tabacum*. Tobacco HR clones were grown and

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stably propagated for 10 months on solid MS culture medium and were used as starting inoculum to establish different batches of HR cultures in liquid medium. During this period, we did not observe significant variations of mAbH10 yields in different culture batches.

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3.4 Characterization of purified mAb H10 from hairy roots

Culture medium was separated from HRs by filtration/centrifugation and clarified supernatant was directly subjected to protein-A affinity chromatography. Eluted fractions (Supporting Information, Fig. S3) were pooled, dialysed against PBS and separated by SDS-PAGE under reducing or non-reducing conditions (Fig. 4A). Average yields from three independent purifications were 1.6 ± 0.4 mg/L for mAb H10Nt from *N. tabacum* HR and 1.5 ± 0.19 mg/L for mAb H10ΔXTFT from ΔXTFT *N. benthamiana* HR, corresponding to about 60% recovery of secreted antibody (Fig 4C). Reducing SDS-PAGE analysis of the purified antibodies revealed, as expected, two major bands at about 50 kDa (HC) and 25 kDa (LC), together with an additional degradation product (~ 23 kDa) (Supporting Information, Fig. S3). A control mAb H10 purified from agro-infiltrated *N. benthamiana* leaves showed the same pattern of bands observed for the HR derived antibodies. In the case of non-reducing SDS-PAGE, a band at about 150 kDa corresponding to the assembled antibody was present in both tobacco HR purifications. Additional faint (at about 100 kDa) and stronger bands (at about 40 kDa; indicated by a black triangle in Fig. 4A) demonstrated the presence of degradation products/assembly intermediates. The observed pattern of bands was similar to that of mAb H10 control from agro-infiltrated leaves (Fig. 4A).

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The Antibody was also purified from the root biomass of the induced HRs. Clarified root extracts were passed through protein-A column and eluted fractions were separated by SDS-PAGE under reducing conditions (Fig. S3). Average yields from three purifications were 16.2 ± 1.7 µg/g FW for mAb H10Nt from *N. tabacum* HR and 12.4 ± 1.5 µg/g FW for mAb

H10ΔXTFT from ΔXTFT *N. benthamiana* HR (Fig. 4C). Band pattern was identical to that observed for antibodies purified from culture medium.

The integrity of both antibody versions was estimated by their ability to bind the antigen human TNC. ELISA plates were coated with recombinant C domain of human TNC (Fig. 4B), and incubated with purified antibodies in serial dilutions. Both antibodies recognized the antigen in a concentration dependent manner, thus demonstrating functional activity in a similar range.

Glycan analyses of H10Nt-Fc by LC-ESI-MS revealed the presence of a single glycan species, *i.e.* plant-typical GnGnXF structures (Fig. 5). In contrast, H10ΔXTFT carried GnGn structures lacking xylose and core α 1,3-fucose (Fig. 5).

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4 Discussion

In literature, there are only a few examples of the successful expression of monoclonal antibodies in HRs. All were developed from stable *N. tabacum* transgenic plants [10, 7]. In this study, we evaluated the production of a tumour targeting mAb in tobacco HRs by using two different approaches. Hairy roots were either generated 'classically' by *A. rhizogenes* infection of a transgenic *N. tabacum* line expressing the antibody or by co-infection of glyco-engineered ΔXTFT *N. benthamiana* with a mix of recombinant *A. rhizogenes* strains bearing the antibody *hc* and *lc* genes. We showed that the percentage of clones expressing detectable amounts of recombinant protein was higher in *N. tabacum* (60% of the clones were positive in Western blot analysis) than in co-transformed ΔXTFT *N. benthamiana* (20% of the clones were positive). This result is merely indicative, due to the low number of HR clones screened, and does not allow for a thorough comparison of the two different approaches. It simply demonstrates that co-infection of leaf discs with a mix of *A. rhizogenes* carrying antibody HC and LC coding sequences, although at a lower efficiency, can be used for the rapid establishment (approx. 2 month) of stable HR clones expressing functional mAbs. A previous work based on the same multiple gene co-transformation strategy, *i.e.* transgenes located in different plasmids and transformed in different batches of *A. rhizogenes*, was used to co-express GUS and GFP in *N. tabacum* hairy roots. In this case a co-transformation efficiency of 65% was obtained, while a higher value (82%) was obtained when the reporter genes were inserted in two different T-DNAs within the same binary vector [4]. Comparison of the two best mAb expressors in liquid culture showed a faster growth (nearly three-fold) in the *N. benthamiana* clone, compared to the *N. tabacum* one. Starting from an inoculum size of ~0.2 g in a 40 mL culture, the FW biomass value of ΔXTFT *N. benthamiana* HRs was 11 g (275 g/L) after 25 days, while in the case of *N. tabacum* it was lower (100 g/L), but comparable to that already described by other groups [11]. It has been previously demonstrated that transgene expression in HRs is strongly

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influenced by positional effects (genes are randomly inserted in different regions of the genomic DNA), affecting not only protein expression but also hairy root growth and morphology [28, 4]. This accounts for the strong variation in protein expression and growth that we and other groups observed among individual transgenic HRs expressing monoclonal antibodies in different plant species [8,11].

A major advantage of HRs is the possibility to secrete the recombinant proteins in the culture medium. Previous literature showed that the addition of protein stabilizing agents (PVP and gelatin) and KNO_3 ~~enhances antibody accumulation~~ in the medium improved antibody recovery [7,~~,~~ 9]. Moreover, some authors also highlighted the importance of the auxin NAA in increasing rhizosecretion of recombinant proteins [29]. An optimised protocol was recently developed leading to high yield accumulation of a mAb in *N. tabacum* HRs, which is based on KNO_3 and NAA to induce rhizosecretion and PVP as protein stabilizing agent [11]. We used a similar protocol to compare mAb H10 secretion in the two best expressing HR clones and the concentration of secreted antibody was similar in both *N. tabacum* (2.7 mg/L) and ΔXTFT *N. benthamiana* (2.24 mg/L) with ~~accumulation levels~~levels comparable to ~~what~~ those previously reported in literature [11]. Maximum antibody accumulation was typically achieved 8 days post-induction (33 days after inoculation) and, most interestingly, antibody accumulation in the medium before induction was hardly detectable (Fig. 3C and D). Overall, our results showed that the amount of functional antibody secreted in the medium by *N. benthamiana* clone 15 represents about 20% of total antibody. This amount is slightly lower but comparable to that of *N. tabacum* clone 6 (35% of total antibody), indicating that there is no major difference in trafficking ability of the two mAb H10 glyco-variants. Significant variations in the amount of secreted antibody in three different *N. tabacum* HR clones expressing –mAb M12 were reported in a previous work with levels ranging from 25 to 57% of the total antibody yield [11].

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~~We achieved levels of about 27 µg in *N. tabacum* and 8 µg in ΔXTFT of secreted antibody per g of root FW in 40 ml cultures. This indicates that, although ΔXTFT HRs have a higher growth rate, antibody secretory efficiency per g of root FW is lower compared to *N. tabacum*.~~

Microscopy analysis of roots after induction revealed a peculiar phenotype with the formation of swollen root tips, root hair proliferation and elongation. This phenomenon was already described by Häkkinen and colleagues who observed the formation of lateral root primordia in the swollen root tip, associated with morphological changes in the inner cortex and pericycle cells [11]. A similar root morphology was also revealed in hydroponic tobacco cultures rhizosecreting a human monoclonal IgG after NAA induction [30]. ~~On this basis, it is conceivable to speculate that these cellular modifications could be significantly involved in the increased protein synthesis and secretion in roots.~~

Antibody purification from culture medium was performed using a two-step protocol in which the medium was clarified by filtration/centrifugation and directly passed through a protein A column with no need for pH adjustment or DNAse treatment. Purification yield~~s~~ of H10 from liquid medium cultures of ΔXTFT *N. benthamiana* and *N. tabacum* HR clones were similar, in the range of 1.5 mg/L (Fig. 4C). It is interesting to note that the amount of antibody purified from the HR spent medium represents about 60% of the total secreted antibody while only 30% of the total intracellular antibody was recovered after protein A purification of root extracts. Purification yields from culture medium are slightly lower than those described by Hakkinen and colleagues which reported yields of 5.9 mg/L for mAb M12, reflecting possible lower antibody degradation and higher accumulation levels in the culture medium [11]. It is important to note that the performance of the protein-A affinity column used for antibody purification from the medium remained unaltered even after several purification cycles, thus indicating that the filtered HR culture medium can be directly used in the downstream processing without the need of further clarification steps. As expected, mAb H10 purification

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yields in HRs are sensibly lower compared to those obtained by using transient expression systems based on *N. benthamiana* agroinfiltration [27]; the latter yielded up to 75 mg/Kg FW of the antibody. Nevertheless, HR cultures still represent and advantageous system in terms of manufacturing and downstream processing for the contained root growth in sterile conditions and the secretion of the recombinant protein in the culture medium.

Degradation of recombinant proteins in the HR culture medium represents a major problem and previous studies demonstrated that the addition of PVP or gelatin can enhance antibody stability [7,9]. In particular, the addition of PVP had stabilizing effects on HR-secreted mAb M12 increasing accumulation by two times [11]. Previous studies reported that the susceptibility of recombinant antibodies to plant proteolysis is largely dependent on their primary sequence including the variable domains [25, 21]. Degradation profile of mAb H10 has been extensively studied in different plant species as well as production systems. This molecule was shown to be particularly susceptible to proteolytic cleavage in the proximity of the interdomain antibody sequences [33]. In particular, a previous study from our group demonstrated that mAb H10 purified from leaves of the transgenic tobacco line 7.6 was almost completely degraded leading to the formation of Fab fragments [13]. Reducing SDS-PAGE analysis of purified mAb H10 from ΔXTFT *N. benthamiana* and *N. tabacum* HRs, and from agro-infiltrated *N. benthamiana* leaves revealed a similar pattern with two major bands, corresponding to the HC and LC and a band at a lower molecular mass associated ~~to~~ with HC degradation as a result ~~resulting from the hydrolysis of a peptide bond~~of a proteolytic event close to the hinge region [13]. Identical patterns for the three purified antibodies were also observed in non-reducing conditions, with a major degradation product at about 40 kDa. In a previous work, a similar degradation product was described and characterized as a functional antibody Fab fragment deriving from specific cleavage in the antibody hinge region [32]. Identical band patterns for the two HR clones were also observed in Western blot analysis of root extracts under non-reducing conditions using an anti-LC antibody (Fig. 2). A strong signal

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at about 45 kDa was obtained suggestive for the formation of LC homodimers. The occurance of LC dimers in *N. benthamiana* had been already described in a recent work in which dimerized monoclonal camelidae variable heavy chain domain antibodies (VHH) were obtained through the C-terminal fusion of a k light chain domain [34]. Non-reducing analysis using anti-HC antibody revealed the presence of an intense band at about 100 kDa and a fainter one at about 50 kDa possibly representing HC dimers and monomers, respectively. The presence of free LC and HC polypeptides in HR clones indicated that they are not synthesized in stoichiometric ratios. A marked difference was observed in the presence of free HC and LC homodimers between the two co-transformed ΔXTFT *N. benthamiana* hairy root clones 11 and 15 (Fig. S2). Since HC and LC coding genes were located in separate plasmids and transformed in different batches of *A. rhizogenes*, further used for hairy roots co-transformation, it is very likely that transgenes were inserted in these two clones in different regions of genomic DNA and possibly in different copy numbers, thus accounting for the differential accumulation of monomeric HC and LC.

It is interesting to note that no significant differences in band patterns were observed between the two HR secreted antibodies or mAb H10 produced in *N. benthamiana* leaves, indicating that equivalent proteolytic events take place in both species and production systems. A similar evidence was observed for analogous proteolytic activity of *A. thaliana* or *N. tabacum* rhizosecretomes on a human IgG [35].

Plant-derived mAbs differ from their mammalian counterpart in their Fc N-glycan composition. Non-human glycosylation may represent a limitation in their therapeutic application since plant typical complex glycan structures moieties may cause immunogenic reactions [35,36] and may also interfere with Fcγ receptors binding [19, 37]. This has a negative impact on immunotherapeutic mAbs, since their effector functions, such as antibody dependent cell cytotoxicity (ADCC), rely on the interaction with Fcγ receptors [39]. Recent

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studies demonstrated that antibodies lacking plant specific xylose and fucose have enhanced binding to Fcγ receptors and improved biological activity [40].

With the aim of improving mAb H10 immunotherapeutic potential we have demonstrated here that it is possible to obtain HRs from ΔXTFT *N. benthamiana* producing mAbs with a human-~~type~~ compatible glycosylation pattern. Häkkinen and colleagues previously characterized the glycosylation profile of mAb M12 obtained in *N. tabacum* HRs, showing that the secreted antibody carries mainly GnGnXF plant typical complex-type glycans [11]. In this work, we have shown that mAb H10Nt has a similar profile to that observed for mAb M12 (GnGnXF), while mAb H10ΔXTFT carries predominantly GnGn structures (>90%). To our knowledge, this is the first example of a functional glyco-modified mAb obtained from HRs lacking typical plant-type sugars, thus representing a step towards the exploitation of root cultures for the production of 'next generation' human therapeutic antibodies.

In conclusion, we have shown here that it is possible to rapidly establish HR cultures secreting full size mAbs with a targeted glycosylation profile by using a simple *A. rhizogenes* infection protocol of ΔXTFT *N. benthamiana* leaf segments. As reported in literature, maximum antibody secretory yields in HRs are in the range of few milligrams per liter of culture medium. To our view, this is the major limitation of this production system, especially if compared to the high-yield methods (hundreds of milligrams per Kg of FW) based on transient expression systems using whole plants. For this reason, efforts have to be focused on devising novel expression vectors as well as cultivation and induction protocols to enhance recombinant protein production and secretion, making stably transformed HRs a competitive production system of glyco-modified antibodies for cancer immunotherapy.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figures

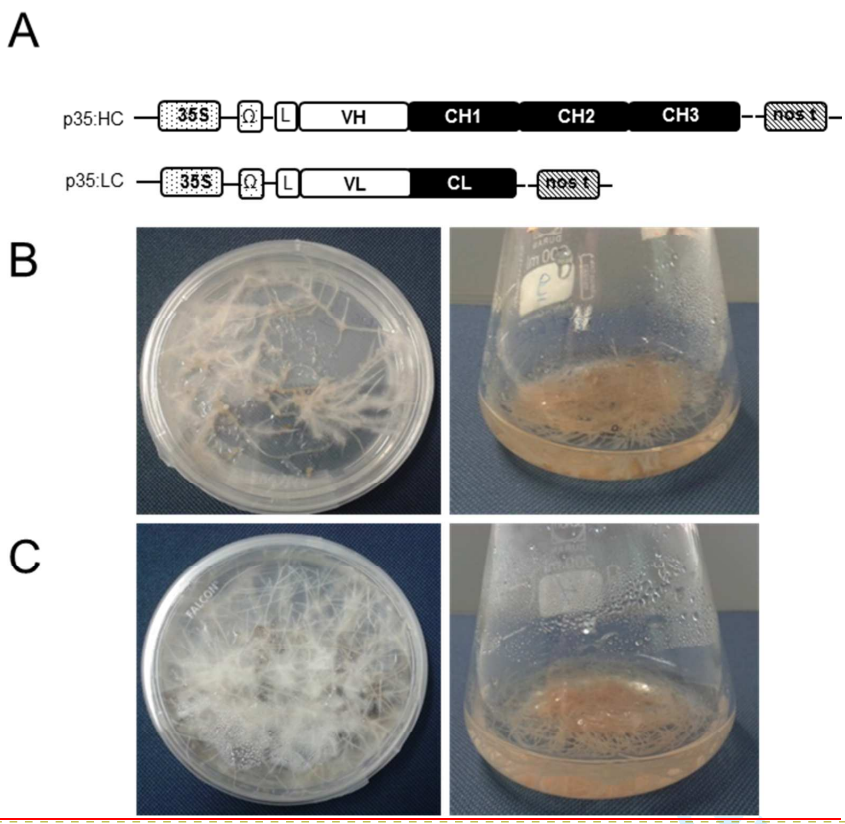

Figure 1

Generation of HRs expressing mAb H10. (**A**) ΔXTFT *N. benthamiana* HRs expressing mAb H10 were obtained by leaf disc co-infection with *Agrobacterium rhizogenes* strains bearing p35-HC and p35-LC plant expression vectors. Both HC and LC coding sequences were under the control of the Cauliflower Mosaic Virus 35S promoter (35S) and the translation enhancer sequence of Tobacco Mosaic Virus (Ω). L: signal peptide sequence derived from an embryonic mouse immunoglobulin HC coding gene. Solid and liquid medium HR cultures expressing the mAb H10 antibody; ΔXTFT *N. benthamiana* clone 15 (**B**) and *N. tabacum* clone 6 (**C**).

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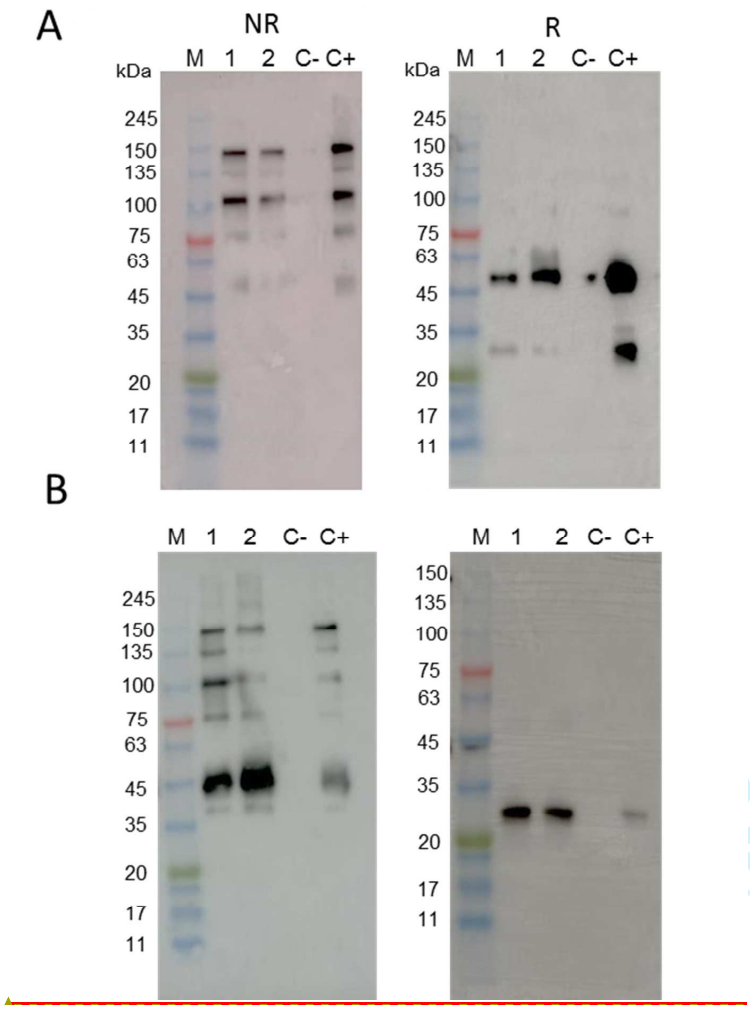

Figure 2

Western blot analysis of HR clones. *N. tabacum* clone 6 (1) and ΔXTFT *N. benthamiana* clone 15 (2) were analysed using anti-HC (A) and anti-LC (B) antibodies under non-reducing (NR) and reducing (R) conditions. Extraction was performed on 0.1 g of root tissue for all samples and 20 µl of each extract were loaded on 4-15% SDS-PAGE gel. (C+), mAb H10 purified from agro-infiltrated *N. benthamiana* leaves. C-: HR clone expressing an unrelated protein (antimicrobial peptide); M: Molecular mass markers.

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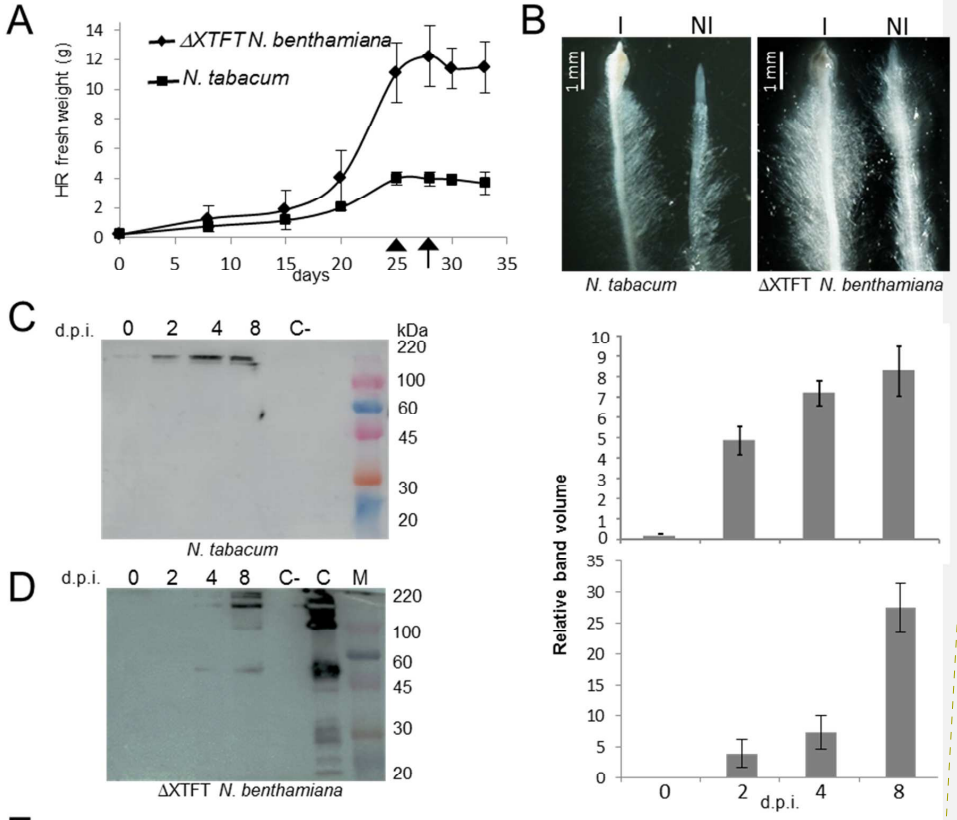

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Antibody	Concentration in the medium (mg/L)	Intracellular antibody (µg/g FW)	Total antibody (mg)
H10ΔXTFT	2.24 ± 0.19	31.3 ± 5	0.47 ± 0.03
H10Nt	2.70 ± 0.32	48.7 ± 7.7	0.3 ± 0.08

Figure 3

Analysis of mAb H10 accumulation in liquid medium of HR cultures. (**A**) The growth rate of ΔXTFT *N. benthamiana* clone 15 and *N. tabacum* clone 6 was measured as root FW at different time points before and after the induction of mAb H10 secretion in the culture medium. The secretion of mAb H10 was triggered by the addition of KNO_3, NAA and PVP twenty-five days after inoculum (indicated by an arrow). Three days after the first induction (28 days after inoculum, see arrow), flasks were supplemented again with NAA and PVP, and

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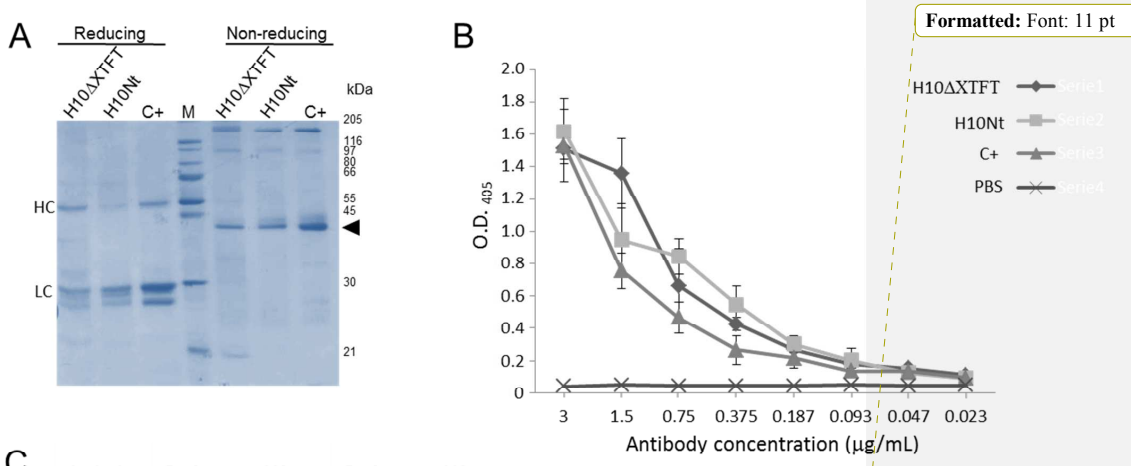
typically left for another 5 days before collection of the spent culture medium for antibody purification (33 days after inoculum). Data represent the average values of three independent experiments ± SD. (**B**) A typical microscopy analysis showing phenotypic differences between induced (I) and non-induced (NI) HRs in both *N. tabacum* and ΔXTFT *N. benthamiana*. Induced HRs show typical swollen root tips and enhanced root hair proliferation. Antibody secretion in culture medium of *N. tabacum* clone 6 (**C**, left panel) and ΔXTFT *N. benthamiana* clone 15 (**D**, left panel) was assayed by Western blot analysis using an anti-*γ* chain antibody. Twenty microliters of culture medium were collected at different time points before (day 0) and after induction of antibody secretion (day 2, 4 and 8). Samples were separated by non-reducing 12% SDS-PAGE gels. C+: H10 purified from agro-infiltrated *N. benthamiana* leaves. C- : culture medium from *N. benthamiana* or *N. tabacum* HRs transformed with an irrelevant protein. Post-induction accumulation of mAb H10 in culture medium of *N. tabacum* (**C**, right panel) and ΔXTFT *N. benthamiana* HRs (**D**, right panel) was evaluated by densitometry analysis of band volumes (values are the mean ± SD of two independent experiments). **(E)** Quantitative TNC-binding ELISA was used to calculate antibody concentration in the HR culture medium and in root biomass (intracellular antibody) eight days after induction (33 days from inoculum); values shown in the panel are the mean ± SD obtained from three independent HR cultures. Total antibody is the total amount of antibody (secreted antibody + intracellular antibody) in the system, taking into account the amount of biomass present and the residual volume of each HR culture. All values were calculated from HR cultures in 250 mL flasks with an initial medium volume of 40 mL.



Antibody	Purification yield from medium (mg/L)	Purification yield from roots (µg/g FW)
H10ΔXTFT	1.5 ± 0.19	12.4 ± 1.5
H10Nt	1.6 ± 0.4	16.2 ± 1.7

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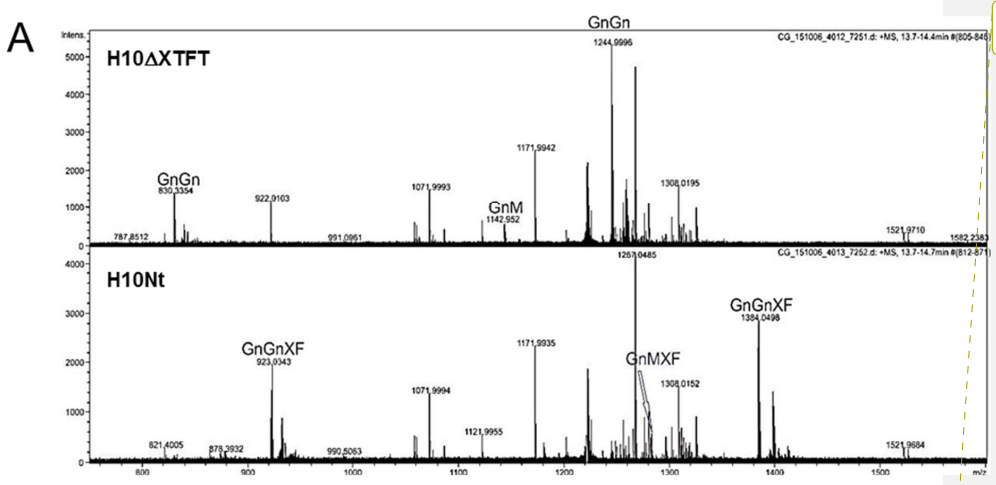
Figure 4

Purification of HR produced antibodies. The mAb H10 was purified from culture medium (80 mL) with a protein-A affinity chromatography column. Two micrograms of each purified antibody were loaded on 12% SDS-PAGE gels under reducing and non-reducing conditions. (**A**) The bands corresponding to mAb H10ΔXTFT from *N. benthamiana* and mAb H10Nt from *N. tabacum* were revealed using Coomassie blue staining. C+: mAb H10 purified from agro-infiltrated leaves of *N. benthamiana*. M: molecular mass standards. HC and LC are indicated as well as a major degradation fragment at about 40 kDa (black triangle). (**B**) Antigen-binding analysis of mAbH10Nt and mAb H10ΔXTFT. Purified antibodies were added to TNC coated plates at serial dilutions starting from a concentration of 3 µg/mL. An anti-human γ chain HRP-conjugated antibody was used for detection (data represent mean ± SD of three experiments). C+: H10 purified from agro-infiltrated *N. benthamiana* leaves. C-: PBS. (C) Antibody yield from hairy roots culture medium and biomass (from HR cultures in 250 mL flasks with an

initial medium volume of 40 mL sampled 8 days post-induction) is the mean value obtained from three independent purifications .



Name	GnGn	GnGnXF	others
H10ΔXTFT	93%	n.d.	7%
H10Nt	n.d.	93%	7%

n.d.: not detected ● Man ■ GlcNAc ▲ Fuc ☆ Xyl

Figure 5

Glycan analysis of antibodies purified from hairy root culture medium.

(A) Liquid chromatography-electrospray mass spectrometry (LC-ESI-MS) of tryptic glycopeptides of H10 purified from hairy roots culture medium of ΔXTFT *N. benthamiana* (H10ΔXTFT) and *N. tabacum* (H10Nt). Doubly and triply charged ions of N-glycosylated variants of the expected tryptic fragments are indicated. (B) Relative amounts (%) of N-glycans in the purified H10ΔXTFT and H10Nt.

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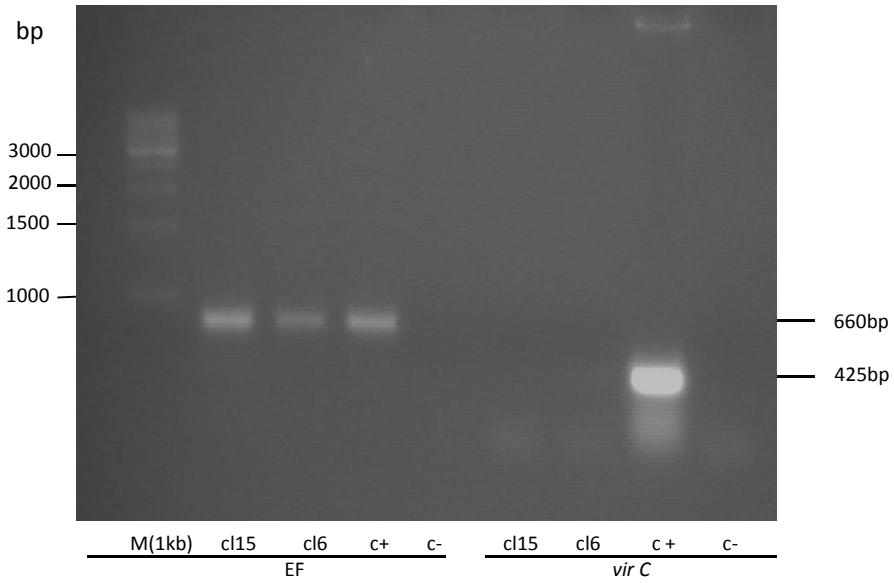
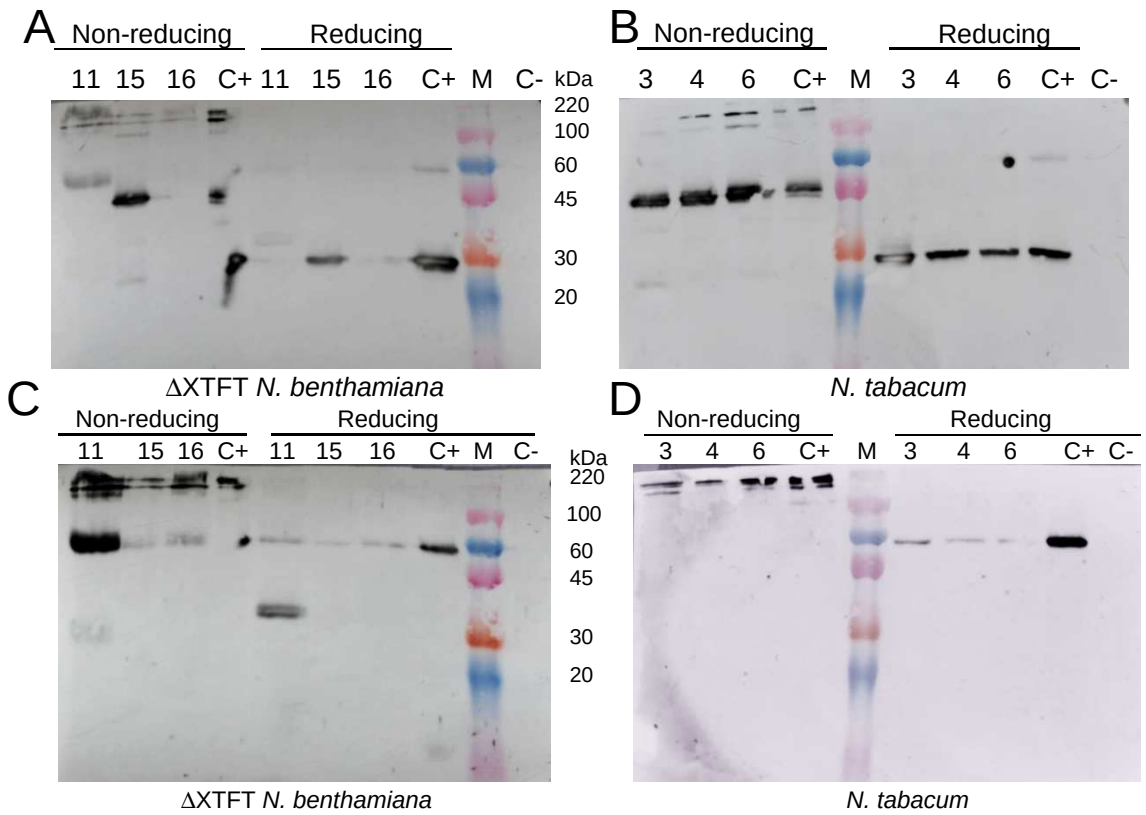
Figure S1



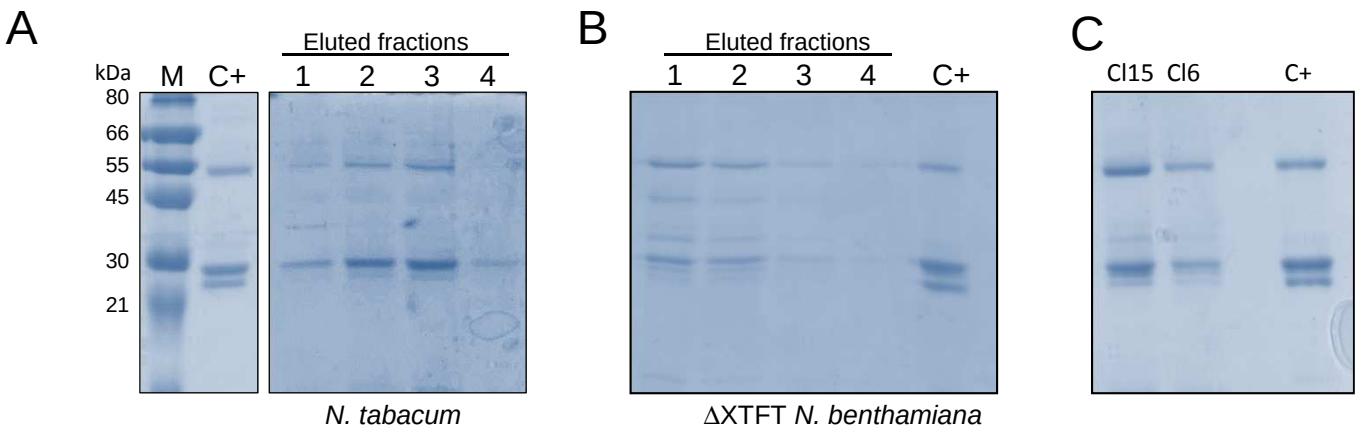
PCR analysis of genomic DNA extracted from ΔXTFT *N.benthamiana* (cl 15) and *N.tabacum* (cl 6) HRs using *Agrobacterium rhizogenes* specific *virC* primers (virC1-for 5' AATGCGTCTCTCTCGTGCAT-3; virC1-rev 5'-AAACCGACCACTAACGCGAT-3'). An expected amplification product of 425 bp was observed in the C+ positive control (*A. rhizogenes* colony). No amplification product was observed in both HR clones indicating the complete eradication of *Agrobacterium rhizogenes* by cefotaxime treatment. As an internal control of DNA amplification EF primers (EF1-UP 5'-ATTGTGGTCATTGGTCATGT-3'; EF1-DW 5'-CCAATCTTGTAAACATCCTG- 3') that amplify the plant elongation factor gene were used. The amplification of a 660 bp fragment was observed in the two HR clones as well as in the positive control (C+). C+: genomic DNA extracted from *N. benthamiana* leaves. C- : no DNA was added in the PCR reactions using *vir C* or EF primers. M: Molecular Weight Marker (1Kb, Promega).

Figure S2



Western blot analysis of selected HRclones. (**A**) ΔXTFT *N. benthamiana* (clones 11,15 and 16); (**B**) *N. tabacum* (clones 3, 4 and 6) were analysed using an anti-LC antibody in reducing and non-reducing conditions. Extraction was performed on 0.1 g of root tissue for all samples and 20 µl of each extract were loaded on 12% SDS-PAGE gel. As positive control, mAb H10 purified from agroinfiltrated *N. benthamiana* leaves was used (C+) . C-: HR clone expressing an unrelated protein (antimicrobial peptide) was used as negative control; M: Molecular mass Markers (Color burst™).

Figure S3



The mAb H10 from culture medium was purified with protein A affinity chromatography and eluted fractions (1 to 4) were run on 12% SDS-PAGE gels under reducing conditions. A typical Coomassie stained gel showing protein A column elution fractions after purification from HR culture medium of *N. tabacum* clone 6 (**A**) and ΔXTFT *N. benthamiana* clone 15 (**B**). The mAbH10 from hairy root extracts of ΔXTFT *N. benthamiana* clone 15 (**Cl15**) and *N. tabacum* clone 6 (**Cl6**) was purified using the same protocol (**C**). C+: mAb H10 purified from agroinfiltrated leaves of *N. benthamiana*. M: protein molecular mass standard (RPN 5800, Amersham Biosciences).