

Accumulation of secreted antibodies in plant cell cultures varies according to the isotype, host species and culture conditions

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Summary

Nicotiana tabacum suspension cells have been widely used to produce monoclonal antibodies, but the yield of secreted antibodies is usually low probably because of proteolytic degradation. Most IgGs that have been expressed in suspension cells have been of the human IgG1 isotype. In this study, we examined whether other isotypes displayed the same sensitivity to proteolytic degradation and whether the choice of plant host species mattered. Human serum IgG displayed different degradation profiles when incubated in spent culture medium from *N. tabacum*, *Nicotiana benthamiana* or *Arabidopsis thaliana* suspension cells. Zymography showed that the protease profile was host species dependent. Three human isotypes, IgG1, IgG2 and IgG4, and a mouse IgG2a were provided with the same heavy- and light-chain variable regions from an anti-human IgM antibody and expressed in *N. tabacum* cv. BY-2 and *A. thaliana* cv. Col-0 cells. Although all tested isotypes were detected in the extracellular medium using SDS-PAGE and a functional ELISA, up to 10-fold differences in the level of intact antibody were found according to the isotype expressed, to the host species and to the culture conditions. In the best combination (BY-2 cells secreting human IgG1), we reported accumulation of more than 30 mg/L of intact antibody in culture medium. The possibility of using IgG constant regions as a scaffold to allow stable accumulation of antibodies with different variable regions was demonstrated for human IgG2 and mouse IgG2a.

Keywords: antibody, isotype, suspension cells, protease, *Nicotiana*, *Arabidopsis*.

Introduction

The therapeutic recombinant protein market is expanding rapidly and is expected to reach 160 billion dollars in 2013 (Huang and McDonald, 2012), and the fastest growing category of therapeutic drugs is antibodies (Sheridan, 2010). Although pharmaceutical monoclonal antibodies are still mainly produced in mammalian cells, plant-expressed antibodies have been under investigation for more than 20 years (De Muynck *et al.*, 2010; Whaley *et al.*, 2011) and are currently undergoing clinical trials (Twyman *et al.*, 2012; Yusibov *et al.*, 2011). Plants represent a powerful alternative protein expression platform due to their low cost of production, inherent safety, easy upscaling and ability to carry out post-translational modifications (Paul and Ma, 2011; Xu *et al.*, 2012). In addition, plant cell culture offers relevant advantages. As they grow in contained sterile bioreactors, plant suspensions are of significant biosafety (no microbes, insects or mammalian pathogens), have low risk of transgenic release in the environment and meet current good manufacturing practice production requirements. Although more expensive than production in plants, the plant cell culture allows shorter production cycles as well as higher consistency and reproducibility of the antibody. Besides, secreted antibody in the extracellular medium allows simply recovery and downstream purification (Doran, 2013; Hellwig

et al., 2004; Huang and McDonald, 2012; McDonald and Huang, 2009; Xu *et al.*, 2011).

The major current limitation of suspension cells for antibody production is the low amount of protein that accumulates in the extracellular culture medium compared with that using mammalian expression systems. In several studies reporting the expression of mouse IgG1 and IgG2b or human IgG1 in *Nicotiana tabacum* suspension cells (Table S1 and references herein), the concentration of secreted IgG in a standard medium did not exceed 7.5 mg/L (Sharp and Doran, 2001a). However, optimization of the culture medium composition resulted in higher yields, up to 107 mg/L (Vasilev *et al.*, 2013).

Secreted IgGs are degraded by plant proteolytic enzymes and/or are unstable in culture medium (Benchabane *et al.*, 2008; Calinski *et al.*, 2009; Doran, 2013; Hellwig *et al.*, 2004). Whether different IgG isotypes from different species show the same behaviour when produced in suspension cells has not been investigated. Most IgGs that have been expressed in *N. tabacum* suspension cells or whole plants have been of the mouse and human IgG1 isotypes (De Muynck *et al.*, 2010). Although they were expressed in the same host, it is difficult to compare yields, because these studies were run independently. Another unresolved question is whether antibody yield depends on the host species. Almost all studies of antibody expression in plant suspension cells have been performed using *N. tabacum*, and it

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is therefore not known whether other species would be more appropriate.

In this study, we addressed the question of whether the level of antibody accumulation depends on the isotype and host species. We focused on human isotypes because, unlike mouse or rat antibodies, they are not immunogenic when used in human therapy. We selected the frameworks corresponding to human IgG1, IgG2 and IgG4, because these three isotypes are being developed as therapeutic antibodies (Salfeld, 2007). The mouse IgG2a framework was also tested for the first time in plant suspension cells. To be able to quantitatively compare the stability of different constant (C) regions, the four antibodies were provided with the same heavy (H)- and light (L)-chain variable (V) regions from Lo-BM2, a human–rat chimeric monoclonal antibody recognizing human IgM (De Muynck *et al.*, 2009). This antibody (IgG1 isotype) was previously expressed in *N. tabacum* plants and BY-2 cells. Intact antibody and similar degradation products were observed in both the leaf intercellular fluid and the BY-2 extracellular medium. However, the yield of intact antibody was higher in the former. The Lo-BM2 variable regions combined with the constant regions of the four isotypes selected were expressed in both *N. tabacum* BY-2 and *A. thaliana* Col-0 cells, which displayed clear differences in their extracellular proteolytic profile. Our results showed that the level of antibody in the extracellular medium of the transgenic cell lines largely varied, depending on both the IgG isotype and the host species. This opens the way to the design of isotype scaffolds adapted to the appropriate host species.

Results

Proteolytic activity of the extracellular medium is species dependent

A major reason for the low yield of recombinant antibodies in plant suspension cells is proteolytic degradation by proteases secreted into the extracellular medium (Doran, 2013). We therefore examined whether suspension cells from different species would have the same extracellular protease profile by analysing the proteolytic activity of the extracellular medium from *N. tabacum* BY-2, *Nicotiana benthamiana* and *Arabidopsis thaliana* cv. Col-0 suspension cells. We performed an in-gel assay (zymography) using extracellular medium harvested after 7 days of culture with gelatin as substrate. As shown in Figure 1a, the gelatin proteolytic activity profiles of *N. tabacum* and *N. benthamiana*, two very close species, were qualitatively similar, but clearly differed from the *A. thaliana* profile. These data show that the extracellular gelatin proteolytic activity is species dependent. As the zymogram only reveals those proteases that can be renatured after SDS-PAGE and that are able to degrade gelatin, we cannot predict whether the antibody susceptibility to extracellular proteases varies according to the species. To assess antibody degradation in the different culture media, we incubated commercial human serum IgG, that is, a mixture of different isotypes, for 24 h in spent culture medium from the three species. As shown in Figure 1b, higher degradation, with a similar degradation fragment around 120 kDa, was observed in medium from the two *Nicotiana* species than in that from *A. thaliana*.

Differential accumulation of antibodies in *A. thaliana* and *N. tabacum* cell lines

Incubating purified human serum IgG in spent culture medium does not necessarily mimic what happens when the antibody is

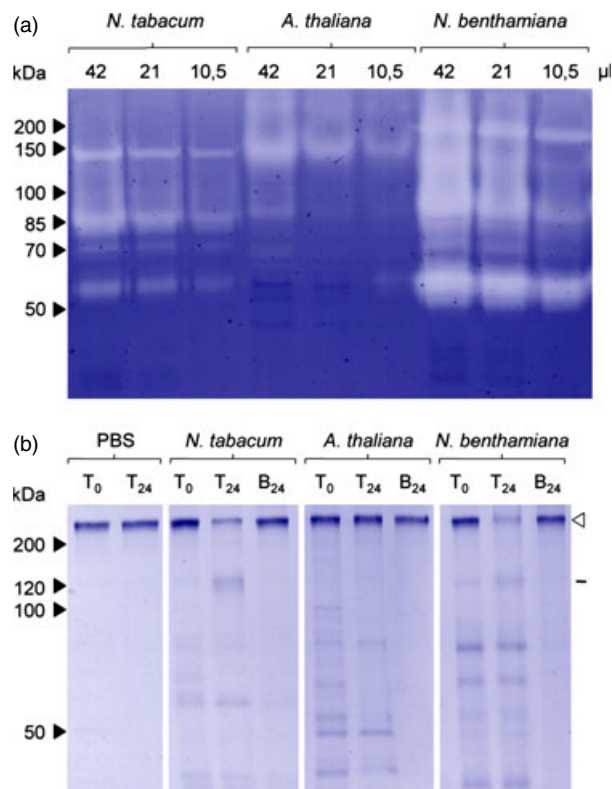


Figure 1 Proteolytic activity in the extracellular medium of *Nicotiana tabacum*, *Arabidopsis thaliana* and *Nicotiana benthamiana* suspension cells. (a) Gelatin zymogram of 42, 21 or 10.5 µl of extracellular medium harvested at day 7 diluted to 0.25 g of cells/L. (b) Human serum IgG (1.25 µg, Sigma) was incubated in 25 µl of PBS or extracellular medium (day 7 culture) from the indicated species for 0 h (T₀) or 24 h (T₂₄); then, the samples were analysed by non-reducing SDS-PAGE. B₂₄ indicates incubation of IgG for 24 h in boiled extracellular medium (control). The white arrowhead indicates full-size IgG and the dash a degradation fragment. Size markers are indicated on the left as black arrowheads.

synthesized within the plant cell and is eventually secreted into the extracellular medium. For instance, glycosylation differs between animal and plant cells and may affect IgG folding or stability or protect a site from proteases. We also could not exclude the possibility that proteolytic degradation takes place within the cell or in the apoplast before the IgG crosses the cell wall. We therefore generated cell lines expressing three different human antibody isotypes, IgG1, IgG2 and IgG4. IgG3 was not included in the study because its half-life in serum is lower than that of the other isotypes and it is not considered interesting as a means of therapy (Correia, 2010). We have already expressed Lo-BM2, a human–rat chimeric IgG1 antibody that recognizes human IgM, in *N. tabacum* BY-2 suspension cells (De Muynck *et al.*, 2009). To avoid any interference from the V region, all the antibodies were provided with the V region of Lo-BM2 by the production of cDNAs coding for the C regions of IgG2 and IgG4 fused to the V region of Lo-BM2. Mouse IgG2a, an isotype not yet produced in plant suspension cells, was also included for comparison. In addition, we compared antibody expression in two species, *A. thaliana*, which gave less antibody degradation in the spent medium (Figure 1b), and *N. tabacum*, which, although *N. benthamiana* and *N. tabacum* gave a qualitatively similar proteolytic profile, showed less intense proteolytic activity than *N. benthamiana*.

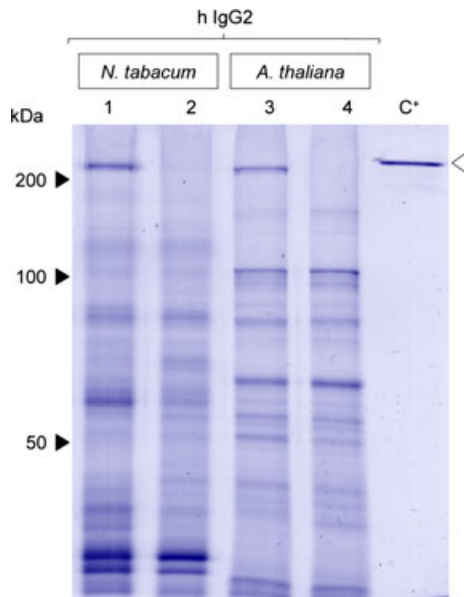


Figure 2 Electrophoretic analysis of human IgG2 in the extracellular medium. Non-reducing SDS-PAGE of 42 μ L of extracellular medium from transgenic *Nicotiana tabacum* or *Arabidopsis thaliana* cells expressing (lanes 1 and 3) or not expressing (lanes 2 and 4) human IgG2. The white arrowhead indicates full-sized human IgG2 (Sigma); the black arrowheads are size markers.

The four constructs provided with a strong constitutive transcription promoter, En2pPMA4 (De Muynck *et al.*, 2009), were introduced into *N. tabacum* and *A. thaliana* using an *Agrobacterium tumefaciens* binary plasmid. Between 17 and 54 transformed calli were obtained and transferred into 4 mL liquid cultures and then SDS-PAGE analysis and ELISA on immobilized IgM were performed on the extracellular medium harvested after 8 days of culture. As an example, Figure 2 shows an SDS-PAGE analysis (under non-reducing conditions) of the extracellular medium of *N. tabacum* and *A. thaliana* lines expressing (lanes 1 and 3) or not expressing (lanes 2 and 4) human IgG2. A band migrating at the same apparent molecular mass as full-size control human IgG2 was clearly observed in the positive clones on the Coomassie blue-stained gel, and its identity was confirmed by Western blotting and mass spectrometry (data not shown). This analysis showed that the antibody expressed in suspension cells represented a major protein in the extracellular medium. To compare the level of expression, we relied on an ELISA using coated IgM and anti-Fc polyclonal antibodies. We thus preferentially measured full-size functional antibodies, but some degradation fragments or partially assembled antibodies were possibly detected as well. Comparison of the three human IgGs in *N. tabacum* (Figure 3a, left) clearly showed large and statistically significant differences in the mean values, in the order IgG1 (10.0 mg/L) > IgG2 (5.4 mg/L) > IgG4 (0.9 mg/L), whereas in *A. thaliana* (Figure 3a, right), the level of expression of the different isotypes was more even and the differences observed

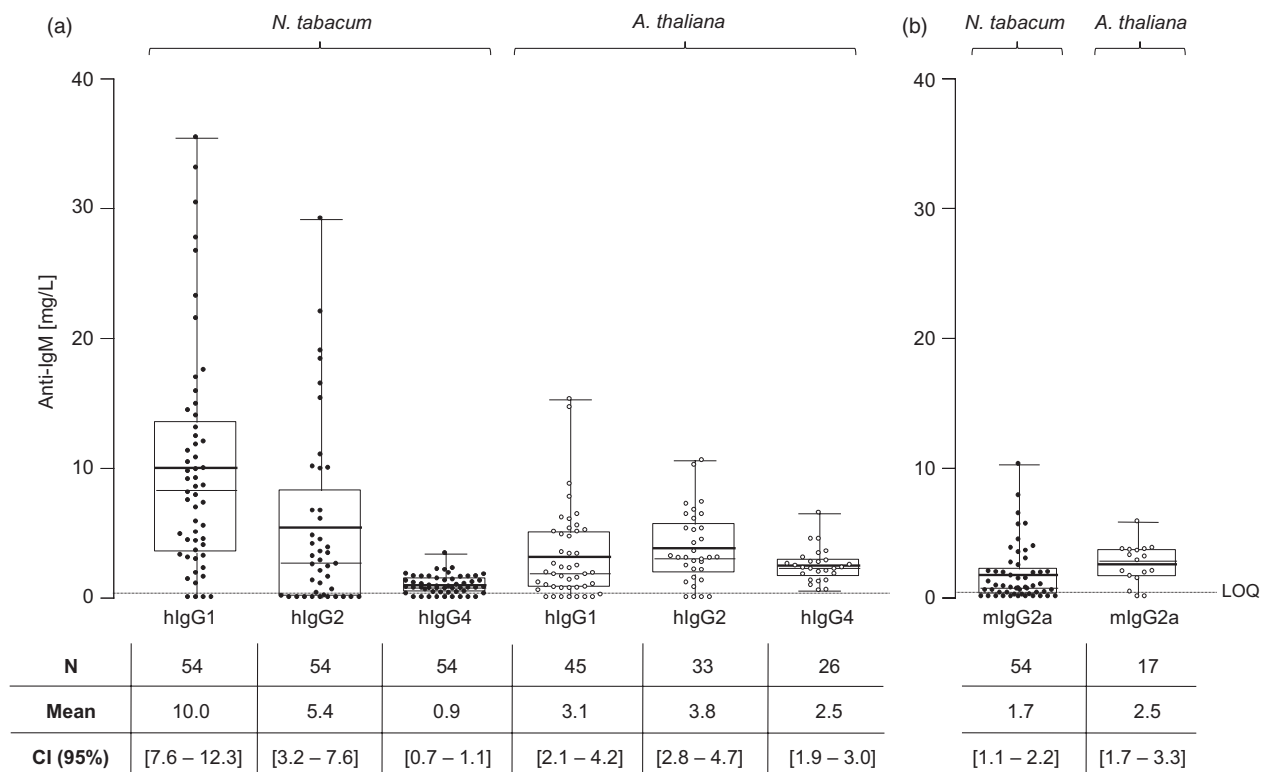


Figure 3 Accumulation levels of antibodies secreted by transgenic *Nicotiana tabacum* or *Arabidopsis thaliana* cells. The extracellular medium (EM) at day 8 of culture from the different cell lines expressing human IgG1, IgG2 or IgG4 (a) or mouse IgG2a (b) was analysed by ELISA on human IgM-coated plates using anti-human or mouse IgG Fc antibodies. Each dot indicates the concentration measured for one clone, black dots for *N. tabacum* clones and white dots for *A. thaliana* clones. The mean value is indicated by the bold horizontal line, and the box and whisker plots indicate, from bottom to top, the minimum, the 0.25, 0.5 and 0.75 quartiles, and the maximum. The limit of quantification (LOQ) is represented by the large horizontal line (0.32 mg/L). The values below the graph are the number of lines tested (N), the mean value and the values for the lower and upper 95% confidence intervals.

were not statistically different. Comparison of the levels of each human IgG isotype in the two hosts revealed another significant difference, as the level of expression of IgG1 and IgG2 was higher in *N. tabacum* than in *A. thaliana*, while the opposite was true for IgG4.

No significant difference was observed in mouse IgG2a expression in *N. tabacum* and *A. thaliana* (Figure 3b). Direct comparison of the expression of the mouse IgG2a and those of the three human isotypes should be made with caution, because the standards and the secondary antibodies used in the ELISA were different, but it appears that it was expressed at a significantly lower level than human IgG1 and human IgG2 in both plant species.

Antibodies have been purified from the extracellular medium of *N. tabacum* suspension cells (Holland *et al.*, 2010). We therefore tested the purification of human IgG2 expressed in *A. thaliana*. Affinity chromatography (protein G) resulted in a purified fraction essentially containing the antibody heavy and light chains (Figure S1). A 25-fold enrichment of the IgG2 binding activity was determined by ELISA (Figure S1). This enrichment might be underestimated because elution from protein G under acidic conditions might denature a fraction of the antibody.

Proteolytic degradation of antibodies

Differences in accumulation level might be attributed to differences in synthesis or stability. As the same transcription promoter was used for all the constructs, it was more likely that the variations were due to protein stability and, in this case, degradation by proteases would be expected to play an important role. To address this point, we selected, for each isotype, the two most highly expressing clones in each plant system and compared the extracellular medium collected after 3, 5, 7 and 10 days of culture by SDS-PAGE under non-reducing conditions and by Western blotting. The data were reproducible for both clones; the results for one of these two highly expressing clones are shown in Figure 4. In the same experiment, we also compared results using cultures of 4 mL in six-well plates and 50 mL in 250-mL Erlenmeyer flasks. In the 4-mL cultures (Figure 4, upper panel), antibodies were detected by Coomassie blue staining and Western blotting in the culture medium of both host species. However, at day 10, human IgG4 and mouse IgG2a were no longer detected in *N. tabacum* suspensions, whereas all were still detected in *A. thaliana*. In 50 mL *N. tabacum* cultures (Figure 4, lower panel), intact human IgG1 and mouse IgG2a were detected at a low level from day 3 up to day 7 or 5, respectively, and a strong degradation product band around 120 kDa accumulated rapidly from day 5. In the case of human IgG4, only a degradation fragment at around 100 kDa was observed over the entire culture period, whereas human IgG2 was well expressed until day 7 and was then degraded. In 50 mL *A. thaliana* cultures, human IgG4 and mouse IgG2a were barely detectable even by Western blotting at any time, while intact human IgG1 and IgG2 were detected from day 3 until day 10. It thus appears that 50 mL cultures in 250-mL Erlenmeyer flasks result in poorer antibody stability than 4 mL cultures. However, in cultures of both sizes, variations in stability were seen for the different antibody isotypes and the different host species.

From the above data, we can conclude that the amount of antibody in the extracellular medium varies widely according to the isotype, host species and culture conditions. Human IgG2 was clearly the most stable, because it was detected with little visible

degradation in the medium from both plant species and under both culture conditions. In contrast, human IgG1 accumulated partly as degraded forms, especially in BY-2 cells, while intact human IgG4 was barely detectable in 50 mL cultures. This somehow contrasts with the screening data (Figure 3) that showed a higher expression level for IgG1 than for IgG2. Considering that the ELISA was performed using coated IgM and anti-Fc polyclonal antibodies, a possible explanation is that partly degraded fragments were considered as positive. Another hypothesis is that full-size antibody observed on the gel might not be active in the ELISA.

The variation in the amount of a given protein detected corresponds to the balance between its synthesis and its degradation. The observation that, in most cases, the antibody was no longer present in the medium at the end of the culture suggested its degradation by extracellular proteases, a phenomenon observed previously (Doran, 2006a, 2013). We therefore used zymography to evaluate the time course of proteolysis, loading the same culture volume on the gel. As shown in Figure 5a, as expected, proteolysis increased with time of culture. As already seen in Figure 1, the *N. tabacum* and *A. thaliana* profiles differed, but, in addition, differences were also seen depending on the culture conditions (small or large culture volume). Although zymography studies are limited, as they only detect proteases that can renature after SDS-PAGE and are active on gelatin, the results showed differences in proteolysis which depended on the host species and culture conditions. Because of zymography limitations, we performed a spiking assay consisting in incubating human IgG2 purified from *N. tabacum* extracellular medium for 24 h in spent culture medium from *N. tabacum* and *A. thaliana* wild-type cells cultivated for 7 days in 4 or 50 mL (Figure 5b). For both species, the degradation of IgG2 was more pronounced in the spent culture medium collected from 4 mL (six-well plate) than from 50 mL (Erlenmeyer). This suggests that the spiking experiment did not fully reflect the fate of an antibody that is synthesized within, and secreted from, plant cells.

We then examined whether the decline and disappearance of the antibodies at days 7 and 10 were caused by slowdown of protein synthesis in stationary phase. We addressed this indirectly by performing a Western blot analysis on both the extracellular medium and cell extracts of IgG2- and IgG4-expressing *N. tabacum* lines grown in 4 or 50 mL cultures for 5, 7 or 10 days. As shown in Figure 6a,b, while the profile in the external medium was similar to that described above, the cell extract was found to contain full-size IgG2 and IgG4 at all time points, including day 10, suggesting that synthesis was still occurring. Interestingly, while IgG2 seemed to be more stable than IgG4 in the extracellular medium, IgG2 degradation products were observed in the cell extracts for both 4 mL (Figure 6a) and 50 mL (Figure 6b) cultures. We cannot exclude the possibility that intracellular degradation of IgG4 also occurred, but to such an extent that short fragments were not revealed by Western blotting. To then demonstrate that the absence of IgG4 in the 50-mL culture medium was not due to secretion impairment, we added BSA during the culture. BSA can indeed prevent the loss of secreted recombinant proteins due to protein degradation or adsorption (Doran, 2013). In the presence of BSA, IgG2 was still observed up to 10 days (stationary phase), while IgG4, which was barely observed in the absence of BSA, was clearly identified, including at day 10 (Figure 6c).

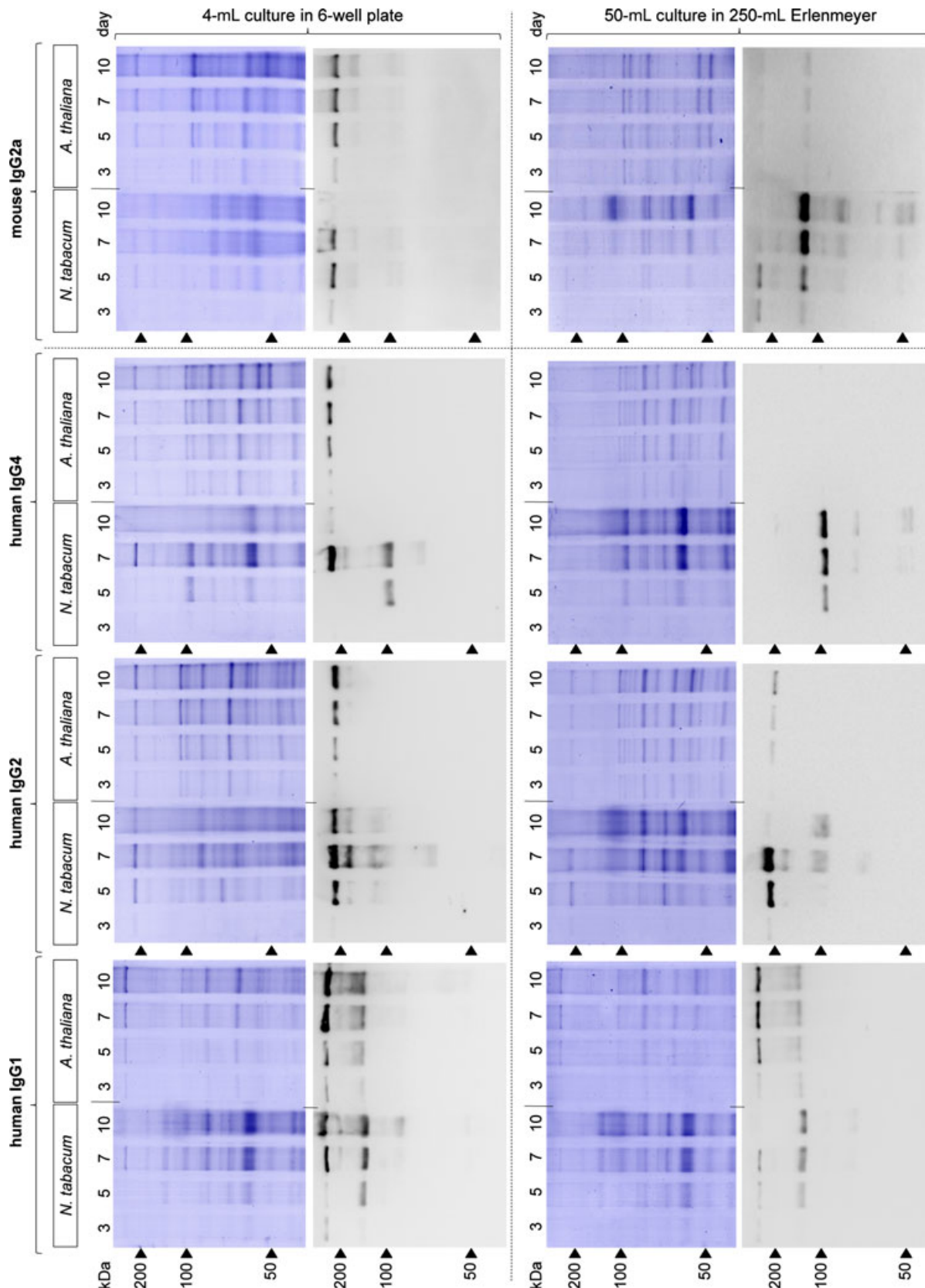


Figure 4 Time course of antibody accumulation in the extracellular medium of *Nicotiana tabacum* or *Arabidopsis thaliana* cells. *N. tabacum* or *A. thaliana* lines expressing the indicated antibody were grown in either 4 mL of medium in six-well plates or in 50 mL of medium in 250-mL Erlenmeyer flasks. The extracellular medium (42 μ L) collected at day 3, 5, 7 or 10 of culture was analysed by non-reducing SDS-PAGE and either stained with Coomassie blue (upper panel) or tested by Western blotting using anti-human or anti-mouse IgG antibodies (lower panel). The black arrowheads are size markers.

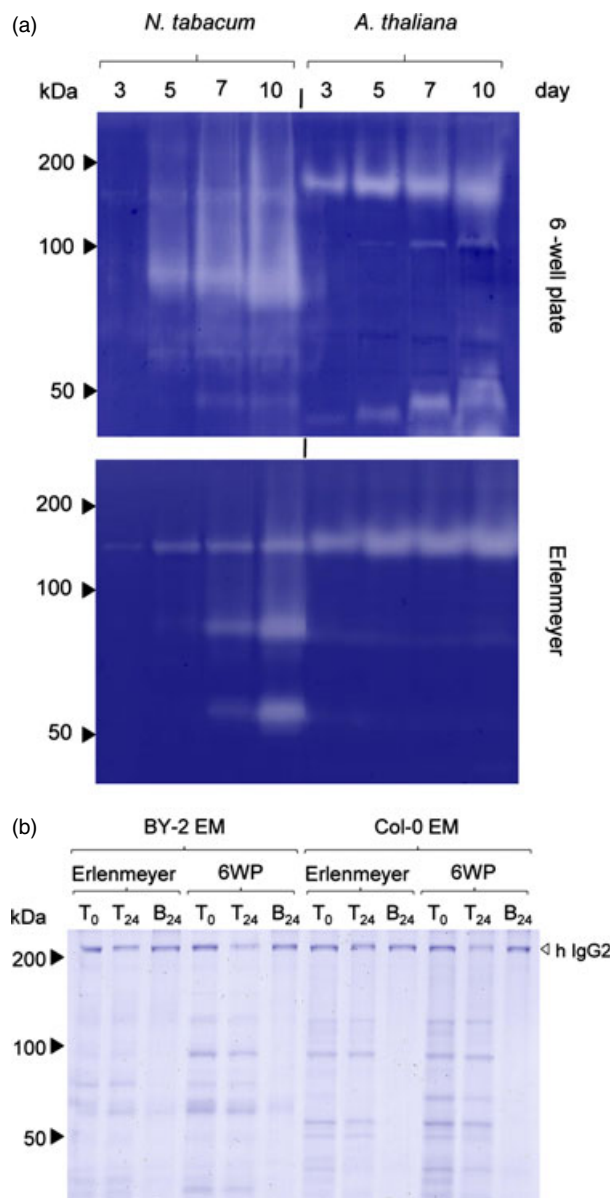


Figure 5 Proteolytic activity in the extracellular medium of *Nicotiana tabacum* and *Arabidopsis thaliana* cells. (a) Mouse IgG2a-expressing *N. tabacum* or *A. thaliana* cells were grown in 4 mL of medium in six-well plates (upper panel) or in 50 mL of medium in Erlenmeyer flasks (lower panel); then, the extracellular medium was collected on the indicated day of culture and a 42-µL sample analysed by zymography. (b) Human IgG2 purified from *N. tabacum* extracellular medium (1 µg) was incubated in 50 µL of extracellular medium (day 7 of culture) from *N. tabacum* cultivated in 4 mL (six-well plate) or 50 mL (Erlenmeyer) and *A. thaliana* cells cultivated in 4 mL (six-well plate) or 50 mL (Erlenmeyer) for 0 h (T₀) or 24 h (T₂₄); then, the samples were analysed by non-reducing SDS-PAGE. B₂₄ indicates incubation of IgG for 24 h in boiled extracellular medium (control). The white arrowhead indicates full-size human IgG2. Size markers are indicated on the left as black arrowheads.

The human IgG2 C regions can be used as scaffold to produce another idiotypic

Of the antibodies tested, human IgG2 was the most stable in both 4 and 50 mL cultures of *N. tabacum*, and we therefore wondered

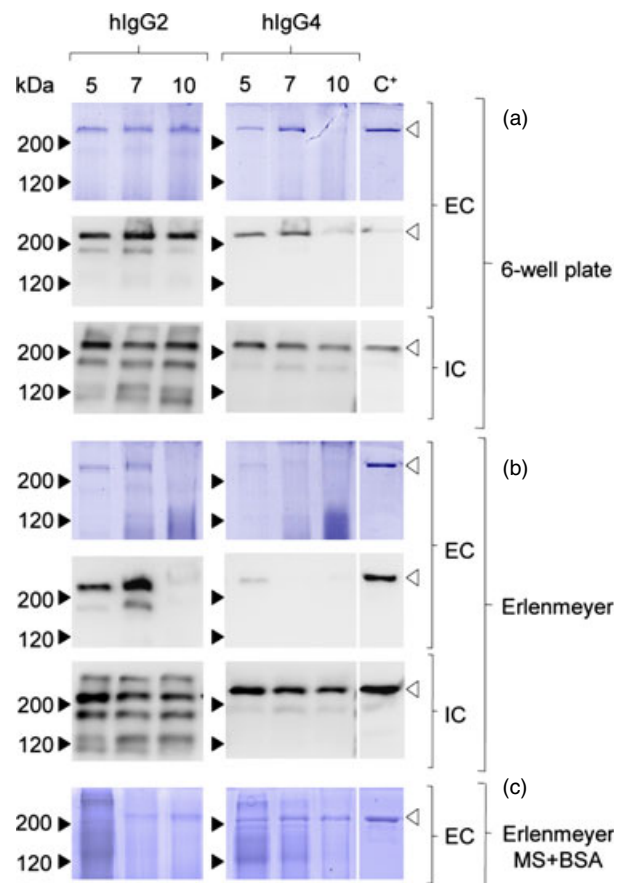


Figure 6 Extra- and intracellular expression of antibodies. *Nicotiana tabacum* cells expressing human IgG2 or IgG4 were grown in (a) 4 mL of medium in six-well plates or (b) in 50 mL of medium in 250-mL Erlenmeyer flasks; then, 25 µL of extracellular medium (EC) and 30 µg of the intracellular protein extract (IC) on the indicated day of culture were analysed by non-reducing SDS-PAGE and Western blotting using anti-human IgG antibody. (c) The same lines were grown in 50 mL of medium supplemented with 0.1% BSA in 250-mL Erlenmeyer flasks. The white arrowhead indicates full-sized human IgG (Sigma). The black arrowheads are size markers.

whether the IgG2 C regions could be used as a scaffold onto which different V regions could be grafted. To test this concept, we replaced the V regions of Lo-BM2 with those from an antibody recognizing human insulin in the human IgG2 and mouse IgG2a scaffolds, transformed *N. tabacum* cells with both constructs, grew selected clones in 4 mL in six-well plates and 50-mL Erlenmeyer flasks for 6 days and analysed the extracellular medium by SDS-PAGE under non-denaturing conditions. As shown in Figure 7, a band corresponding to intact antibody was seen in 4 mL cultures for both scaffolds, but only human IgG2 scaffold allowed accumulation of full-size antibody in 50 mL conditions, showing that, under these more challenging conditions, the IgG2 scaffold can be used to produce another idiotypic.

Discussion

Various mouse and human antibodies have been produced in plant suspension cells. However, the antibody levels reported have always been relatively low, from undetectable to 7.5 mg/L

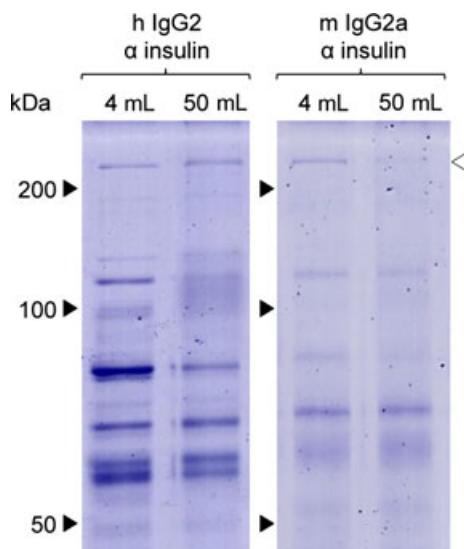


Figure 7 Expression analysis of human IgG2 and mouse IgG2a constant regions coupled to anti-insulin variable regions. *Nicotiana tabacum* cells expressing human IgG2 and mouse IgG2a constant regions coupled to anti-insulin variable regions were grown in 4 mL (six-well plate) or 50 mL (Erlenmeyer). Cells were collected after 6 days of culture, and a 42- μ L sample was analysed by non-reducing SDS-PAGE. The white arrowheads indicate full-sized IgG. The black arrowheads are molecular mass markers.

in a non-optimized medium (Table S1 and references herein). Naturally, caution is required when one quantitatively compares antibody levels obtained in different reports under different experimental conditions. However, to our knowledge, the presence of an antibody in the extracellular medium has never been shown directly on a Coomassie blue-stained gel. This low abundance is partly explained by proteolytic degradation. In this report, a functional ELISA showed that several isotypes were expressed by certain clones at levels up to 10 mg/L (up to 30 mg/L for human IgG1). As a consequence, a band corresponding to the full-size antibody could be clearly observed on a Coomassie blue-stained gel. There are several reasons that might explain this high level of accumulation: (i) the transcription promoter used was a chimeric promoter composed of a *N. plumbaginifolia* promoter (NpPMA4) reinforced by two copies of the CaMV 35S enhancer (De Muynck *et al.*, 2009); (ii) higher accumulation was obtained in 4 mL cultures in six-well plates than in 50 mL cultures in Erlenmeyer flasks, conditions similar to those used in most previous studies (De Muynck *et al.*, 2009; Fischer *et al.*, 1999; López *et al.*, 2010; Sharp and Doran, 2001a); (iii) because proteolytic degradation was shown to increase over time, the stage when the culture medium is collected for antibody measurement is crucial, as shown previously (De Muynck *et al.*, 2009; Holland *et al.*, 2010; Sharp and Doran, 2001b); and (iv) we screened a large number of transformants (up to 54). Because of the position effect, there is a large variation in the expression level for the same construct (as shown in Figure 3) and screening of a low number of transgenic lines might result in a high-expression clone being missed. For instance, five of the 54 IgG1-expressing clones displayed an expression level above 25 mg/L. Table S2 shows the probability of finding one or more of these five clones depending on the number of clones screened. Screening of 10 clones would result in a probability of 0.73 of finding one of

these five clones, while screening of 20 would result in a probability of 0.95 of finding one of the five and a probability of 0.73 of finding a second. It is therefore essential to screen a large number of clones to select a high expressor. To help with this step, the co-expression of a visual marker, such as the fluorescent protein DsRed, can be used for easy pre-screening (Holland *et al.*, 2010; Kirchhoff *et al.*, 2012), as long as a good correlation is found between the expression of the reporter gene and that of the gene of interest. Finally, the antibody yields reported here might be further improved by optimizing the growth conditions. For instance, increasing the concentration, and changing the type, of the nitrogen source has been shown to markedly increase antibody accumulation in the culture medium (Holland *et al.*, 2010; Ullisch *et al.*, 2012). Very recently, a study based on fractional factorial designs showed that extensive optimization of medium composition of *N. tabacum* cells resulted in strong improvement of the level of a human IgG1 antibody in the extracellular medium (Vasilev *et al.*, 2013). It will be of major interest to test whether these optimized conditions might improve the stability of the various isotypes in 50 mL cultures.

Another finding in this study was the large variation in accumulation of antibodies of different isotypes. In *N. tabacum*, the species most often used for antibody expression, the mean expression level in 4 mL cultures varied from 10.0 (IgG1) to 0.9 (IgG4) mg/L. In this study, as the same plasmid, including the same initiation codon context, was used in all cases, the synthesis rate of all isotypes is expected to be similar, as suggested by the similar intracellular level of IgG2 and IgG4 in *N. tabacum* (Figure 6a,b). There is no obvious reason why some antibodies would be impaired in secretion because they all share the same signal peptide. Moreover, as the different isotypes have approximately the same size, we do not expect major differences in the last step consisting in cell wall crossing. The observation that BSA added to the culture medium improved IgG2 and IgG4 accumulation in the medium supports the hypothesis that antibodies are actually secreted but become partly undetected because of adsorption to the vessel or degradation by extracellular proteases (Doran, 2013). This hypothesis was further supported by the observation that full-size IgG was observed at certain stages of *N. tabacum* cell culture and then disappeared, while degradation products were still observed. Human isotypes are known to behave differently in human serum due to their structures. For instance, while the intrachain disulphide bonds of IgG1, IgG2 and IgG4 are similar, the interchain bonds show differences (Correia, 2010). Actually, human antibodies are known to exhibit isotype-specific degradation when exposed *in vitro* to proteases (Baici *et al.*, 1982; Berasain *et al.*, 2003; Brezski and Jordan, 2010), so it is not unexpected to see variations when they are expressed in plant suspension cells. A previous study on Lo-BM2 (IgG1 isotype) showed that the degradation profile was similar in BY-2 cells as in whole plants, although the yield differed (De Muynck *et al.*, 2009). It would be interesting to determine whether this would be true for all the isotypes studied in this report.

Of the three human IgGs tested, IgG4 showed the lowest level of functional antibody in both *N. tabacum* and *A. thaliana*. This isotype is known to form labile inter-heavy-chain disulphide bonds, resulting in the creation of half antibody molecules (Schuurman *et al.*, 2001). We identified an IgG4 fragment with an apparent molecular mass of around 100 kDa that accumulated in both 4 and 50 mL cultures of *N. tabacum* cells. This might reflect the accumulation of an half IgG4 molecule

(72 kDa + carbohydrate moiety) and, besides degradation, explain the low level of functional antibody seen in the ELISA. Alternatively, it might represent a fully assembled antibody that has been degraded at either the N- or C-terminal; however, this is unlikely for the 100 kDa fragment, as its mass spectrometry analysis identified several heavy- and light-chain peptides (Figure S2), the presence of which shows that the minimal compatible size would be 120 kDa without taking the carbohydrate into account. This size is too large compared with that observed on the gel. Thus, the hypothesis of an half IgG4 molecule deserves further investigation.

One important conclusion from this study is that a given isotype has different stabilities in the two host species and that different isotypes have different stabilities in the same culture system. For instance, all the isotypes displayed lower stability at the end of the 4-mL cultures of *N. tabacum* compared with those using *A. thaliana*. This is in agreement with the observations that proteolytic activity increased over culture time and that the gelatin proteolytic profiles clearly differed between these species (Figures 1a and 5a). The IgG expression level at day 10 was higher in 4 mL (six-well plate) than in 50 mL (Erlenmeyer) cultures. This observation is not in agreement with the higher proteolytic activity or with the stronger degradation of purified IgG2 in a spent medium of 4 mL cultures. However, this comparison needs to be analysed with caution. First, zymography only provides a partial picture of the protease complement in the extracellular medium. Many proteases have been identified in extracellular medium from cultures of *N. tabacum* (Navarre et al., 2012) and *A. thaliana* (Borderies et al., 2003), and the identities of all of those involved in antibody degradation are not yet known. Second, incubation in a spent medium does not reflect the dynamics of a cell culture. For instance, it is possible that degradation already occurs in the secretory pathway or in the apoplast before the antibody crosses the wall. Strategies to reduce the loss of secreted proteins have been implemented (Benchabane et al., 2008; Doran, 2013; Hellwig et al., 2004; Sharp and Doran, 2001b; Tsoi and Doran, 2002). Our data suggest that another strategy for the expression of any protein would be to test different host species to identify a protease-compatible extracellular environment. Another and non-exclusive explanation as to why different types of culture (six-well plates or Erlenmeyer flasks) result in different levels of antibody accumulation in the medium is related to adsorption. Antibody adsorption to glass vessels has been reported (Doran, 2006b), and the adsorption level depends on the type of culture vessel used (plastic, glass, stainless steel) (Kwon et al., 2012). The comprehensive study of differences in the antibody levels will require a more detailed analysis of the culture parameters (e.g. aeration, agitation, pH change, flask type and medium composition). These parameters could then be exploited to scale up cultures to achieve better yields in large culture volumes.

Human IgG1 is the isotype most frequently chosen for the development of therapeutic antibodies, as its Fc region can trigger effector functions. However, IgG2 and IgG4 are also valuable therapeutic candidates (Salfeld, 2007). According to our data, the choice of a suitable host or culture condition is required to optimize the production of each of these isotypes. Our data also showed that, once an interesting constant scaffold has been identified, the variable regions can be exchanged. The N-site of the CH2 region is conserved in all human and mouse isotypes, while an extra N-site is present in the CH3 region of human IgG3 and mouse IgG1 isotypes. Because glycosylation is known to have

an influence on IgG stability and activity, it would be of particular interest to perform a comparative analysis of the glycosylation profile of the different isotypes in *N. tabacum* and *A. thaliana* hosts. Glyco-engineering is an obligatory step for therapeutic applications, and tools are available for the humanization of antibodies (Gomord et al., 2010; Loos and Steinkellner, 2012). The design of different isotype scaffolds and their use in the most appropriate host should therefore lead to optimal production of antibodies in plant cells.

Experimental procedures

Plant cell cultures

Nicotiana tabacum cv. Bright yellow 2 (BY-2) (Nagata et al., 1992) suspension cells were grown in the dark at 25 °C with agitation on a rotary shaker (90 rpm) in liquid MS medium [0.44% Murashige and Skoog salts (MP BIOMEDICALS, Solon, OH), 3% sucrose, 0.02% KH_2PO_4 , $2.5 \cdot 10^{-4}\%$ thiamine, $5 \cdot 10^{-3}\%$ myo-inositol and $2 \cdot 10^{-5}\%$ 2,4-D, pH 5.8 (KOH)] supplemented when indicated with 0.1% BSA (Sigma, St-Louis, MO). Cultures were grown either in 50 mL of medium in a 250-mL Erlenmeyer flask or in 4 mL of medium in six-well plates (GREINER BIO-ONE, Frickenhausen, Germany), and a 5% inoculum was transferred each week into fresh medium. Transformed cells were grown in medium supplemented with 100 µg/mL of kanamycin.

Arabidopsis thaliana cv. Col-0 (Axelos et al., 1992) suspension cells were grown in liquid medium [0.32% Gamborg B5 (Duchefa Biochemie, Haarlem, The Netherlands), 3% sucrose, 0.05% MES, $2 \cdot 10^{-4}\%$ 2,4D, pH 5.8 (KOH)] and were diluted (10%) weekly into fresh medium. Transformed cells were grown in medium supplemented with 20 µg/mL of hygromycin.

After 7 days of culture, *N. tabacum* and *A. thaliana* cell mass reached 0.22 ± 0.02 g/mL and 0.12 ± 0.03 g/mL, respectively, in six-well plates and 0.20 ± 0.03 g/mL and 0.11 ± 0.01 g/mL, respectively, in Erlenmeyer (means $\pm$ SD).

For both species, calli were transferred monthly onto solid medium (0.8% agar).

Gene constructs and plant cell transformation

cDNAs coding for the constant (C) region of the heavy (H) or kappa light (L) chain of mouse IgG2a (P01863), human IgG2 (P01859) or human IgG4 (P01861) fused to the variable regions of monoclonal antibody Lo-BM2 (De Muynck et al., 2009) were synthesized (GenScript, Piscataway, NJ) with codons optimized for *A. thaliana*. For *N. tabacum* transformation, they were cloned separately into expression cassettes containing a strong constitutive transcription promoter, En2pPMA4 (De Muynck et al., 2009), and the *nos* terminator. The H-chain cassette was cloned into the pAUX3131 vector and the light chain into the pAUX3132 (Goderis et al., 2002) vector, and the two cassettes were then introduced in tandem into the binary vector pPZP-RCS2 (Goderis et al., 2002) provided with the *nptII* marker gene (kanamycin resistance) to generate the final constructs pPZP-RCS2-nptII-MIgG2aLoBM2, pPZP-RCS2-nptII-HIgG2-LoBM2 and pPZP-RCS2-nptII-HIgG4-LoBM2. The binary plasmid pPZP-RCS2-nptII-HIgG1-LoBM2 was obtained previously (De Muynck et al., 2009). For *A. thaliana* transformation, the same constructs were generated except that the *nptII* marker gene was replaced by the *hptI* gene, conferring resistance to hygromycin, yielding four more binary plasmids: pPZP-RCS2-hptI-MIgG2aLoBM2, pPZP-RCS2-hptI-HIgG1-LoBM2, pPZP-RCS2-hptI-HIgG2-LoBM2 and pPZP-RCS2-

hptl-HlgG4-LoBM2. The eight binary vectors were transferred into *Agrobacterium tumefaciens* LBA4404virG (van der Fits *et al.*, 2000) by electroporation. *N. tabacum* BY-2 and *A. thaliana* Col-0 cells were transformed as described previously (De Muynck *et al.*, 2009). The variable regions (VH + VL) corresponding to a monoclonal antibody recognizing human insulin were cloned from a non-commercial mouse hybridoma, grafted to human IgG2 and mouse IgG2a constant region (H + L) sequences and assembled in two binary plasmids pPZP-RCS2-nptII-MlgG2a-INS and pPZP-RCS2-nptII-HlgG2-INS.

Screening using an indirect functional ELISA

Between 17 and 54 calli were selected from the cells transformed to express each of the four antibody variants and were subcultured three times in liquid culture before harvesting the extracellular medium for ELISA. Microtitre plates (96-well) (GREINER BIO-ONE) were coated overnight at 4 °C with 100 µL/well of human IgM (5 µg/mL) (Calbiochem, San Diego, CA) in carbonate buffer [71 mM NaHCO₃, 35 mM Na₂CO₃, pH 9.5 (NaOH)]. The plates were then washed three times with washing buffer (PBS, 0.1% Tween 20), blocked for 1 h at 25 °C with 200 µL/well of blocking buffer (PBS, 5% non-fat milk) and washed three times with washing buffer and then 100 µL of PBS or 100 µL of doubling dilutions in PBS of the culture supernatant from each transgenic line or the wild-type strain as negative control and antibody LoBM2 produced in NS0 cells (De Muynck *et al.*, 2009) diluted in PBS as the positive control. The plates were incubated for 1.5 h at 37 °C, washed three times and incubated for 1 h at 25 °C with 100 µL/well of the corresponding horseradish peroxidase (HRP)-conjugated anti-human or anti-mouse IgG Fc region antibodies (Sigma, 1/10 000). After four washes, 100 µL of o-phenylenediamine (OPD, Sigma) peroxidase substrate in citrate buffer (0.05 M Na₂HPO₄, 0.025 M citric acid, pH 5.0) was added; then, after 15 min at 20 °C, 50 µL of stopping solution (1 M H₂SO₄) was added and the absorbance measured at 490 nm (Model 550, Microplate Reader; Bio-Rad, Hercules, CA). For the detection of the mouse IgG2a, the positive control was mouse IgG2a (Sigma) directly coated on the plate instead of IgM.

SDS-PAGE and Western blotting

Proteins were boiled for 5 min in non-reducing SDS loading buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue) supplemented with 1 mM PMSF and protease inhibitor cocktail (1 µg/mL each of leupeptin, aprotinin, antipain, chymostatin and pepstatin), centrifuged for 5 min at 13 000 rpm and separated by SDS-PAGE (8% polyacrylamide). For colloidal Coomassie blue staining, gels were incubated for 1 h at room temperature in fixation solution (50% ethanol, 2% phosphoric acid), washed three times with water, stained overnight in staining solution [34% methanol, 17% ammonium sulphate, 3% phosphoric acid, 700 mg/L of Coomassie Brilliant Blue G-250 (SERVA, Heidelberg, Germany)] and destained in water.

For Western blotting, proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA) using a semi-dry device (Trans-blot semi-dry electrophoretic transfer cell, Bio-Rad); then, the membrane was blocked for 1 h at room temperature with 3% (w/v) milk powder (GLORIA, Lima, Peru) in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) and then incubated for 1 h at room temperature with HRP-conjugated antibodies against whole-mouse IgG (Sigma, 1 : 10 000 dilution in TBST) or against whole-human IgG (Sigma, 1 : 10 000 dilution in TBST). In a preliminary study, we checked that the anti-human IgG Fc region

antibody used in ELISA gave similar signals with all three human IgGs tested. Signals were quantified using a Kodak Image Station 4000R (Eastman Kodak company, Rochester, NY).

Cell extract preparation

Plant cells (2 mL of culture) were filtered on three layers of Miracloth (Calbiochem), resuspended in 700 µL of homogenization buffer [50 mM Tris-HCl, pH 8.5, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride (PMSF) and a protease inhibitor cocktail (1 µg/mL each of leupeptin, aprotinin, antipain, chymostatin and pepstatin)], and ground at 4 °C in a Precellys 24 (Bertin Technologies, Saint-Quentin, France) for 3 × 45 s at 5000 rpm with a 1-min break using 500 mg of glass beads (0.85–1.23 mm). The homogenate was centrifuged at 4 °C at 10 000 g for 5 min, and the supernatant was centrifuged for 15 min at 4 °C at 130 000 g to obtain the soluble protein fraction (supernatant). The protein content was measured (Bradford, 1976) and aliquots analysed by SDS-PAGE and Western blotting.

Zymography

Proteins were incubated for 20 min at room temperature in non-reducing SDS loading buffer and electrophoresed on a 8% polyacrylamide gel containing 0.05% (w/v) gelatin, which was then washed for 3 × 20 min at room temperature in 2.5% Triton X-100 in distilled water, incubated for 16 h at 37 °C in 10 mM MES, 5 mM CaCl₂, 1% Triton X-100, pH 5.8 (KOH), and stained as described previously.

Purification of IgG

Extracellular medium (150 mL) of a 6-day-old culture was filtered on Miracloth, supplemented with 15 mL of 200 mM Na₂HPO₄, pH 7.9 (H₃PO₄), and filtered successively on a 1-µm and 0.45-µm filter. The filtrate was loaded on a HiTrap Protein G (1 mL, GE Healthcare), washed with 200 mM Na₂HPO₄, pH 7.0, and eluted with 0.1 M glycine, pH 2.0 (HCl). Elution fractions were supplemented with 0.1 volume of 1 M Tris, pH 9.0 (HCl).

IgG spiking test

Nicotiana benthamiana, *N. tabacum* and *A. thaliana* extracellular mediums from 7-day-old cultures in 4 mL (six-well plate) or 50 mL (Erlenmeyer) were harvested, then purified human IgG2 expressed in *N. tabacum* or commercial seric human IgGs (Sigma) were added, and the mixture was incubated at 37 °C for 24 h. As controls, incubations were performed for 0 h or 24 h in a medium that had been boiled for 5 min prior to IgG addition. In the latter case, proteins aggregated by the heat treatment were removed by centrifugation. Degradation of IgG was examined by SDS-PAGE.

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Supporting information

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

Figure S1. Purification of IgG2 secreted from *A. thaliana*.

Figure S2. Mass spectrometry analysis of an IgG4 degradation fragment.

Table S1. Fully monoclonal IgG isotypes secreted in plant suspension cells.

Table S2. Probability of obtaining at least $\times$ high-expression transformants when sampling n transformants of 54.