Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 97–108 doi: 10.1111/pbi.12360

Rice endosperm produces an underglycosylated and potent form of the HIV-neutralizing monoclonal antibody 2G12

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Received 12 November 2014;
revised 8 February 2015;
accepted 16 February 2015.
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Keywords: rice, endosperm, 2G12, HIV, microbicides, glycosylation.

Summary

Protein microbicides against HIV can help to prevent infection but they are required in large, repetitive doses. This makes current fermenter-based production systems prohibitively expensive. Plants are advantageous as production platforms because they offer a safe, economical and scalable alternative, and cereals such as rice are particularly attractive because they could allow pharmaceutical proteins to be produced economically and on a large scale in developing countries. Pharmaceutical proteins can also be stored as unprocessed seed, circumventing the need for a cold chain. Here, we report the development of transgenic rice plants expressing the HIV-neutralizing antibody 2G12 in the endosperm. Surprisingly for an antibody expressed in plants, the heavy chain was predominantly aglycosylated. Nevertheless, the heavy and light chains assembled into functional antibodies with more potent HIV-neutralizing activity than other plant-derived forms of 2G12 bearing typical high-mannose or plant complex-type glycans. Immunolocalization experiments showed that the assembled antibody accumulated predominantly in protein storage vacuoles but also induced the formation of novel, spherical storage compartments surrounded by ribosomes indicating that they originated from the endoplasmic reticulum. The comparison of wild-type and transgenic plants at the transcriptomic and proteomic levels indicated that endogenous genes related to starch biosynthesis were down-regulated in the endosperm of the transgenic plants, whereas genes encoding prolamin and glutaredoxin-C8 were up-regulated. Our data provide insight into factors that affect the functional efficacy of neutralizing antibodies in plants and the impact of recombinant proteins on endogenous gene expression.

Introduction

More than 35 million people are currently living with HIV/AIDS (UNAIDS, 2013). Most of the infected population lives in sub-Saharan Africa, where the disease caused more than 1.8 million deaths in 2012. Although the number of deaths caused by HIV/AIDS has declined over the last few years due to the deployment of antiretroviral drugs, the virus continues to spread because there are no effective vaccines, and prophylaxis still remains reliant on barrier methods or abstinence, both of which are characterized by poor compliance. Microbicides containing antiretroviral drugs and/or HIV-neutralizing antibodies could potentially be used to prevent the transmission of HIV if sufficient quantities could be supplied to protect the at-risk population (Ramessar *et al.*, 2010).

HIV-1 entry into susceptible cells requires the envelope (Env) protein, a trimer of gp120/gp41 heterodimers with gp120 acting as the external surface subunit that engages cellular receptors, and gp41 as the transmembrane subunit that mediates membrane fusion (Poignard *et al.*, 2001). The gp120 protein interacts with CD4 on the surface of lymphocytes and then with the coreceptor CCR5 or CXCR4, so antibodies against gp120/gp41 can be used to prevent the uptake of HIV into target cells. A panel of broadly neutralizing anti-HIV antibodies has been described, and these differ in terms of the HIV strains and clades that are neutralized and the potency of neutralization *in vitro* (reviewed by McCoy and Weiss, 2013). However, only a few of the antibodies have been tested in preclinical studies for their ability to prevent the transmission of HIV, or the related model virus SHIV, in animal experiments (Ramessar *et al.*, 2010).

HIV-neutralizing antibodies 2G12, 2F5 and 4E10 have been produced in Chinese hamster ovary (CHO) cells for prophylactic and therapeutic use (Hofmann-Lehmann *et al.*, 2001; Stiegler *et al.*, 2002) but although yields of 5–10 g/L can be achieved in commercial antibody manufacturing processes, the drawbacks of this platform include the high costs of fermentation

and downstream processing infrastructure as required for compliance with good manufacturing practice (GMP), and the high running costs, which translate into a correspondingly high cost of goods. CHO cells are therefore economically suitable for the manufacture of injectable antibodies for specific forms of cancer (small patient populations, low doses) but not for microbicide applications where the target population numbers in the millions and large, repetitive doses of multiple antibodies are required (Ma *et al.*, 2013). The cost and capacity aspects make this platform unfeasible to deploy in developing countries because 100–1000 kg of each antibody would be required annually and each microbicide would require 2–3 antibodies targeting different epitopes to prevent the emergence of 'escapes' (Ramessar *et al.*, 2010). CHO cells also have low capacity/scalability, and safety risks arise due to the potential for contamination with viruses (Chu and Robinson, 2001; Stöger *et al.*, 2000, 2002, 2005).

Plants could potentially be developed into a platform for the large-scale production of prophylactic antibodies because they are inexpensive to cultivate, more scalable than any other platform and, for these reasons, should be easy to deploy in developing countries (Fischer *et al.*, 2004; Ma *et al.*, 2003; Paul *et al.*, 2013; Ramessar *et al.*, 2010). Technoeconomic evaluation in tobacco, maize and rice has shown that upstream production costs can be reduced to 5% of the cost of fermentation, mainly because plants can be grown in greenhouses that do not need to be GMP ready (Tusé *et al.*, 2014). Although pioneering work has been carried out using tobacco, cereal seeds are likely to be the most suitable platform for deployment in such areas because the infrastructure for large-scale cultivation and harvesting is already in place and the dry seeds favour product stability (Lotter-Stark *et al.*, 2012; Ramessar *et al.*, 2008c). Cereal seeds also have GRAS status which means that crude seed extracts containing antibodies could be used to produce microbicides, removing the need for downstream processing which accounts for up to 80% of overall production costs in plants, and this minimal processing concept has already been demonstrated for other proteins (Ning *et al.*, 2008; Xie *et al.*, 2008). Rice as a platform for molecular farming provides unique advantages in terms of protein storage compartments derived from the endomembrane system in the seeds (Drakakaki *et al.*, 2006; Khan *et al.*, 2012). Several reports have described the economic viability of production processes based on maize and rice, even when downstream processing is included (Evangelista *et al.*, 1998; Nandi *et al.*, 2005). In sub-Saharan Africa, which suffers the greatest HIV burden in the world, rice is not grown as widely as maize, and the latter has already been investigated as a potential source of HIV-neutralizing antibodies (Rademacher *et al.*, 2008; Ramessar *et al.*, 2008b). However, pharmaceutical transgenic crops would be grown in containment rather than in the field, at least initially, so it makes sense to compare the productivity of different cereals and the activity of the antibodies produced therein to find the most promising cultivation system for antibody production in containment (Kuo *et al.*, 2013; Ramessar *et al.*, 2008a).

Here, we investigated the accumulation of antibody 2G12 in rice endosperm focusing on its sequestration into subcellular storage compartments. We also investigated the glycosylation of the antibody heavy chain and its impact on the binding behaviour and HIV-neutralizing properties of the assembled antibody. The impact of our results on the production of prophylactic, HIV-neutralizing antibodies in plants is discussed.

Results

Generation of transgenic rice plants expressing the HIV-neutralizing antibody 2G12

Rice embryos were transformed by particle bombardment with constructs containing the coding sequences for the 2G12 heavy and light chains, under the control of the rice glutelin-1 promoter (Ramessar *et al.*, 2008b) plus a third construct containing the selectable marker *hpt* (Christou and Ford, 1995). Embryo-derived callus was selected on hygromycin-supplemented medium, and 20 independent transformants were regenerated and transferred to the greenhouse. Leaves and seeds from these plants were analysed by PCR to confirm the presence of the transgene, by dot blot to confirm transgene expression and by immunoglobulin-specific sandwich ELISA to confirm the presence of correctly assembled 2G12. Six independent lines expressing 2G12 at the highest levels were self-pollinated to produce T1 and T2 seeds for more detailed analysis.

Confirmation of antibody assembly in T1 transgenic endosperm

The presence of correctly assembled 2G12 in T1 rice endosperm was confirmed using the immunoglobulin-specific sandwich ELISA described above. The antibody content of the endosperm was determined by calculating the concentrations from titration curves based on positive controls spiked with known concentrations of the same antibody produced in maize, which is immunologically identical to the commercial CHO standard (Figure 1). We used nontransformed rice endosperm as a negative control to rule out cross-reaction between the immunoglobulin-specific detection antibody and endogenous plant proteins. Among the six independent lines expressing both antibody chains, we selected the two lines (4 and 14) producing the highest levels of assembled 2G12 for more detailed investigation. The yield of 2G12 in the best performing line (14) was 37.168 μg/g in the T1 generation, but this increased to 46.4 μg/g in the T3 generation after two rounds of self-pollination.

Accumulation of 2G12 in rice endosperm induces the formation of novel protein bodies

The localization of 2G12 in the endosperm of lines 4 and 14 was investigated by fluorescence microscopy and transmission electron microscopy (TEM). Colocalization with glutelin indicated that most of the antibody accumulates in the protein storage vacuoles (glutelin bodies, PB-II), but additional labelling was detected outside these organelles (Figure 2a–c). Consistent with this result, TEM showed that most of the antibody had accumulated in glutelin bodies and only a small amount in the ER-derived prolamin bodies (also known as type-I protein bodies, PB-I). In the transgenic endosperm, we also observed novel protein bodies that were studded with ribosomes although these bodies did not show significant labelling (Figure 3a–b). There was no evidence of 2G12 labelling in the untransformed control plants.

The 2G12 heavy chain in rice endosperm is predominantly aglycosylated

The 2G12 antibody was purified from rice endosperm extracts by protein A affinity chromatography. Neither the flow through nor the wash fractions contained measurable amounts of the 2G12 heavy and light chains, indicating that the antibody assembled

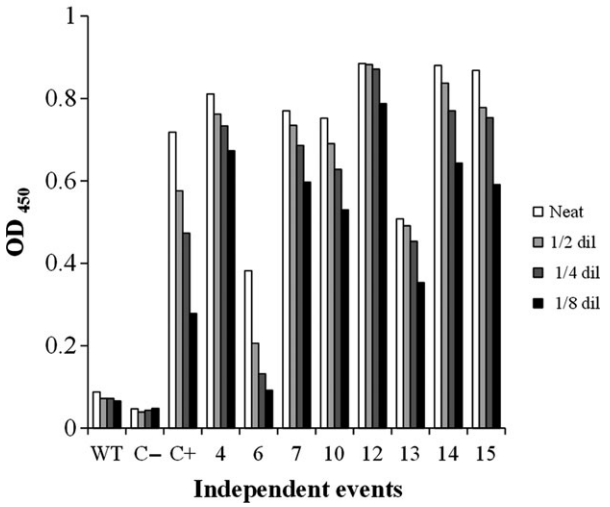


Figure 1 The expression of 2G12 in mature rice endosperm. An immunoglobulin-specific sandwich ELISA procedure was used to screen endosperm extracts from independent transgenic lines for 2G12. The plates were coated with an anti-human κ-chain antibody, and captured 2G12 was detected with an anti-human IgG antibody labelled with horseradish peroxidase. Four serial dilutions were loaded per sample (neat, 1/2, 1/4 and 1/8). WT = wild-type extracts; C^- = negative control (PBS); C^+ = 2G12 purified from maize (500 ng/mL) as positive control. OD = optical density at 450 nm.

correctly *in planta* and was retained efficiently on the resin. The final concentration of pure antibody from the T1 generation was 34 μg/g dry seed weight, representing a recovery of 97%, but after protocol optimization and two rounds of self-pollination to produce T3 homozygous seeds, the antibody concentration increased to 42 μg/g dry seed weight, representing a recovery of 96%. The presence of the heavy and light chains was confirmed by reducing SDS-PAGE (50- and 25-kDa bands). No degradation products were present, suggesting the antibody remained stable in the seeds (Figure 4).

The purified antibody heavy chain was digested with trypsin and analysed by MALDI-TOF mass spectrometry. Remarkably, we found that 50% of the EEQYNSTYR peptide, which contains the specific NSx glycan acceptor sequence responsible for heavy chain glycosylation, was completely aglycosylated. Among the remainder, 19% and 14% contained vacuolar-type N-glycans (MUXF and MMXF, respectively), 13% carried a single N-acetylglucosamine (GlcNAc) attached to the asparagine residue, suggesting the presence of ENGase activity in the early part of the secretory pathway, and the remaining 4% carried high-mannose glycans of the Man_7 configuration (Figure 5).

The underglycosylated ^{OS}2G12 shows normal binding activity but enhanced HIV-neutralization activity relative to ^{NT}2G12

Because 50% of the 2G12 heavy chain peptide was aglycosylated in rice, statistically (assuming random association between chains), it is likely that 25% of the assembled antibody (^{OS}2G12) is fully aglycosylated, 50% is hemiglycosylated and 25% is fully glycosylated, with only a minor fraction containing complex-type glycans on both chains. The ability of the antibody to bind gp120 was tested *in vitro* using an antigen-specific ELISA with both the purified antibody and the crude extracts of each rice line. The capture reagent was gp120-IIIB and the bound

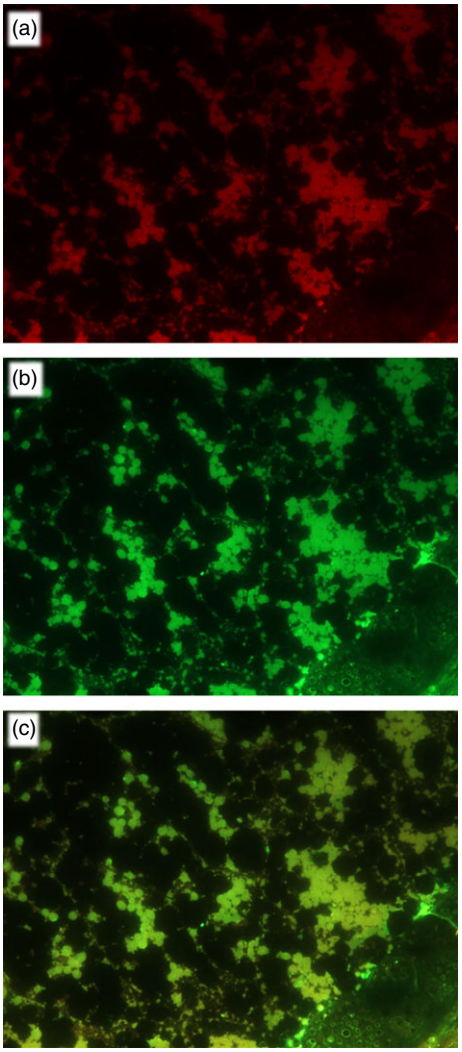


Figure 2 Localization of 2G12 and the seed storage protein glutelin in rice endosperm. Immunofluorescence microscopy confirmed that 2G12 (line 4) accumulated in the protein storage vacuoles (glutelin bodies, PB-II) and to a lesser extent in prolamin bodies (PB-I). (a) Red fluorescence shows the localization of 2G12; (b) Green fluorescence shows the localization of glutelin; (c) Yellow fluorescence shows the colocalization of 2G12 and glutelin. Antibody 2G12 was detected with a polyclonal rabbit anti-HC or anti-LC antiserum diluted 1 : 100 and a secondary AlexaFluor 594-conjugated goat anti-rabbit IgG (red fluorophore). An antiglutelin antibody (green fluorophore) was used to detect endogenous glutelin.

2G12 was detected using a mixture of anti-human gamma and anti-human kappa antibodies, which bind the heavy and light chains, respectively. The transgenic endosperm extracts showed higher gp120-binding activity than wild-type endosperm extracts as expected, which confirmed that the assembled 2G12 produced in the transgenic endosperm was functional. The binding activity of the crude extracts was consistent with the concentration of antibody relative to the purified ^{OS}2G12, which demonstrated near identical binding activity to the 2G12 produced in maize (^{ZM}2G12) which was used as a positive control (Figure 6).

The HIV-neutralizing activity of the purified ^{OS}2G12 was compared to the same antibodies produced in and purified from CHO cells (^{CHO}2G12) and tobacco (^{NT}2G12) and to an unrelated pure antibody recognizing a different antigen (antirabies antibody

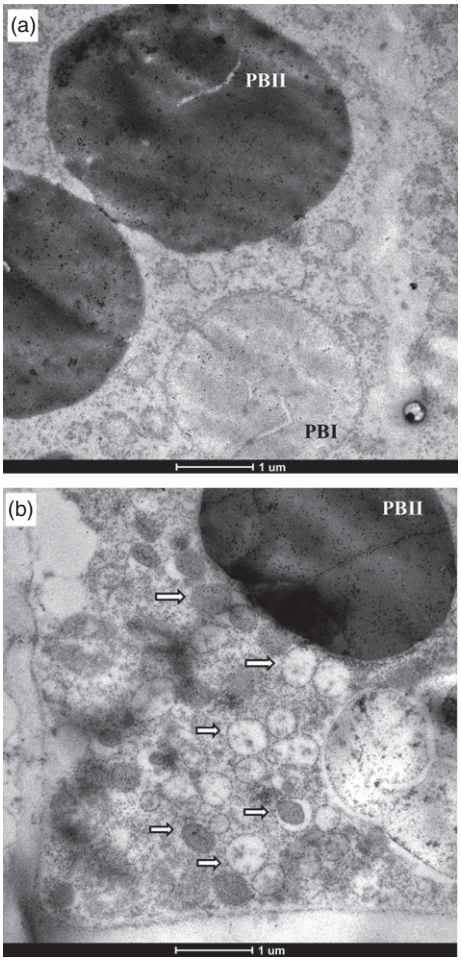


Figure 3 Localization of 2G12 in the rice endosperm by electron microscopy. Ultrathin sections of immature rice seeds (line 14) revealed (a) the accumulation of 2G12 mostly in PB-II structures and more sparsely in PB-I structures, (b) the presence of novel bodies surrounded by ribosomes (white arrows). Bar = 500 nm. Antibody 2G12 was detected with a polyclonal goat anti-HC or anti-LC antibody (diluted 1 : 100 in PBS) and a secondary donkey anti-goat IgG coupled to 15-nm colloidal gold diluted 1 : 30 in PBS.

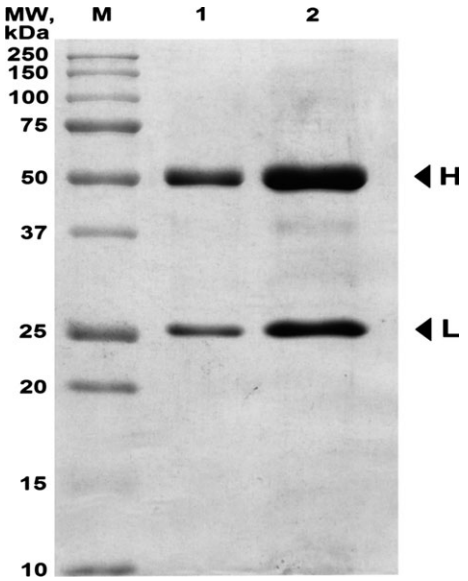


Figure 4 SDS-PAGE analysis of 2G12 purified from rice endosperm. Fractions eluting from the protein A column were separated by SDS-PAGE (12% polyacrylamide) under reducing conditions, and the gels were stained with Coomassie Brilliant Blue. The heavy (H) and light (L) chains were identified, and no degradation products were observed.
M = molecular weight markers. Lanes 1 and 2 were loaded with 2 and 5 μg of 2G12 (line 14), respectively.

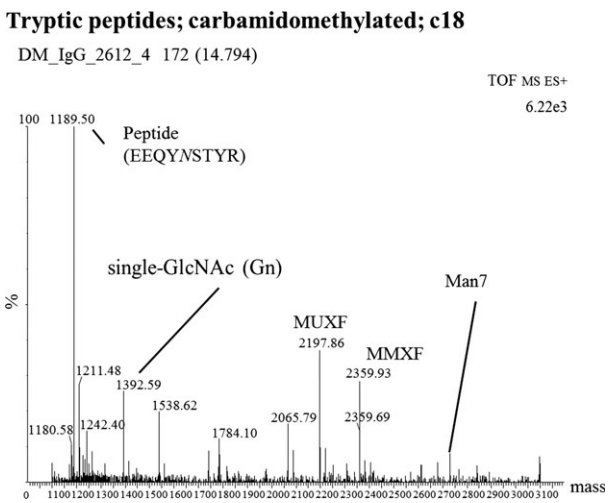


Figure 5 Glycosylation profile of 2G12 purified from rice endosperm. Deconvoluted mass spectrum (*m/z* = 1100–3100) of the tryptic glycopeptide derived from the 2G12 heavy chain (band H shown in Fig. 4). MMXF = paucimannosidic structures, Man_7 = mannose 7.

E559) all at starting concentrations of 100 μg/mL. The potency of HIV neutralization was determined using a syncytium inhibition assay with HIV-1 BaL. The 50% inhibitory concentration (IC_{50}) was 1.197 μg/mL for ^{OS}2G12, 0.083 μg/mL for ^{CHO}2G12 and 7.889 μg/mL for ^{NT}2G12, demonstrating that ^{CHO}2G12 was 14 times more potent than ^{OS}2G12, but ^{OS}2G12 was approximately seven times more potent than ^{NT}2G12, which was a significant difference at $P < 0.05$ (Figure 7).

Proteomic analysis and transcript profiling reveal genes modulated by 2G12 expression in rice endosperm

Comparative proteomic analysis was carried out using transgenic lines 7, 10, 12 and 14 and a wild-type control, revealing two major up-regulated proteins and two major down-regulated proteins in the transgenic plants (Table 1). One of the up-regulated proteins was the storage protein prolamin and the other was the enzyme glutaredoxin-C8. Both the down-regulated proteins were related to starch synthesis and storage. We also measured the abundance of the corresponding transcripts in the same four transgenic lines by quantitative real-time RT-PCR to correlate the mRNA and protein levels of the modulated genes. Accordingly, we found that the transcript levels of endogenous starch-branching enzyme (GLGB _ORYSJ) and ADP-glucose pyrophosphorylase (GLGS _ORYSJ) were down-regulated in all the transgenic lines compared to wild-type plants (Figure S1 a–b) and that the expression levels of endogenous glutaredoxin-C8 (GRXC8 _ORYSJ) and prolamin PPROL 14E (PRO7_ORYSJ) were up-regulated in all the transgenic lines compared to wild-type plants (Figure S1 c–d).

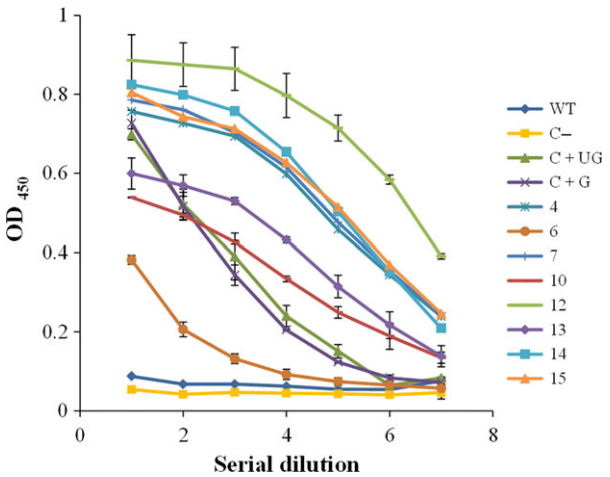
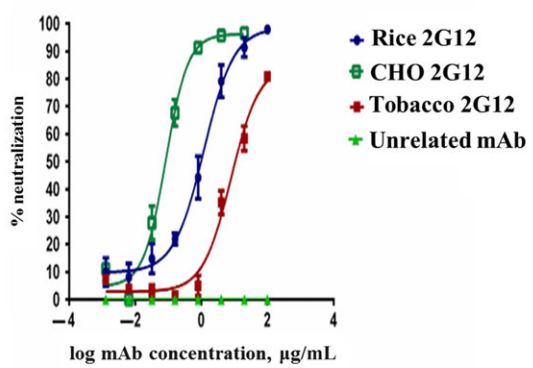


Figure 6 Antigen-binding activity of crude extracts from rice endosperm and purified 2G12. ELISA was used to determine the binding activity of purified 2G12 and serially diluted crude extracts of rice endosperm expressing the same protein. Plates were coated with the HIV gp120 protein, and bound 2G12 was detected with a mixture of anti-human κ-chain and anti-human IgG antibodies labelled with horseradish peroxidase. Values represent the average of two experiments, and bars indicate standard deviations. The binding kinetics showed a concentration-dependent relationship. All the crude extracts showed higher binding kinetics compared to wild-type extracts (negative control) and purified 2G12 (positive control). WT = wild-type extracts; C^{-} = negative control (PBS); C^{+UG} = underglycosylated 2G12 purified from rice (500 ng/mL) as a positive control; C^{+G} = glycosylated 2G12 purified from maize (500 ng/mL) as a positive control; OD = optical density at 450 nm.



	IC_{50} titer (µg/mL)			
Virus	Clade	Rice 2G12	CHO 2G12	Tobacco 2G12
HIV-1 BaL	B	1.197	0.083	7.889

Figure 7 *In vitro* virus neutralization efficacy of 2G12 from different sources. HIV neutralization was measured using a syncytium inhibition assay (Montefiori, 2005; Wei *et al.*, 2003) with HIV-1 strain BaL, representing clade B (HIV-1 is divided into four groups (M, N, O and P), and within group M, there are at least nine distinct subtypes (or clades) named A, B, C, D, F, G, H, J and K). An unrelated plant-derived antirabies antibody E559 was used as a negative control.

Discussion

Protein microbicides against HIV can help to prevent the transmission of the virus, as demonstrated in a number of preclinical and clinical studies (De Clercq, 2007; Nutan and Gupta, 2011) concerning anionic polymers and polyanions (El-Sadr *et al.*, 2006; Joshi *et al.*, 2006; McCormack *et al.*, 2005; Patton *et al.*, 2006), CCR5 inhibitors (Kawamura *et al.*, 2003) and fusion inhibitors (Ramessar *et al.*, 2010). However, the production and distribution of therapeutic proteins using traditional CHO-based fermenters are challenging and expensive in key HIV-endemic areas because there is only a limited amount of GMP-ready production infrastructure and the cold chain is often unreliable (Fischer *et al.*, 2012; Ma *et al.*, 2013; Rybicki, 2014).

Therapeutic proteins have been expressed in plants, including a large number of vaccine candidates and recombinant antibodies, so the principle of pharmaceutical molecular farming is well established (Ramessar *et al.*, 2008a; Twyman *et al.*, 2005). Plants have many technoeconomic advantages over fermenter-based systems because upstream production does not require expensive GMP facilities and can be achieved using greenhouses which cost less to build and operate and are much more scalable, thereby providing an opportunity for the production of antibodies as 'commodities' despite the lower yields achieved in plants (Hood *et al.*, 2002; Ma *et al.*, 2003; Paul *et al.*, 2013; Ramessar *et al.*, 2008a). Although many of the economic advantages of plants are offset by the high costs of downstream processing, cereal seeds such as rice and maize are suitable for minimal processing strategies because they have GRAS status, so pharmaceutical products can be orally administered as whole seeds, flakes or flour (Ning *et al.*, 2008; Xie *et al.*, 2008) and also made into pastes for topical applications (Paul *et al.*, 2013; Ramessar *et al.*, 2008a).

Several detailed economic studies have been carried out for recombinant proteins produced in rice seeds. For example, He *et al.* (2011) have shown that rice seeds are cost-effective for the production of recombinant human serum albumin, which like microbicide antibodies is also required on a large scale. Zhang *et al.* (2012) developed a cost-effective process for the production and downstream processing of recombinant human α1-antitrypsin. Furthermore, An *et al.* (2013) showed that rice endosperm cells can express atypical proteins such as hydrophobic proteins or those with a low molecular mass. Nandi *et al.* (2005) described the production and processing of recombinant human lactoferrin in rice, revealing that the cost of goods could be reduced to as little as $US 5.90 per gram with a productivity of 600 kg per year. The authors of this study represent the US

Table 1 The two most strongly up-regulated and down-regulated proteins in each transgenic line (fold increase/decrease) compared to wild-type rice plants. Fold changes of <2 were considered relevant if comparable shifts were shown in the transcriptome profile

Protein	10:WT Fold increase	7:WT Fold increase	12:WT Fold increase	14:WT Fold increase	Average
PRO7_ORYSJ	1.96	2.79	2.41	2.50	2.42
GRXC8_ORYSJ	1.42	1.08	1.57	1.54	1.40

Protein	10:WT Fold decrease	7:WT Fold decrease	12:WT Fold decrease	14:WT Fold decrease	Average
GLGB_ORYSJ	1.17	1.49	1.18	1.41	1.31
GLGS_ORYSJ	1.07	1.32	0.95	1.11	1.11

biotechnology company Ventria Bioscience (Fort Collins, CO), and lactoferrin is one of a range of rice-derived recombinant proteins that are already marketed as technical reagents (through their InVitria division) and some of which (including lactoferrin) are also being developed for clinical applications. Rice and other cereal seeds are particularly suitable for developing country applications because they maintain the stability of recombinant proteins accumulating in the endosperm so that a cold chain is not necessary for product distribution, and the economy of large-scale production makes the product affordable without detracting from its safety or performance (Fischer *et al.*, 2012; Sabalza *et al.*, 2013).

We expressed the HIV-neutralizing antibody 2G12 in transgenic rice endosperm to evaluate the potential of rice seeds as a vehicle for inexpensive microbicide production. We also investigated the biochemical and cellular processes that are influenced by the accumulation of a heterologous recombinant protein such as 2G12 in the endosperm to gain a better understanding of factors that might control the accumulation of recombinant proteins in plants. We recovered transgenic rice plants and confirmed the presence and strong expression of the transgenes in six independent lines, ultimately choosing two lines with the highest antibody yields for more detailed analysis. The yields of 2G12 in rice were similar to those reported for 2G12 in other cereal seeds and better than those reported for other antibodies, although as stated earlier CHO cells can achieve antibody yields of 5–10 g/L albeit with more limited scalability and a lower overall capacity. We achieved a maximum yield of 42 μg/g dry seed weight in T3 seeds, which compares well with the 30 μg/g initially achieved for 2G12 in maize (Rademacher *et al.*, 2008) although this was subsequently improved to 75 μg/g by the selective breeding of high-performance lines and then to 100 μg/g by subjecting the best-performing line to a dedifferentiation–regeneration cycle (Ramessar *et al.*, 2008b). Our results for 2G12 were far better than those we reported for HIV-neutralizing antibody 2F5 in maize seeds, where the maximum accumulation was 0.80 μg/mL (Sabalza *et al.*, 2012). We are the first to report the expression of a full-size immunoglobulin in rice, so there are no prior studies to compare. Our data also suggest that the 2G12 yields in rice could also be improved beyond 42 μg/g dry seed weight by selective breeding.

The localization of 2G12 in the transgenic endosperm was investigated by immunohistochemistry and TEM, revealing that most of the antibody accumulated in PB-II compartments and to a lesser extent in PB-I compartments. The subcellular location of a recombinant protein can influence its yield, structure and functionality, but it is difficult to predict the localization of recombinant proteins in the endosperm of cereal seeds because protein trafficking appears to be influenced not only by the species and tissue but also by intrinsic properties of the expressed protein (Drakakaki *et al.*, 2006). When a 2G12 antibody carrying a C-terminal KDEL tag was expressed in maize endosperm, it accumulated within ER-derived zein bodies, but no significant labelling was observed in storage vacuoles or any other cell compartment (Rademacher *et al.*, 2008). In contrast, an untagged version of 2G12 accumulated in the storage vacuoles and, to a much lesser extent, also in zein bodies (Ramessar *et al.*, 2008b). The expression of recombinant fungal phytase in rice resulted in the accumulation of phytase in both types of protein bodies (PB-I and PB-II), but no additional labelling was observed in the apoplast or other cell compartments (Drakakaki *et al.*, 2006). Our rice plants were transformed with the untagged version of 2G12, and the results were similar to those reported in maize for the same version of the antibody (Ramessar *et al.*, 2008b). Immunohistochemistry showed that the signal for 2G12 overlapped with that for glutelin, indicating that most of the antibody co-accumulated with this storage protein in PB-II compartments, also known as glutelin bodies. More detailed analysis by TEM confirmed that most of the 2G12 was localized in PB-II compartments and a smaller amount in PB-I compartments, but we also observed novel protein bodies that were not present in untransformed seeds, suggesting the compartments were formed *de novo* as a consequence of antibody expression even though they did not show significant 2G12 labelling. The novel protein bodies were unusual in architecture, featuring prominent studs in the membrane reflecting the presence of ribosomes. The induction of novel protein bodies in transgenic rice cells has been reported before (Torres *et al.*, 1999; Yang *et al.*, 2012). In our transgenic lines, the antibody was able to induce novel protein bodies without the benefit of a KDEL tag, suggesting that the protein was abundant enough in the rough ER to induce the budding of protein bodies even without a dedicated retention sequence. Green fluorescent protein fused to rice prolamin has also been shown to induce the formation of protein body-like structures in transgenic rice endosperm, although the protein ultimately accumulated in PB-I compartments (Saito *et al.*, 2009).

Further information about the intracellular trafficking of recombinant proteins can be gathered by analysing the glycan profile if the protein contains canonical N-glycan acceptor sites. The heavy chain of IgG antibodies carries an acceptor site at residue N297 of the Fc domain, and the structure of the glycan affects antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), which can thus be modulated by choosing an appropriate production platform (Jung *et al.*, 2011) or by deliberately controlling the glycan structures (Forthal *et al.*, 2010). Antibodies expressed in plants typically comprise a mixture of glycoforms, ranging from the absence of any glycans (aglycosylated heavy chain) to high-mannose and complex-type glycans, and antibodies with single oligosaccharides resulting from extensive glycan trimming (Baez *et al.*, 2000; De Wilde *et al.*, 1998; Hristodorov *et al.*, 2013; Khoudi *et al.*, 1999; Ma *et al.*, 1994). The aglycosylated fraction is usually a minor component. For example, 13.3% of 2G12 was aglycosylated when the KDEL-tagged version was expressed in standard maize and only 10.6% was aglycosylated in a sweetcorn variety (Rademacher *et al.*, 2008), whereas in the absence of a KDEL tag, the aglycosylated fraction was 11.5% (Ramessar *et al.*, 2008b). Surprisingly, we found that the aglycosylated fraction of the 2G12 heavy chain in transgenic rice endosperm represented 50% of the total, the remainder comprising vacuolar-type complex N-glycans (19% MUXF and 14% MMXF), 13% individual N-acetylglucosamine (GlcNAc) residues confirming the presence of ENGase activity, and only 4% oligomannose glycans (Man_7) as expected for ER-resident proteins, suggesting that the recombinant protein was directed through distinct trafficking pathways characterized by different glycan signatures. In most previous investigations, the predominant glycoforms were the complex type (if the protein passed through the secretory pathway to the apoplast or the storage vacuole) or the oligomannose type (if the protein was retrieved to the ER lumen). Only one previous study reported a prevalent aglycosylated fraction and that involved the expression of an scFv-Fc antibody in Arabidopsis seeds, where most of the antibodies carried oligomannose glycans but 35–40% were aglycosylated (Van Dro-

ogenbroeck *et al.*, 2007). The authors proposed that the glycosylation machinery may have been saturated at the high expression levels they achieved, but we observed no change in the steady-state mRNA levels of BiP and PDI, two proteins that are usually up-regulated when the secretory pathway is under heavy load. Indeed, we even noted a slight decline in the level of *PDI* mRNA in some transgenic lines. These results suggest that a large proportion of the heavy chain is cotranslationally imported into a region of the ER that lacks glycosyltransferases and accumulates in protein bodies without further modification. The remaining proportion appears to follow the canonical pathway, undergoing initial glycosylation in the ER lumen followed by either complex modifications in the Golgi body and/or trimming back to a single GlcNAc residue. Interestingly, Zhang *et al.* (2012) found that nearly 70% of their rice-derived recombinant human α1-antitrypsin was aglycosylated, which they suggested might reflect the trafficking of the protein to the vacuole through a latent PAC pathway, thus providing a strategy for the production of minimally glycosylated recombinant proteins in plants (Shimada *et al.*, 1997, 2002). This contrasts with the untagged version of 2G12 expressed in maize, which also accumulated in the storage vacuoles but underwent a higher degree of glycosylation (Ramessar *et al.*, 2008b).

The glycosylation of 2G12 is not only important from a protein trafficking and accumulation perspective but also because it can affect the functionality of the protein once it has been purified. We tested the antigen-binding efficiency of the crude extracts of different rice lines expressing ^{OS}2G12 against purified ^{OS}2G12 and ^{ZM}2G12 as a control. We found that pure ^{OS}2G12 and ^{ZM}2G12 bound to gp120 with near identical efficiency, and because various glycosylated forms of ^{ZM}2G12, ^{NT}2G12 and ^{CHO}2G12 have previously been shown to bind gp120 in a near identical manner, this suggests that glycosylation has a negligible impact on epitope recognition or binding (Forthal *et al.*, 2010; Rademacher *et al.*, 2008). In contrast, we found that ^{OS}2G12 was more potent than ^{NT}2G12 in our HIV-neutralization assays, suggesting that the specific glycan profile of the rice-derived antibody is advantageous for the relevant effector functions of the protein, perhaps by affecting the efficiency of interactions with different Fc receptors, as previously shown for different glycoforms of 2G12 produced in tobacco (Forthal *et al.*, 2010). The tobacco antibody we used was untagged and was therefore secreted to the apoplast and endowed predominantly with complex-type plant glycans.

The IC_{50} of ^{CHO}2G12 (with mammalian glycans) was lower than that of purified ^{OS}2G12, which was in turn lower than that of purified ^{NT}2G12. Although glycans do not appear to be required to establish the domain-exchange structure that binds the mannose cluster on gp210 or for any other aspect of antigen binding (Calarese *et al.*, 2003), there is increasing evidence that glycans affect the effector functions of many antibodies, influencing not only HIV-neutralization (Forthal *et al.*, 2010) but also the ability to neutralize anthrax LeTx (Mett *et al.*, 2011). However, other studies have shown the equivalence or near equivalence of aglycosylated and glycosylated variants of the same antibody produced in CHO cells (Hristodorov *et al.*, 2013a), maize (Baez *et al.*, 2000) and soybean (Zeitlin *et al.*, 1998), so it appears that plants should be evaluated on a case-by-case basis to determine the most suitable glycan profiles for specific prophylactic or therapeutic applications. In the context of HIV, syncytium inhibition assays offer indicative IC_{50} values, but it should be borne in mind that the assays show high variability and two- to threefold differences within an experiment should not be regarded as significant. Interlaboratory variation is even higher, particularly when different HIV strains and assay protocols are used, so there is little value in the direct comparison of IC_{50} values reported in different studies, particularly where different forms of the antibody are also used. This explains why our IC_{50} data for ^{NT}2G12 and ^{CHO}2G12 are inconsistent with those published in previous reports (Forthal *et al.*, 2010; Rademacher *et al.*, 2008).

We also investigated the wider effects of high-level antibody production by carrying out a proteomic analysis of transgenic and wild-type endosperm tissue and analysing the transcripts corresponding to the four differentially expressed proteins we identified. The two down-regulated proteins were both related to starch biosynthesis, that is ADP-glucose pyrophosphorylase (GLGS) and starch-branching enzyme (GLGB). GLGS controls a rate-limiting step in starch biosynthesis involving carbon partitioning between starch and sucrose in photosynthetic tissues (Heldt *et al.*, 1977; Preiss *et al.*, 1991). It also regulates starch biosynthesis in nonphotosynthetic sink organs such as seeds, and in cereals, the major form of this enzyme is located in the cytoplasm (Denyer *et al.*, 1996; Sikka *et al.*, 2001; Thorbjornsen *et al.*, 1996). GLGB is the only enzyme that can introduce α-1,6-glucosidic linkages into α-glucans, and it is required for amylopectin biosynthesis. Plants express two classes of this enzyme (BEI and BEII) with different functions (Nakamura *et al.*, 2003). Rice and maize have two isoforms of the BEII enzyme (BEIIa and BEIIb), the latter expressed only in the endosperm (Abe *et al.*, 2014). The down-regulation of GLGS and GLGB in the transgenic plants was confirmed at both the mRNA and protein levels, suggesting that the accumulation of 2G12 has a significant impact on starch metabolism and results in the accumulation of less starch in the seeds. Accordingly, we found that weight of the transgenic seeds was ~22% lower than the wild-type seeds, potentially indicating that the expression of recombinant proteins in the rice endosperm can disrupt the balance between protein and carbohydrate storage. Seed viability and germination were not affected.

This hypothesis was supported by the greater abundance of PRO7, a seed storage protein and GRXC8 (glutaredoxin-C8), an enzyme that scavenges reactive oxygen species (Lin *et al.*, 2014). In rice, prolamins accumulate in the PB-I compartments which bud directly from the ER. The greater abundance of prolamin in the transgenic endosperm may be related to the down-regulation of carbohydrate metabolism discussed above and/or may reflect the disruption of protein trafficking in the cell due to the accumulation of 2G12 in the ER, resulting in a compensatory mechanism as the cell attempts to sort several abundant proteins into different compartments. The concentration of 2G12 in PB-II compartments and the genesis of novel ER-derived protein bodies may generate feedback at the level of storage protein deposition that results in the up-regulation of particular prolamin genes. The surge of proteins through the endomembrane system is likely to cause local stress that induces the production of reactive oxygen species, resulting in the production of glutaredoxin-C8 as a stress response. These data suggest that yields of proteins could be improved in rice seeds by regulating protein/carbohydrate metabolism and protecting the endosperm from abiotic stress.

Experimental procedures

Cloning and construct design

All constructs were based on the binary vector pTRA, a derivative of pPAM (GenBank Accession Number AY027531). The vector

contains two tobacco *RB7* scaffold attachment regions that flank the expression cassette. The coding regions of the 2G12 heavy and light chains (obtained from Polymun, Vienna, Austria) included N-terminal signal sequences targeting the secretory pathway. The expression cassette comprised the endosperm-specific rice glutelin-1 promoter, the maize ubiquitin-1 first intron, the *Tobacco etch virus* 5′ leader that acts as a translational enhancer, the coding region and the *Cauliflower mosaic virus* 35S terminator, resulting in final constructs pTRAgtiGH and pTRAgtiGL. A separate vector was used to provide the *hpt* selectable marker (Christou and Ford, 1995).

Transformation and regeneration of transgenic rice plants

Seven-day-old mature rice zygotic embryos (*Oryza sativa* cv. Nipponbarre) were transferred to osmotic medium (4.4 g/L MS powder supplemented with 0.3 g/L casein hydrolysate, 0.5 g/L proline, 72.8 g/L mannitol and 30 g/L sucrose) 4 h before bombardment with 10 mg gold particles coated with the 2G12 heavy and light chain constructs and the selectable marker *hpt* at a 3 : 3 : 1 ratio (Sudhakar *et al.*, 1998; Valdez *et al.*, 1998). The embryos were returned to osmotic medium for 12 h before selection on MS medium (4.4 g/L MS powder, 0.3 g/L casein, 0.5 g/L proline and 30 g/L sucrose) supplemented with 50 mg/L hygromycin and 2.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark for 2–3 weeks. Transgenic plantlets were regenerated and hardened off in soil. Plants were grown in the greenhouse and growth chamber at 28/20 °C day/night temperature with a 10-h photoperiod and 60–90% relative humidity for the first 50 days, followed by maintenance at 21/18 °C day/night temperature with a 16-h photoperiod thereafter.

Characterization of rice plants and transgene expression

Genomic DNA was isolated from rice leaves by phenol extraction (Edwards *et al.*, 1991) and 50 ng aliquots were used for PCR with three primers designed to generate overlapping products to confirm transgene integrity. GoTaq DNA polymerase was used with the program recommended by the manufacturer (Promega, Fitchburg, Wisconsin). The positive lines were then screened for transgene expression by dot blot. The total soluble protein (TSP) was extracted in three volumes (v/w) of phosphate-buffered saline (PBS). The samples were centrifuged twice (15 min, 4 °C, 13 000 ***g***), and 4 μL of supernatant was spotted onto a nitrocellulose membrane (Amersham; GE Healthcare, Buckinghamshire, UK). The membrane was blocked for 1 h with 5% blocking buffer and probed overnight, shaking at room temperature with alkaline phosphatase-labelled antibodies against the heavy and light chains (Sigma-Aldrich, St. Louis, Missouri): goat anti-human IgG Fc chain (Sigma A9544) and goat anti-human kappa light chain (Sigma A3813), each diluted 1 : 1000 from stock. The blots were washed three times with PBS supplemented with 0.2% Tween-20 (PBST), and the antibody was detected using Sigma FAST™ BCIP/NBT in distilled water.

Confirmation of antibody assembly and accumulation (ELISA)

Three seeds representing each event were analysed by ELISA to quantify 2G12 expression, using goat anti-human kappa light chain (Sigma K3502) or goat anti-human IgG Fc chain (Sigma K2136) as capture antibodies. These were diluted 1 : 200 and coated directly onto 96-well Nunc-Immuno Maxisorp surface plates (Nalge Nunc, Penfield, NY) and incubated overnight at 4 °C. The plates were blocked with 5% nonfat milk for 2 h and then washed with PBST. Serial dilutions of the samples were added to the wells and incubated for 2–3 h at room temperature. After washing, a horseradish peroxidase (HRP)-conjugated sheep anti-human kappa chain antiserum (The Binding Site, Birmingham, UK) was added to the plates at a 1 : 1000 dilution and incubated at room temperature for 2 h before the signal was detected with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma). After 4–5 min, the signal was quantified by measuring the absorbance at 450 nm.

Fluorescence microscopy

Sections of immature rice endosperm (20 days after pollination) were mounted on glass slides and pre-incubated in 5% (w/v) bovine serum albumin (BSA Fraction V) in phosphate buffer (0.1 M, pH 7.4) and then incubated with polyclonal rabbit anti-HC or anti-LC antiserum diluted 1 : 100. Sections were then treated with the secondary antibody diluted in phosphate buffer (0.1 M, pH 7.4). An AlexaFluor 594-conjugated goat anti-rabbit IgG (red fluorophore) was used to detect 2G12, and an antiglutelin antibody (green fluorophore) was used to detect endogenous glutelins.

Transmission electron microscopy

Developing rice seeds (20 days after pollination) were fixed and processed as described by Arcalis *et al.* (2004). Immature seeds from wild-type and transgenic rice plants were cut into small pieces with a razor blade under PBS (pH 7.4) and fixed in 4% (w/v) paraformaldehyde with 0.5% (v/v) glutaraldehyde in PBS overnight at 4 °C. After several washes in PBS, the samples were dehydrated through an ethanol series (50%, 70%, 90%, 100%) at 4 °C and progressively embedded in LR White resin (25% in ethanol, 50%, 75% and pure resin for 3 h each) followed by an additional step with fresh 100% resin. Blocks were mounted in Beem capsules and polymerized overnight at 60 °C. Sections showing silver interference colours were collected on gold grids, pre-incubated in 5% (w/v) BSA in PBS for 15 min at room temperature and then incubated with either polyclonal goat anti-HC or anti-LC (1 : 100 in PBS) for 2 h at room temperature. After three washes for 10 min in PBST, sections were incubated with the secondary antibody (donkey anti-goat IgG coupled to 15-nm colloidal gold) diluted 1 : 30 in PBS and incubated for 1 h at room temperature. After two washes for 10 min in PBS and two in distilled water, samples were air-dried and the sections were observed using a FEI Tecnai G2 transmission electron microscope.

Protein A affinity chromatography

Rice seeds were ground to a fine powder, and 5 g of seed powder was extracted overnight at 4 °C in five volumes of buffer (1× PBS, 5 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.4). Insoluble material was removed by centrifuging twice at 8000 ***g*** for 30 min at 4 °C. The supernatant was filtered and the antibody concentration in the crude extract determined by ELISA. The sample was loaded onto a protein A affinity column (ceramic HyperDF) at a flow rate of 2 mL/min. The column was washed with PBST and PBS, and the antibody was eluted in 100 mM glycine (pH 3.6) containing 100 mM fructose and buffered with 0.1 volumes of 1 M sodium acetate (pH 4.75). Antibody-containing fractions were identified by the droplet Bradford method (Bradford, 1976), and 2G12 concentrations were determined by ELISA. Fractions containing >50 μg/mL were pooled and dialysed

for 2 days against 10 mM sodium acetate (pH 4.75) containing 1 mM EDTA and 0.1 mM 2-mercaptoethanol (omitted in the final dialysis step). The dialysed antibody was concentrated by ultrafiltration using spin columns with a 30-kDa molecular weight cut-off (MWCO). Protein extracts were separated by SDS-PAGE (12% polyacrylamide) using precast Bis–Tris Nu-PAGE gels (Invitrogen, Carlsbad, CA).

Glycan analysis

The antibody heavy chain band from the Coomassie-stained SDS-PAGE gel was destained, carbamidomethylated, digested with trypsin and extracted from gel pieces as described (Shukla *et al.*, 2007). The peptides were separated by capillary reversed-phase chromatography with a Q-TOF Ultima Global mass spectrometer (Waters, Manchester, UK) for detection (Van Droogenbroeck *et al.*, 2007). The MS data from the tryptic peptides were analysed as previously described (Van Droogenbroeck *et al.*, 2007) and compared with data sets generated by *in silico* tryptic digestion of the 2G12 coding region, using the PeptideMass program (http://web.expasy.org/peptide_mass/). Based on the tryptic peptide data sets, tryptic glycopeptide data sets were generated by adding glycan mass increments to the masses of the two identified glycopeptides.

In vitro binding to gp120 (ELISA)

The specific antigen-binding activity of 2G12 was determined by coating ELISA wells with 100 ng recombinant gp120 from HIV strain IIIB, provided by the MRC Centralized Facility for AIDS Reagents, Potters Bar, UK. After washing with PBST and blocking with BSA, serial dilutions of 2G12 were added and the amount of bound antibody was determined using a mix of HRP-conjugated antigamma chain and HRP-conjugated antikappa chain (The Binding Site) at a 1 : 1000 dilution, washing with PBS, developing the signal with TMB substrate and reading the absorbance at 450 nm.

Syncytium inhibition assay for HIV neutralization

HIV-1 neutralization was assessed using a syncytium inhibition assay. Ten twofold serial dilutions (starting concentration 100 μg/mL) of ^{OS}2G12, ^{CHO}2G12, ^{NT}2G12 and a non-neutralizing control were pre-incubated with HIV-1 BaL at 10 half-maximum tissue culture infectious doses (Terman and Bertram, 1985) per mL for 1 h at 37 °C. $CD4^+$ human AA-2 cells were added at a density of 4×10^5 cells/mL and incubated for a further 5 days. We analysed eight replicates per antibody dilution step. The presence of one or more syncytia per well after 5 days was scored as positive for infection. The 50% inhibitory concentrations (IC_{50}) were calculated using the concentrations present during the antibody–virus pre-incubation step (Montefiori, 2005; Wei *et al.*, 2003).

Comparative proteomic analysis

We sampled 24 T4 seeds from line 10, 36 T4 seeds from lines 7, 12 and 14, and 48 wild-type seeds, thus providing four independent samples of transgenic seeds expressing 2G12. Approximately 100 mg of seed powder was vortexed with 500 μL buffer (20 mM Tris–HCl, pH 8.3, 1.5 mM KCl, 10 mM DTT, 1 mM PMSF and 0.1% (v/v) SDS) at room temperature for 10 min and centrifuged for 5 min at 10 000 ***g***. The protein content of the supernatant was measured using a NanoDrop (Thermo Fisher Scientific, Waltham, Massachusetts), and 100 μg of TSP was precipitated using 1 : 4 cold acetone. After centrifugation as above, the pellet was dried at room temperature for 10 min and dissolved in 50 μL 50 mM NH_4HCO_3 to a final concentration of 2 μg/μL and then mixed with 10 μL 50 mM ammonium bicarbonate and 25 μL 0.2% (v/v) RapiGEST™ at 80 °C for 15 min before adding 2.5 μL 100 mM DTT and incubating at 60 °C for a further 30 min. After centrifuging as above, samples were mixed with 2.5 μL 300 mM iodoacetamide in the dark at room temperature for 30 min and then with 10 μL at 37 °C overnight. RapiGEST was precipitated with 10 μL 5% TFA for 90 min at 37 °C, and the samples were centrifuged at 10 000 ***g*** at 6 °C for 30 min. The supernatants were transferred to Total Recovery vials (Waters, Milford, Massachusetts), freeze-dried and resuspended in 100 μL 200 mM ammonium formate containing 50 fmol/μL Waters protein standards digested with rabbit phosphorylase B. The final protein concentration was 1 μg/μL.

The tryptic peptides were fractionated using a nanoACQUITY™ system (Waters). First-dimension separation was carried out using an XBridge™ reversed-phase column (300 μm × 50 mm, 5 μm C18 resin) with 20 mM ammonium formate as mobile phase A and acetonitrile as mobile phase B. Second-dimension separation was carried out using a Symmetry precolumn (300 μm × 50 mm, 5 μm C18 resin) and a HSST3 reversed-phase column (75 μm × 150 mm, 1.8 μm C18 resin) with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B. The tryptic peptides were analysed using a Synapt G2 HDMS™ mass spectrometer (Waters) with a hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight (oa-TOF) geometry in positive nanoelectrospray ion mode. Exact mass retention time (EMRT) nanoLC-MSE data (Silva *et al.*, 2006) were collected in alternating low and elevated energy mode at 3 eV and 12–45 eV, respectively, with a continuum spectra acquisition time of 1.5 s and a 0.1-s interscan delay.

MS data were processed using ProteinLynx Global Server (PLGS) v2.5 (Waters). Proteins were identified using the ion accounting algorithm and a search of the UniProt *Oryza* spp database with MassPREP digestion standards (MPDS). Identifications and quantitative data packaging were generated using dedicated algorithms (Li *et al.*, 2009; Silva *et al.*, 2006) and a search against a species-specific UniProt database. The intensity measurements were normalized to those representing peptides of the digested internal standard (Murad and Rech, 2012).

Quantitative real-time PCR

RNA was isolated from rice endosperm 25 days after pollination as described by Li and Trick (2005) with corrections and modifications. Extraction buffer I was 100 mM Tris (pH 8.0), 150 mM LiCl, 50 mM EDTA, 1.5% SDS, 1.5% 2-mercaptoethanol, and extraction buffer II was 4.2 M guanidine thiocyanate, 1 M sodium acetate, (pH 4.0), 0.5% lauryl sarcosine, 25 mM sodium citrate (pH 7.0). The samples were processed on a Bio-Rad CFX96 system using the Quantitect reverse transcription kit (Qiagen, Hilden, Germany) in 25 μL reactions comprising 10 ng cDNA, 1× iQ SYBR Green Supermix (Bio-Rad, Hercules, California) and 0.2 μM forward and reverse primers. To calculate relative expression levels, serial dilutions (60–0.096 ng) were used to produce standard curves for each gene. PCRs were carried out in triplicate using 96-well optical reaction plates, initially heating for 3 min at 95 °C followed by 40 cycles of 95 °C for 15 s, 50 °C for GLGB, 50.7 °C for GRXC8 and PRO7 and 58.3 °C for GLGS for 1 min, and 72 °C for 20 s. Amplification specificity was confirmed by melt curve analysis on the final PCR products in the temperature range 50–90 °C with fluorescence acquired after each 0.5 °C

increment. The fluorescence threshold value and gene expression data were calculated using CFX96 system software. Values represent the mean of three replicates ± SD. The forward and reverse primers for each gene are shown in Table S1.

Acknowledgements

The authors would like to acknowledge funding from the Ministerio de Ciencia e Innovación, Spain (BIO2012-35359), the Centre CONSOLIDER on Agrigenomics funded by MICINN, Spain, and COST Action FA0804 (Molecular farming: plants as a production platform for high value proteins). The authors report no conflict of interests.

References

Abe, N., Asai, H., Yago, H., Oitome, N.F., Itoh, R., Crofts, N., Nakamura, Y. and Fujita, N. (2014) Relationships between starch synthase I and branching enzyme isozymes determined using double mutant rice lines. *BMC Plant Biol.* **14**, 80.

An, N., Ou, J., Jiang, D., Zhang, L., Liu, J., Fu, K., Dai, Y. and Yang, D. (2013) Expression of a functional recombinant human basic fibroblast growth factor from transgenic rice seeds. *Int. J. Mol. Sci.* **14**, 3556–3567.

Arcalis, E., Marcel, S., Altmann, F., Kolarich, D., Drakakaki, G., Fischer, R., Christou, P. and Stoger, E. (2004) Unexpected deposition patterns of recombinant proteins in post-endoplasmic reticulum compartments of wheat endosperm. *Plant Physiol.* **136**, 3457–3466.

Baez, J., Russell, D., Craig, J. and Cass, P.L. (2000) Corn seed production of therapeutic proteins moves forward. *BioPharm. Int.* **13**, 50–54.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Calarese, D.A., Scanlan, C.N., Zwick, M.B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M.R., Stanfield, R.L., Roux, K.H., Kelly, J.W., Rudd, P.M., Dwek, R.A., Katinger, H., Burton, D.R. and Wilson, I.A. (2003) Antibody domain exchange is an immunological solution to carbohydrate cluster recognition. *Science*, **300**, 2065–2071.

Christou, P. and Ford, T.L. (1995) The impact of selection parameters on the phenotype and genotype of transgenic rice callus and plants. *Transgenic Res.* **4**, 44–51.

Chu, L. and Robinson, D.K. (2001) Industrial choices for protein production by large-scale cell culture. *Curr. Opin. Biotechnol.* **12**, 180–187.

De Clercq, E. (2007) The design of drugs for HIV and HCV. *Nat. Rev. Drug Discov.* **6**, 1001–1018.

De Wilde, C., De Rycke, R., Beeckman, T., De Neve, M., Van Montagu, M., Engler, G. and Depicker, A. (1998) Accumulation pattern of IgG antibodies and Fab fragments in transgenic Arabidopsis thaliana plants. *Plant Cell Physiol.* **39**, 639–646.

Denyer, K., Dunlap, F., Thorbjørnsen, T., Keeling, P. and Smith, A.M. (1996) The major form of ADP-glucose pyrophosphorylase in maize endosperm is extra-plastidial. *Plant Physiol.* **112**, 779–785.

Drakakaki, G., Marcel, S., Arcalis, E., Altmann, F., Gonzalez-Melendi, P., Fischer, R., Christou, P. and Stoger, E. (2006) The intracellular fate of a recombinant protein is tissue dependent. *Plant Physiol.* **141**, 578–586.

Edwards, K., Johnstone, C. and Thompson, C. (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* **19**, 1349.

El-Sadr, W.M., Mayer, K.H., Maslankowski, L., Hoesley, C., Justman, J., Gai, F., Mauck, C., Absalon, J., Morrow, K., Mâsse, B., Soto-Torres, L. and Kwiecien, A. (2006) Safety and acceptability of cellulose sulfate as a vaginal microbicide in HIV-infected women. *AIDS*, **20**, 1109–1116.

Evangelista, R.L., Kusnadi, A.R., Howard, J.A. and Nikolov, Z.L. (1998) Process and economic evaluation of the extraction and purification of recombinant beta-glucuronidase from transgenic corn. *Biotechnol. Prog.* **14**, 607–614.

Fischer, R., Stöger, E., Schillberg, S., Christou, P. and Twyman, R.M. (2004) Plant-based production of biopharmaceuticals. *Curr. Opin. Plant Biol.* **7**, 152–158.

Fischer, R., Schillberg, S., Hellwig, S., Twyman, R.M. and Drossard, J. (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnol. Adv.* **30**, 434–439.

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

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

Heldt, H.W., Chong, C.J., Maronde, D., Herold, A., Stankovic, Z.S., Walker, D.A., Kraminer, A., Kirk, M.R. and Heber, U. (1977) Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. *Plant Physiol.* **59**, 1146–1155.

Hofmann-Lehmann, R., Vlasak, J., Rasmussen, R.A., Smith, B.A., Baba, T.W., Liska, V., Ferrantelli, F., Montefiori, D.C., McClure, H.M., Anderson, D.C., Bernacky, B.J., Rizvi, T.A., Schmidt, R., Hill, L.R., Keeling, M.E., Katinger, H., Stiegler, G., Cavacini, L.A., Posner, M.R., Chou, T.C., Andersen, J. and Ruprecht, R.M. (2001) Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. *J. Virol.* **75**, 7470–7480.

Hood, E.E., Woodard, S.L. and Horn, M.E. (2002) Monoclonal antibody manufacturing in transgenic plants: myths and realities. *Curr. Opin. Biotechnol.* **13**, 630–635.

Hristodorov, D., Fischer, R., Joerissen, H., Müller-Tiemann, B., Apeler, H. and Linden, L. (2013) Generation and comparative characterization of glycosylated and aglycosylated human IgG1 antibodies. *Mol. Biotechnol.* **53**, 326–335.

Joshi, S., Dutta, S., Bell, B., Profy, A., Kuruc, J., Fang, G., Cianciola, M., Maslankowski, L., Soto-Torres, L., Panchanadikar, A., Mehendale, S. and Reynolds, S.J. (2006) Documenting intermenstrual bleeding in a vaginal microbicide study: case reports and lessons learned. *AIDS Res. Hum. Retroviruses*, **22**, 294–296.

Jung, S.T., Kang, T.H., Kelton, W. and Georgiou, G. (2011) Bypassing glycosylation: engineering aglycosylated full-length IgG antibodies for human therapy. *Curr. Opin. Biotechnol.* **22**, 858–867.

Kawamura, T., Gulden, F.O., Sugaya, M., McNamara, D.T., Borris, D.L., Lederman, M.M., Orenstein, J.M., Zimmerman, P.A. and Blauvelt, A. (2003) R5 HIV productively infects Langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. *Proc. Natl Acad. Sci. USA*, **100**, 8401–8406.

Khan, I., Twyman, R.M., Arcalis, E. and Stoger, E. (2012) Using storage organelles for the accumulation and encapsulation of recombinant proteins. *Biotechnol. J.* **7**, 1099–1108.

Khoudi, H., Laberge, S., Ferullo, J.M., Bazin, R., Darveau, A., Castonguay, Y., Allard, G., Lemieux, R. and Vézina, L.P. (1999) Production of a diagnostic monoclonal antibody in perennial alfalfa plants. *Biotechnol. Bioeng.* **64**, 135–143.

Kuo, Y.C., Tan, C.C., Ku, J.T., Hsu, W.C., Su, S.C., Lu, C.A. and Huang, L.F. (2013) Improving pharmaceutical protein production in *Oryza sativa*. *Int. J. Mol. Sci.* **14**, 8719–8739.

Li, Z. and Trick, H.N. (2005) Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch. *Biotechniques*, **38**, 872, 874, 876.

Li, G.Z., Vissers, J.P., Silva, J.C., Golick, D., Gorenstein, M.V. and Geromanos, S.J. (2009) Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics*, **9**, 1696–1719.

Lin, Z., Zhang, X., Yang, X., Li, G., Tang, S., Wang, S., Ding, Y. and Liu, Z. (2014) Proteomic analysis of proteins related to rice grain chalkiness using iTRAQ and a novel comparison system based on a notched-belly mutant with white-belly. *BMC Plant Biol.* **14**, 163.

Lotter-Stark, H.C., Rybicki, E.P. and Chikwamba, R.K. (2012) Plant made anti-HIV microbicides-a field of opportunity. *Biotechnol. Adv.* **30**, 1614–1626.

Ma, J.K., Lehner, T., Stabila, P., Fux, C.I. and Hiatt, A. (1994) Assembly of monoclonal antibodies with IgG1 and IgA heavy chain domains in transgenic tobacco plants. *Eur. J. Immunol.* **24**, 131–138.

Ma, J.K., Drake, P.M. and Christou, P. (2003) The production of recombinant pharmaceutical proteins in plants. *Nat. Rev. Genet.* **4**, 794–805.

Ma, J.K., Christou, P., Chikwamba, R., Haydon, H., Paul, M., Ferrer, M.P., Ramalingam, S., Rech, E., Rybicki, E., Wigdorowitz, A., Yang, D.C. and Thangaraj, H. (2013) Realising the value of plant molecular pharming to benefit the poor in developing countries and emerging economies. *Plant Biotechnol. J.* **11**, 1029–1033.

McCormack, S., Jespers, V., Low-Beer, N., Gabe, R., Kaganson, N., Chapman, A., Nunn, A., Lacey, C. and Van Damme, L. (2005) A dose-ranging phase I study of dextrin sulphate, a vaginal microbicide, in HIV-negative and HIV-positive female volunteers. *Sex. Transm. Dis.* **32**, 765–770.

McCoy, L.E. and Weiss, R.A. (2013) Neutralizing antibodies to HIV-1 induced by immunization. *J. Exp. Med.* **210**, 209–223.

Mett, V., Chichester, J.A., Stewart, M.L., Musiychuk, K., Bi, H., Reifsnyder, C.J., Hull, A.K., Albrecht, M.T., Goldman, S., Baillie, L.W.J. and Yusibov, V. (2011) A non-glycosylated, plant-produced human monoclonal antibody against anthrax protective antigen protects mice and non-human primates from B. anthracis spore challenge. *Hum. Vaccin.* **7**, 183–190.

Montefiori, D.C. (2005) Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. *Curr. Protoc. Immunol.* Chapter 12, Unit 12.11.

Murad, A.M. and Rech, E.L. (2012) NanoUPLC-MSE proteomic data assessment of soybean seeds using the Uniprot database. *BMC Biotechnol.* **12**, 82.

Nakamura, Y., Fujita, N., Kubo, A., Rahman, S., Morell, M. and Satoh, H. (2003) Engineering of amylopectin biosynthesis in rice endosperm. *J. Appl. Glycosci.* **50**, 197–200.

Nandi, S., Yalda, D., Lu, S., Nikolov, Z., Misaki, R., Fujiyama, K. and Huang, N. (2005) Process development and economic evaluation of recombinant human lactoferrin expressed in rice grain. *Transgenic Res.* **14**, 237–249.

Ning, T., Xie, T., Qiu, Q., Yang, W., Zhou, S., Zhou, L., Zheng, C., Zhu, Y. and Yang, D. (2008) Oral administration of recombinant human granulocyte-macrophage colony stimulating factor expressed in rice endosperm can increase leukocytes in mice. *Biotechnol. Lett.* **30**, 1679–1686.

Nutan and Gupta, S. (2011) Microbicides: a new hope for HIV prevention. *Indian J. Med. Res.* **134**, 939–949.

Patton, D.L., Cosgrove Sweeney, Y.T., McCarthy, T.D. and Hillier, S.L. (2006) Preclinical safety and efficacy assessments of dendrimer-based (SPL7013) microbicide gel formulations in a nonhuman primate model. *Antimicrob. Agents Chemother.* **50**, 1696–1700.

Paul, M.J., Teh, A.Y.H., Twyman, R.M. and Ma, J.K. (2013) Target product selection - where can Molecular Pharming make the difference? *Curr. Pharm. Des.* **19**, 5478–5485.

Poignard, P., Saphire, E.O., Parren, P.W. and Burton, D.R. (2001) gp120: Biologic aspects of structural features. *Annu. Rev. Immunol.* **19**, 253–274.

Preiss, J., Ball, K., Hutney, J., Smith-White, B., Li, L. and Okita, T.W. (1991) Regulatory mechanisms involved in the biosynthesis of starch. *Pure Appl. Chem.* **63**, 535–544.

Rademacher, T., Sack, M., Arcalis, E., Stadlmann, J., Balzer, S., Altmann, F., Quendler, H., Stiegler, G., Kunert, R., Fischer, R. and Stoger, E. (2008) Recombinant antibody 2G12 produced in maize endosperm efficiently neutralizes HIV-1 and contains predominantly single-GlcNAc N-glycans. *Plant Biotechnol. J.* **6**, 189–201.

Ramessar, K., Capell, T. and Christou, P. (2008a) Molecular pharming in cereal crops. *Phytochem. Rev.* **7**, 579–592.

Ramessar, K., Rademacher, T., Sack, M., Stadlmann, J., Platis, D., Stiegler, G., Labrou, N., Altmann, F., Ma, J.K., Stöger, E., Capell, T. and Christou, P. (2008b) Cost-effective production of a vaginal protein microbicide to prevent HIV transmission. *Proc. Natl Acad. Sci. USA*, **105**, 3727–3732.

Ramessar, K., Sabalza, M., Capell, T. and Christou, P. (2008c) Maize plants: an ideal production platform for effective and safe molecular pharming. *Plant Sci.* **174**, 409–419.

Ramessar, K., Sabalza, M., Miralpeix, B., Capell, T. and Christou, P. (2010) Can microbicides turn the tide against HIV? *Curr. Pharm. Des.* **16**, 468–485.

Rybicki, E.P. (2014) Plant-based vaccines against viruses. *Virol. J.* **11**, 205.

Sabalza, M., Madeira, L., van Dolleweerd, C., Ma, J.K., Capell, T. and Christou, P. (2012) Functional characterization of the recombinant HIV-neutralizing monoclonal antibody 2F5 produced in maize seeds. *Plant Mol. Biol.* **80**, 477–488.

Sabalza, M., Vamvaka, E., Christou, P. and Capell, T. (2013) Seeds as a production system for molecular pharming applications: status and prospects. *Curr. Pharm. Des.* **19**, 5543–5552.

Saito, Y., Kishida, K., Takata, K., Takahashi, H., Shimada, T., Tanaka, K., Morita, S., Satoh, S. and Masumura, T. (2009) A green fluorescent protein fused to rice prolamin forms protein body-like structures in transgenic rice. *J. Exp. Bot.* **60**, 615–627.

Shimada, T., Kuroyanagi, M., Nishimura, M. and Hara-Nishimura, I. (1997) A pumpkin 72-kDa membrane protein of precursor-accumulating vesicles has characteristics of a vacuolar sorting receptor. *Plant Cell Physiol.* **38**, 1414–1420.

Shimada, T., Watanabe, E., Tamura, K., Hayashi, Y., Nishimura, M. and Hara-Nishimura, I. (2002) A vacuolar sorting receptor PV72 on the membrane of vesicles that accumulate precursors of seed storage proteins (PAC vesicles). *Plant Cell Physiol.* **43**, 1086–1095.

Shukla, A.A., Hubbard, B., Tressel, T., Guhan, S. and Low, D. (2007) Downstream processing of monoclonal antibodies-application of platform approaches. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **848**, 28–39.

Sikka, V.K., Choi, S.B., Kavakli, I.H., Sakulsingharoj, C., Gupta, S., Ito, H. and Okita, T.W. (2001) Subcellular compartmentation and allosteric regulation of the rice endosperm ADPglucose pyrophosphorylase. *Plant Sci.* **161**, 461–468.

Silva, J.C., Gorenstein, M.V., Li, G.Z., Vissers, J.P.C. and Geromanos, S.J. (2006) Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol. Cell Proteomics*, **5**, 144–156.

Stiegler, G., Armbruster, C., Vcelar, B., Stoiber, H., Kunert, R., Michael, N.L., Jagodzinski, L.L., Ammann, C., Jäger, W., Jacobson, J., Vetter, N. and Katinger, H. (2002) Antiviral activity of the neutralizing antibodies 2F5 and 2G12 in asymptomatic HIV-1-infected humans: a phase I evaluation. *AIDS*, **16**, 2019–2025.

Stöger, E., Vaquero, C., Torres, E., Sack, M., Nicholson, L., Drossard, J., Williams, S., Keen, D., Perrin, Y., Christou, P. and Fischer, R. (2000) Cereal crops as viable production and storage systems for pharmaceutical scFv antibodies. *Plant Mol. Biol.* **42**, 583–590.

Stöger, E., Sack, M., Perrin, Y., Vaquero, C., Torres, E., Twyman, R.M., Christou, P. and Fischer, R. (2002) Practical considerations for pharmaceutical antibody production in different crop systems. *Mol. Breeding*, **9**, 149–158.

Stöger, E., Ma, J.K., Fischer, R. and Christou, P. (2005) Sowing the seeds of success: pharmaceutical proteins from plants. *Curr. Opin. Biotechnol.* **16**, 167–173.

Sudhakar, D., Duc, L.T., Bong, B.B., Tinjuangjun, P., Maqbool, S.B., Valdez, M., Jefferson, R. and Christou, P. (1998) An efficient rice transformation system utilizing mature seed-derived explants and a portable, inexpensive particle bombardment device. *Transgenic Res.* **7**, 289–294.

Terman, D.S. and Bertram, J.H. (1985) Antitumor effects of immobilized protein A and staphylococcal products: linkage between toxicity and efficacy, and identification of potential tumoricidal reagents. *Eur. J. Cancer Clin. Oncol.* **21**, 1115–1122.

Thorbjornsen, T., Villand, P., Denyer, K., Olsen, O.A. and Smith, A.M. (1996) Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. *Plant J.* **10**, 243–250.

Torres, E., Vaquero, C., Nicholson, L., Sack, M., Stöger, E., Drossard, J., Christou, P., Fischer, R. and Perrin, Y. (1999) Rice cell culture as an alternative production system for functional diagnostic and therapeutic antibodies. *Transgenic Res.* **8**, 441–449.

Tusé, D., Tu, T. and McDonald, K.A. (2014) Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed. Res. Int.* **2014**, 256135.

Twyman, R.M., Schillberg, S. and Fischer, R. (2005) Transgenic plants in the biopharmaceutical market. *Expert Opin. Emerg. Drugs*, **10**, 185–218.

UNAIDS. (2013) *UNAIDS Glob. Rep*. Available at: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/unaids_global_report_2013_en.pdf (Last accessed 7 Jnuary 2014).

Valdez, M., Cabrera-Ponche, J.L., Sudhakar, D., Herrera-Estrella, L. and Christou, P. (1998) Transgenic central American, west African and Asian

elite rice varieties resulting from particle bombardment of foreign DNA into mature seed-derived explants utilizing three different bombardment devices. *Ann. Bot.* **82**, 795–801.

Van Droogenbroeck, B., Cao, J., Stadlmann, J., Altmann, F., Colanesi, S., Hillmer, S., Robinson, D.G., Van Lerberge, E., Terryn, N., Van Montagu, M., Liang, M., Depicker, A. and Jaeger, G.D. (2007) Aberrant localization and underglycosylation of highly accumulating single-chain Fv-Fc antibodies in transgenic Arabidopsis seeds. *Proc. Natl Acad. Sci. USA*, **104**, 1430–1435.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., Komarova, N.L., Nowak, M.A., Hahn, B.H., Kwong, P.D. and Shaw, G.M. (2003) Antibody neutralization and escape by HIV-1. *Nature*, **422**, 307–312.

Xie, T., Qiu, Q., Zhang, W., Ning, T., Yang, W., Zheng, C., Wang, C., Zhu, Y. and Yang, D. (2008) A biologically active rhIGF-1 fusion accumulated in transgenic rice seeds can reduce blood glucose in diabetic mice via oral delivery. *Peptides*, **29**, 1862–1870.

Yang, L., Hirose, S., Suzuki, K., Hiroi, T. and Takaiwa, F. (2012) Expression of hypoallergenic Der f 2 derivatives with altered intramolecular disulphide bonds induces the formation of novel ER-derived protein bodies in transgenic rice seeds. *J. Exp. Bot.* **63**, 2947–2959.

Zeitlin, L., Olmsted, S.S., Moench, T.R., Co, M.S., Martinell, B.J., Paradkar, V.M., Russell, D.R., Queen, C., Cone, R.A. and Whaley, K.J. (1998) A humanized monoclonal antibody produced in transgenic plants for immunoprotection of the vagina against genital herpes. *Nat. Biotechnol.* **16**, 1361–1364.

Zhang, L., Shi, J., Jiang, D., Stupak, J., Ou, Q., An, N., Li, J. and Yang, D. (2012) Expression and characterization of recombinant human alpha-antitrypsin in transgenic rice seed. *J. Biotechnol.* **164**, 300–308.

Supporting information

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

Figure S1 Quantitative real-time RT-PCR analysis of modulated genes.

Table S1 Oligonucleotide sequences of primers used for quantitative real-time RT-PCR analysis.