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Production of recombinant HIV monoclonal antibodies for human health in transgenic maize seeds

K. Ramessar*,[1], E. Stoger, T. Capell* and P. Christou*,*****
*Departament de Produccio Vegetal I Ciencia Forestal (PVCF), Universitat de Lleida, Av. Alcalde Rovira Roure, 177, Lleida, E-25198, Spain
**Institute for Molecular Biotechnology, Biology VII, RWTH Aachen, Worringerweg 1, 52074 Aachen, Germany
***Institucio Catalana de Recerca i Estudis Avançats (ICREA), Spain
[1]Corresponding author: Koreen.Ramessar@pvcf.udl.es

SUMMARY – Monoclonal antibodies are difficult to express in microbial expression systems due to their specific folding requirements and need for post-translational modifications. Plant production systems, apart from being inexpensive with potential for rapid, economical scale-up, have the necessary processing steps for folding and assembly (via the secretory pathway). Cereal seeds offer high stability of proteins when dry and can be easily stored for long periods of time. The objective of this study therefore, was to express the neutralizing HIV monoclonal antibody (C2G12) into the endosperm tissue of maize seeds. The heavy and light chain genes of C2G12 were cloned into suitable transformation vectors containing the rice glutelin-1 promoter for endosperm-specific expression. These genes, together with the *bar* gene for selection, were co-bombarded into immature zygotic M37W maize embryos (an elite South African variety) either as full vector constructs, or minimal transgene cassettes (promoter, open reading frame and terminator). Selection occurred on phospinothricin-containing media and independent transgenic events were accordingly identified and characterized by Southern blot analysis.

Introduction

An estimated 40.3 million people worldwide are living with human immunodeficiency virus (HIV), including 4.9 million people who contracted it in 2005 alone (UNAIDS/WHO, 2005). Human neutralizing monoclonal antibodies (MAbs) that recognize the viral envelope are promising effector molecules for the inhibition of attachment and entry of HIV into susceptible cells. The C2F5 and C2G12 MAbs are two of the three most potent candidates for anti-HIV-1 passive immunotherapy (DSouza *et al*., 1997) and they have previously been shown in combination to protect against live viral challenge in a macaque model (Mascola *et al*., 1999; 2000). CHO derived C2F5 and C2G12 MAbs, included in an initial phase I clinical trial in Austria, were shown to be safe and virologically active in human subjects. Thus, the 2G12 MAb genes were chosen for use in this study.

Due to the high demand for recombinant pharmaceuticals, current production systems, such as bacteria, fungi, cultured insect or mammalian cells and transgenic animals (Benatti *et al*., 1991) are no longer sufficient. Thus, alternative production systems are required and plants provide an attractive system for production of such molecules because the plant secretory pathway is capable of folding, assembling and transporting these with high efficiency (Frigerio *et al*., 2000; Hadlington *et al*., 2003). Therefore, plants ultimately represent an inexpensive and versatile expression system for a wide variety of recombinant proteins, and they offer the potential for rapid and economical scale-up. Crop plants, for example maize, have well established agronomic, harvesting, transport, storage and processing practices. Maize, like other cereal seeds, additionally does not contain toxic metabolites and exhibits high stability of recombinant proteins in dry seeds. This allows for long-term storage and easy distribution prior to processing.

The aim of this study therefore was to engineer maize, via particle bombardment, with the heavy (*HC*) and light chain (*LC*) genes for endosperm specific expression of the 2G12 MAb under the control of the rice glutelin promoter. For selection of transgenic tissues, the *bar* gene conferring herbicide resistance was co-transformed. The transgenic maize line expressing the antibody at the highest level will be used for further downstream analysis and eventually, as the source for the recombinant antibodies envisaged for Phase I clinical trials.

Material and methods

Plant material

Pre-cultured immature zygotic embryos (1.2 - 1.8 mm) of the South African elite, white maize genotype (M37W), were used as explants.

Transformation vectors

The C2G12 heavy and light chain genes were placed on separate plasmid vectors (pTRAgtiGH and pTRAgtiGLF respectively). The vectors contained the rice glutelin-1 promoter for seed specific expression, the maize ubiquitin-1 intron, and a signal peptide upstream of the coding regions. The selectable marker gene (*bar*) was placed on a separate plasmid vector (pTRAuxBar) and was driven by the maize ubiquitin 1 promoter, with its first intron. For preparation of the minimal DNA transgene cassettes (promoter, open-reading frame and terminator), the pTRAgtiGH and pTRAgtiGLF plasmids were digested with *Asc*I and *Pme*I yielding 8788 bp and 8074 bp fragments respectively, while pTRAuxBar was digested with *Asc*I and *Fse*I to yield a 2807 bp fragment.

Microprojectile bombardment

Pre-cultured M37W immature zygotic embryos (IZEs) were pre-treated on osmoticum-containing N6-based callus induction medium (Armstrong and Songstad, 1993) for 3 hours prior to bombardment. The bombardment mixture was prepared by precipitating plasmid DNA onto gold particles and target tissues were bombarded using standard particle bombardment methods (Mehlo *et al*., 2005; Drakakaki *et al*., 2006).

Selection and regeneration

The bombarded IZEs were cultured on osmoticum-N6 media for 16 h post-bombardment before being transferred to N6-callus induction media for 2 days. The IZEs were subsequently transferred to N6-callus induction media supplemented with 3 mg l^{-1} PPT for 4-6 weeks under dark conditions, with subculture to fresh media every 2 weeks. Proliferating white, compact type-I calli were then regenerated on an MS-based media (Murashige & Skoog, 1962) containing 3mg l^{-1} PPT, for 2-4 weeks under full light conditions. Regenerating plantlets were subcultured at 2-3 week intervals, until they developed shoots (greater than 3 cm) and roots (greater than 1cm) before being hardened off in soil and grown under greenhouse conditions. Putative transgenic plants (depending on pollen availability) were either self or cross-pollinated with wildtype M37W pollen.

Southern blot analysis

Genomic DNA (15 μg), extracted from leaves of the transgenic lines using the standard phenol/chloroform method , was digested with *Kpn* I restriction enzyme, separated on a 0.8% (w/v) agarose gel and transferred to positively charged Nylon membranes. Prehybridization and hybridization were carried out at 42°C in DIG Easy Hyb (Roche Diagnostics). Nucleic acids were fixed by UV crosslinking. The membranes were prehybridized for 2 h and then hybridized with the respective probe for 16-20 h. The digoxigenin-labelled PCR probes for the heavy (*HC*) and light chain (*LC*) genes contained 177bp of the end of the rice glutelin-1 promoter, the entire ubiquitin intron including the relevant signal peptides, and the beginning of the specific antibody-chain gene (217bp for the *HC* and 128bp for the *LC*). Membranes were then washed twice at room temperature with 2 × SSC and 0.1% SDS solution for 5 minutes each, and once at 68°C with a 0.5 × SSC and 0.1% SDS solution for 20 minutes. Chemiluminescence detection of nucleic acids was performed using CSPD as substrate (Roche Diagnostics). Before re-probing with the *LC* probe, the membranes were stripped twice in 0.2 M NaOH and 0.1% SDS solution for 15 minutes at 37°C, then rinsed twice in 2 x SSC for 30 minutes each, and air dried for 15 minutes.

Results and discussion

Regeneration of transgenic plants

Embryogenic type-I calli were initiated when bombarded IZEs were cultured on N6-based PPT selection media. This white, compact, cup-shaped calli formed somatic embryos which regenerated to plantlets with shoots and roots. Transgenic plantlets were successfully hardened off in soil. Forty independent events in total (including events from both full plasmid and minimal transgene cassette experiments) were selected for further analysis. Most plants displayed a normal phenotype however; a small number displayed leaf-curling which was attributed to tissue culture stress. All plants were successfully pollinated (either selfed or outcrossed).

Southern blot analysis

Kpn I restriction enzyme used for digestion of genomic DNA, has a unique restriction site on both plasmids and thus cuts the plasmids once. The probe that was used was designed to contain (in addition to part of the *HC* gene), the maize ubiquitin intron. Southern blot analysis (Fig. 1) showed hybridising bands of the digested genomic DNA with the *HC* probe occurring for all, except one (Lane 5), transgenic events. Hybridisation with wild type M37W (Lane WT) also occurs however; this is expected as the probe used contained the ubiquitin intron sequence. This however, can be subtracted from the resulting independent transformation patterns displayed by the different events analysed to yield unique integration patterns for the respective events. Similar results were obtained upon probing with the *LC* probe (data not shown). Southern blot analysis of all independent transformation events tested, were positive for detection with both the *HC* and *LC* probes.

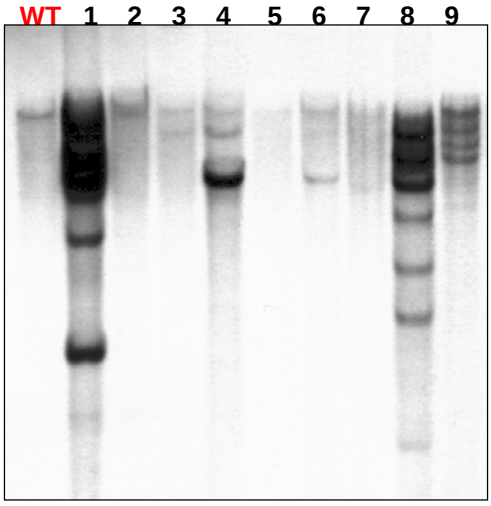

Fig. 1. Southern blot analysis of T_0 transgenic M37W elite white maize plants (with full plasmid constructs). Fifteen micrograms of genomic DNA (purified from leaf material) for all samples, were restricted with the *Kpn*I restriction enzyme; the figure shows hybridisation, upon probing for the detection of the 2G12 heavy chain gene. Lanes 1 to 9 represents independent transgenic events while WT represents wild type, M37W.

Current work

Ongoing work focuses on the detection of the HIV monoclonal antibody (MAb) protein in transgenic seeds through western blot analysis. Biochemical analysis for expression levels (ELISA) and specific antigen-binding activity of these neutralising MAbs produced, are also ongoing. The events identified to

expresses the MAb at the highest levels, will be selected for further studies.

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