Transient expression of a full-size antibody against Hepatitis B surface antigen in plant cell suspension cultures

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INVESTIGACIÓN

ABSTRACT

When generating stably transformed transgenic plants, transient gene expression experiments are especially useful to confirm that the foreign molecule of interest is expressed with an adequate biological activity. In this paper we report the transient expression of a full-size mouse monoclonal antibody against Hepatitis B surface antigen in tobacco cell suspension cultures. Transient expression in tobacco cell cultures was fast, and protein expression could be detected in just a few days. We were able to verify that the gene construction was functional, and that 0.3-0.5 μg of a biologically active antibody was easily purified from 1 mL of cell culture by Protein A chromatography. Recovered recombinant antibodies were sufficient for a detailed characterization by SDS-PAGE, Western-blot, and ELISA. These results allowed us to move on to the large-scale production of the plantibody in stably transformed transgenic plants.

Key words: Plantibody, HBsAg, *Agrobacterium tumefaciens*, cell suspension cultures

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RESUMEN

Expresión transitoria de un anticuerpo completo contra el antígeno de superficie del virus de la Hepatitis B en cultivos de suspensiones celulares de plantas. Al generar plantas transgénicas establemente transformadas, se hace imprescindible confirmar que la molécula foránea de interés es expresada con una adecuada actividad biológica y esto se puede lograr mediante los experimentos de expresión transitoria de genes. En este artículo se reporta la expresión transitoria del anticuerpo monoclonal de ratón contra el antígeno de superficie del virus de la Hepatitis B en suspensiones celulares de tabaco. Mediante la expresión transitoria en cultivo de células se pudo detectar en pocos días la expresión de la proteína de interés; fue posible verificar que la construcción génica era funcional y además se pudieron purificar 0.3-0.5 μg del anticuerpo biológicamente activo a partir de 1 mL del cultivo celular mediante cromatografía de afinidad por proteína A. El recobrado del anticuerpo recombinante fue suficiente para la caracterización detallada por SDS-PAGE, Western-blot y ELISA. Estos resultados nos permitieron abordar la producción, a gran escala, de planticuerpos a partir de plantas transformadas establemente.

Palabras claves: Planticuerpo, HBsAg, *Agrobacterium tumefaciens*, cultivo de suspensiones celulares

*I*ntroduction

Transgenic plants producing recombinant immunoglobulins have been reported as a cost-effective technology for the large-scale production of therapeutic monoclonal antibodies [1, 2, 3, 4]. Antibodies are efficiently folded and assembled "*in planta*" and retain the antigen binding properties of the immunoglobulins produced by hybridoma cells [5, 6, 7, 8].

Regenerating transgenic plants from transformed cells is both labour-intensive and time-consuming. It is thus important to have a method that allows testing antibody expression cassettes before moving into the phase where stably transformed plants are developed. Transient gene expression in plant cells and tissues is such a procedure. This system allows the rapid evaluation of plant-expressed antibodies, with the advantages that expression is not biased by positional effects [9], and that heterologous gene expression is induced at an advanced developmental stage, avoiding potentially negative effects on the plant cells. Transient expression assays are usually applicable to protoplasts and intact cells [9, 10].

Based on the fact that plant cell suspension cultures obtained from transgenic plants are currently employed for antibody production [11, 12, 13], we have now established a transient expression system where cell suspension cultures are infected with recombinant A*grobacterium.* Using the transient expression of a mouse monoclonal antibody against the Hepatitis B virus surface antigen (HBsAg) in tobacco cells in culture as a model, we show that this procedure allows rapid testing of gene functionality, and provides enough immunoglobulin to confirm biological activity, and initiate the characterization of the plantibody.

*M*aterials and methods

Agrobacterium-mediated transient expression system

Agrobacterium tumefaciens strain LBA4404 [14] carrying the binary vector pDHCLC was used to infect tobacco (Petit Havana SR1) cell suspensions. This vector has been previously described by our group [15]. Briefly, the plasmid bears the cassettes P35S-LC-TNOS and P35S-HC-TNOS containing the light and heavy chain genes, respectively, of the CB-Hep.1 anti-HBsAg mouse monoclonal antibody. The construct also harbours the sporamin prepeptide [16] for protein secretion, and the KDEL tetrapeptide for endoplasmatic reticulum protein retention.

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Introduction of pDHCLC into *Agrobacterium tumefaciens* was done by the liquid nitrogen transformation method [17]. Cell suspension cultures, and recombinant *Agrobacterium* cultures for infection were performed essentially as described by An in 1985 [18].

Protein extraction from cell suspension

Cells were collected at 3, 5, and 7 days post-infection (DPI), washed twice with two volumes (w:v) of PBS buffer, and ground in liquid nitrogen to a fine powder with mortar and pestle. Soluble protein was extracted by using 1 mL of extraction buffer (60 mM Tris-HCl, pH 8, 500 mM NaCl, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% Tween 20, 0.1 mM PMSF) per gram of powder. The homogenate was centrifuged for 10 minutes at 16 000 g and protein concentration in the supernatant was determined by the Bradford method [19].

Analyses of anti-HBsAg antibody expression by ELISA

Plates were coated with 100 µL per well of 5 µg/mL of pure recombinant HBsAg (Heber Biotec S.A.) in 50 mM sodium carbonate buffer, pH 9.6. After blocking with 5% skim milk and 0.1% Tween 20 in PBS (PBST), 100 µL of the test sample were applied per well and incubated overnight at 4 ºC. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG diluted 1:30,000 (Boehringer Mannheim) was added to every well and incubated for 1 h at 37 ºC. The colour enzymatic reaction was developed with 4-nitrophenyl phosphate. Optical Densities (O.D.) of the wells were measured at 405 nm. The CB-Hep.1 mouse monoclonal antibody purified from ascites was used as the positive control.

Analyses of expression by Western-blot

Proteins were separated on a 10% SDS-PAGE, under non denaturing conditions, and electroblotted on a PVDF membrane. The anti-HBsAg antibody was detected with rabbit anti-Fab IgG polyclonal antibodies [20], and AP-conjugated anti-rabbit IgG (Boehringer Mannheim).

Purification of the plantibody

The antibody was purified by affinity chromatography using Protein A Sepharose (Pharmacia), starting from the cell suspension protein extracts. The column was equilibrated with extraction buffer and washed with citric acid pH 5.0. Protein was eluted with citric acid pH 3.0, and immediately neutralized with Tris-HCL 2M. Purity of the fractions was analyzed on a 12.5% SDS-PAGE and Coomasie staining.

Results and Discussion

Taking advantage of our previous experience establishing callus cell cultures [13], we envisaged that combining this method with *Agrobaterium* infection would lead to a suitable transient expression system for antibody molecules. This transient gene expression in cell suspension cultures can rapidly verify that the gene construct is functional. In general, transient expression systems are predictive and reflect the accumulation that can be expected in transgenic tobacco plants at least when using the constitutive 35S promoter [21, 22]. Our system is unique for antibody analysis due to the fact that the literature only reports leaves as the source of transient expression of plantibodies [23, 24], using the constitutive promoter 35S.

Expression of the CB-Hep.1 mouse monoclonal antibody in stably transformed tobacco plants has been previously studied in detail by our group [15]. Hence, this molecule was an adequate model to evaluate our transient expression strategy.

The tobacco cell suspension was infected by cocultivation with recombinant *A. tumefaciens*, bearing the plasmid that contains the heavy and light chain antibody genes in tandem expression cassettes, together with the secretion and endoplasmic reticulum (ER) anchorage signal sequences. The selection of ER as an accumulation compartment is derived from our previous experience with single-chain Fv antibody fragments of this antibody [8].

For ELISA assays, protein extracts were taken from the same cell culture at different times of cultivation after A*grobacterium* infection, and tested together with the negative and positive controls (Figure 1). Optimum time for sample collection was five DPI, when D.O. values from tested samples were two-fold higher than the negative control.

Figure 2 demonstrates how the extracts from two different infected cell cultures, collected on the fifth

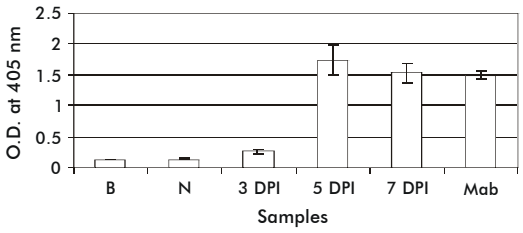

Figure 1. Kinetic analysis of transient anti-HBsAg antibody expression in cultured tobacco cells, as determined by ELISA. B: extraction buffer, N: protein extract from non-infected cell suspension (negative control), 3DPI, 5DPI, 7DPI: samples collected 3, 5 and 7 days post-infection. MAb: 0.1 µg of the anti-HBsAg antibody purified from ascites (positive control). Values are the average of the O.D. obtained from three replicates, with standard deviations (error bars).

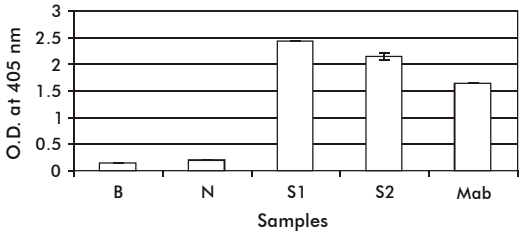

Figure 2. Transient anti-HBsAg antibody expression in cultured tobacco cells, as determined by ELISA. B: extraction buffer, N: protein extract from non-infected cell suspension (negative control), S1 and S2: samples (protein extracts from two different infected cell suspensions). MAb: 0.1 µg of anti-HBsAg antibody purified from ascites (positive control). Values are the average of the O.D. obtained from two independent ELISA experiments with two replicates each. Bars indicate standard deviations.

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DPI, contain functional anti-HBsAg antibodies. Different values in ELISA between S1 and S2 samples are due to the fact that each transient expression experiment is an independent event, and results in different infection (and translation) efficiencies.

We showed that successive cell washes with PBS before protein extraction resulted in an increase in the positive signal in ELISA. Figure 3 depicts the results of an ELISA experiment where non-washed (T1), and washed culture cells (T2) were used as testing material. Washing increased the signal by at least two-fold, thereby improving antigen-antibody recognition. This simple step increases the sensitivity of the system, and may allow the detection of proteins with low expression levels in tobacco cells.

The molecular size expected for the anti-HBsAg plantibody (ca. 150 kDa) was verified by Western-blot, using specific anti-Fab polyclonal antibodies. Samples were run under non-reducing conditions to confirm the assembly of heavy and light chains in an immunoglobulin molecule (Figure 4). No signs of proteolytic degradation were found.

The anti-HBsAg plantibody was purified by Protein A chromatography from the transient expression cell suspension material. The SDS-PAGE (reducing conditions) in Figure 5 is an example of the purification process. The protein bands corresponding to light and heavy antibody chains are not visible in the protein extract from infected cell suspension cultures (lane 1), but can be detected in the elution fraction (lane 2; ca. 25 and 50 kDa, respectively). The yield of the purification process was of about 0.3-0.5 µg/mL of cell suspension.

Conclusion

The evaluated transient expression system proved adequate for the fast determination of the functionality of the antibody gene construction, and the obtainment of purified anti-HBsAg antibodies by standard Protein A chromatography for initial characterization. Experience with several other antibodies will demonstrate the universal usefulness of the method.

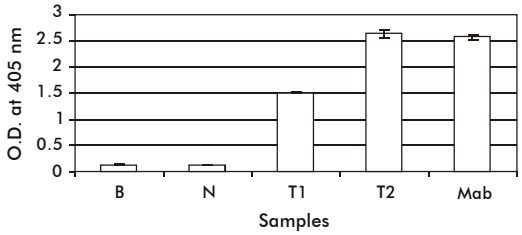

Figure 3. Transient anti-HBsAg antibody expression in washed and non-washed cultured tobacco cells, as determined by ELISA. B: extraction buffer, N: protein extract from non-infected cell suspension (negative control), T1: sample from cells that were not washed before protein extraction, T2: sample from cells washed before protein extraction, MAb: 0.1 μg of anti-HBsAg antibody, purified from ascites (positive control). Values are the average of O.D. obtained from two independent ELISA experiments with two replicates each. Bars indicate standard deviations.

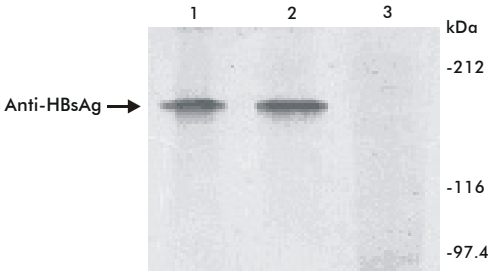

Figure 4. Detection by Western blot of the plantibody in cell suspension cultures. Lane 1: 1 μg of anti-HBsAg purified from ascites, Lane 2: 30 μL of protein extract from infected cell suspension cultures, Lane 3: 30 μL of protein extract from non-infected cell suspension cultures. Molecular weights are indicated on the right side of the Figure.

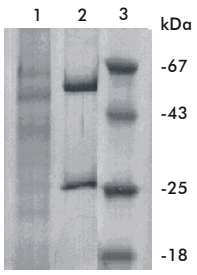

Figure 5. SDS-PAGE of the purified anti-HBsAg from infected plant cell suspension cultures. Lane 1: 30 μL of protein extract from infected cell suspension culture, Lane 2: 30 μL of the elution fraction, Lane 3: molecular weight markers.

The use of plant cell suspension cultures and infection with recombinant *Agrobacterium tumefaciens*, could also be potentially useful for the functional analysis of genetic construction that employ promoters other than 35S, due to the undifferentiated state of the target cell.

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