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Research Brief

Efficient expression of a *Toxoplasma gondii* dense granule Gra4 antigen in tobacco leaves

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ABSTRACT

A His-tagged truncated version of *Toxoplasma gondii* dense granule 4 protein ($Gra4_{163-345}$) was transiently expressed in tobacco leaves. Two genetic constructions were used to accomplish this goal. In one of them, based in a Potato virus X (PVX) amplicon, the sequence encoding His-$Gra4_{163-345}$ was placed under control of an additional PVX coat protein subgenomic promoter. In the other, the same sequence was fused to an apoplastic transport signal and placed under the direction of the *cauliflower mosaic virus* 35S promoter. His-$Gra4_{163-345}$ accumulation in agroinfiltrated tobacco leaves was estimated by Western blot analysis using mouse anti-Gra4 antibody and a seropositive human serum. Here, we demonstrated the feasibility of producing a Gra4 antigen using transient expression methods in plants.

1. Introduction

Within the last years, the use of recombinant technology permitted the development of new diagnostic and immunoprophylatic tools in the field of parasitology. A proper knowledge of the efficacy of different adjuvants and expression systems is important for the design of recombinant vaccines. Within the available expression systems, the most widely used is the expression of recombinant antigens in *Escherichia coli*. However, as some proteins can not be produced by bacteria, other systems based on yeast and insect cells have been developed for heterologous expression. Interestingly, expression of recombinant proteins in plants has recently emerged as a powerful and modern bioreactor system. Plants provide an inexpensive system for large-scale production of recombinant proteins and have many advantages over other expression systems, particularly in terms of feasibility, scaling up and biosafety (Twyman et al., 2003; Ma et al., 2005). In fact, several viral and pharmaceutical proteins, among others, had been successfully expressed, either permanently or transiently in plants (Streatfield, 2007). Despite the potential benefits of expressing antigens in plant-based systems, this strategy received limited attention for the expression of parasite antigens. In 1995, Turpen et al. expressed selected malarial B-cell epitopes inserted in the tobacco mosaic tobamovirus coat protein. Subsequently, Ghosh et al. (2002) reported the cloning and expression of *Plasmodium falciparum* C-terminal region of merozoite surface protein (PfMSP119) in transgenic tobacco plant. We have successfully expressed a parasite protein (the *Toxoplasma gondii* surface antigen SAG1) in tobacco leaves (Clemente et al., 2005). Although, transient expression of SAG1 was moderate compared to other proteins, the immunization of mice with recombinant leaf extracts combined with Freund's adjuvant showed immunoprotective properties against *T. gondii* infection (Clemente et al., 2005). More recently, Wang et al. (2008) expressed *P. yoelii* merozoite surface protein 4/5 (PyMSP4/5) in transgenic plants. TPyMSP4/5 transgenic tobacco induced antigen-specific antibodies in mice following parenteral delivery and also increased antibody responses induced by DNA vaccination when delivered parenterally or orally in a mouse model of malaria infection. As far as we know, these are the only documented experiences in plant-based expression of parasite proteins.

The present work builds on the previous examples in an attempt to develop and optimize the expression of parasite antigens in plants. Here, we report the expression of another *T. gondii* antigen, the dense granule protein 4 (Gra4). Gra4 is a key antigen because it is known to effectively promote expression of IgA and acute IgG antibodies (Mevelec et al., 1998; Nigro et al., 2003; Altcheh et al., 2006). In addition, Gra4 appears as an interesting component for the development of an anti-*Toxoplasma* multiantigenic vaccine, which could be based either in DNA or in recombinant proteins (Martin et al., 2004; Mevelec et al., 2005; Zhang et al., 2007).

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Toxoplasma gondii is an important human and veterinary pathogen (Tenter et al., 2000). This parasite has long been known to be the causative agent of congenital disorders, as well as being responsible for severe or lethal opportunistic diseases in immunocompromised individuals, including cancer patients receiving chemotherapy, transplanted patients and individuals suffering from AIDS and other immunosuppressive disorders (Luft and Remington, 1992; Wong and Remington, 1993). In addition, *T. gondii* is listed as a Category B pathogen in NIAID's organisms of interest for biodefense research. The conventional therapy against toxoplasmosis is rather ineffective, mostly due to two major causes: its severe toxicity to the human host and the resistance of the resting stage (i.e, tissue cysts) (Luft and Remington, 1992; Wong and Remington, 1993; Petersen, 2007).

In this paper, the truncated Gra4 ($Gra4_{163\text{-}345}$) sequence (Martin et al., 2004) was chosen for transient expression in plants, based on a PVX amplicon, which allows cytoplasmic accumulation of the recombinant protein (Clemente et al., 2005). An alternative strategy for plant expression was investigated for antigen secretion into the extracellular space. Both constructs were assayed by agroinfiltration of tobacco leaves.

2. Materials and methods

2.1. Construction of pZPVXGra4 amplicon

The His-tagged Gra4 C-terminal region (encoding from residue 163 to residue 345 at the C-terminal end of the protein) was amplified by polymerase chain reaction (PCR) from plasmid pQE31-$Gra4_{163\text{-}345}$ (Nigro et al., 2003). Amplification was performed using primers 5′-gggcccatgggatctcatcaccatcac-3′ (6xHisFApaI) and 5′-cc cgggttactcttttctcattctttc-3′ (Gra4RSmaI), carrying recognition sites for ApaI and SmaI (underlined), respectively. The resulting DNA fragment includes the six-residue His tag, part of the pQE31 polylinker and the $Gra4_{163\text{-}345}$ sequence. The PCR product was cloned at the ApaI/SmaI sites of pGEM-T Easy (Promega) and analyzed by sequencing. After digestion with the same enzymes, the His-$Gra4_{163\text{-}345}$ DNA fragment was purified from an agarose gel (GXF; Amersham) and cloned into the corresponding sites of pZPVX (Clemente et al., 2005) generating amplicon pZPVXHis-$Gra4_{163\text{-}345}$ (Fig. 1A).

2.2. Construction of pApoGra4 vector

The His-$Gra4_{163\text{-}345}$ sequence was fused to the apoplasmic signal sequence (encoding residues 1–22 of tobacco AP24 osmotin) and placed under the control of the *Cauliflower mosaic virus* (CaMV) duplicate 35S promoter (Clemente et al., 2005) (Fig. 1B). Amplification of the apoplasmic version of His-$Gra4_{163\text{-}345}$ was performed with primers 5′-atcgatatgggcaacttgagatcttc-3′ (ApoFcla) and 5′-tctagattactcttttctcattctttc-3′ (Gra4RXba), carrying recognition sites for ClaI and XbaI (underlined), respectively. The His-tagged $Gra4_{163\text{-}345}$ sequence fused to the apoplastic sequence was amplified with primers ApoFcla and Gra4RXba, using overlapping fragments corresponding to the apoplastic signal and the His-$Gra4_{163\text{-}345}$ coding sequence. This PCR product (Ap-His-$Gra4_{163\text{-}345}$ fragment) was digested with ClaI and XbaI and cloned into the expression cassette of the intermediate plasmid pBPFΩ7. Finally, the entire Ap-His-$Gra4_{163\text{-}345}$ expression cassette was released from the vector and cloned into the binary plant vector pZP200 to obtain the plasmid pAp-His-$Gra4_{163\text{-}345}$ (for details, see Clemente et al., 2005).

2.3. Plant materials

Homozygous transgenic *Nicotiana tabacum cv. Xanthi* (line X-27-8) plants expressing high levels of the *Tobacco etch virus* (TEV) P1/HC-Pro sequence were cultivated in a greenhouse. Developing leaves were harvested eight weeks post-germination and used for vacuum infiltration.

2.4. Agrobacterium-mediated transient expression

Agrobacterium tumefaciens strain GV3101 (Rif^R Gm^R) was transformed with constructions pZPVXHis-$Gra4_{163\text{-}345}$ and pAp-His-$Gra4_{163\text{-}345}$ using the protocol of the freeze–thaw method (Höfgen and Willmitzer, 1988). Growth of recombinant *Agrobacterium* and vacuum infiltration of tobacco leaves was performed as described by Kapila et al. (1997). After infiltration, leaves were incubated with their adaxial side down within *Petri* dishes containing wet Whatman paper for four days (23 °C/16 h photoperiod).

2.5. Plant extracts preparation

About 10 g of tobacco leaves were used for extraction of transiently expressed recombinant proteins. Infiltrated leaves were ground in a mortar with liquid nitrogen to a fine powder. Soluble protein was extracted using 1 ml of urea buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8) per g of leaf material. The homogenate was centrifuged at 13,000 rpm for 10 min and the supernatant was used for expression analyses.

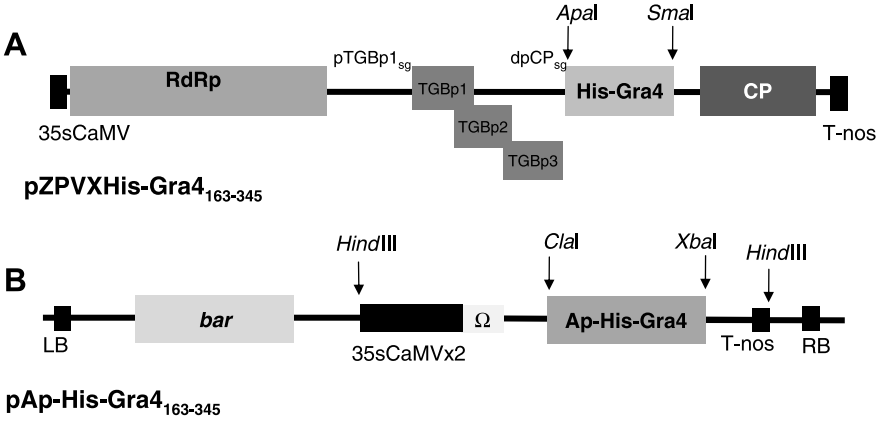

Fig. 1. Schematic representation of constructs used in the *Agrobacterium*-mediated transient expression systems. (A) pZPVXHis-$Gra4_{163\text{-}345}$. RdRp: RNA-dependent RNA-polymerase; TGBp1, TGBp2, TGBp3: triple gene block protein 1, protein 2 and protein 3, respectively; CP: coat protein gene; psgp1: protein 1 subgenomic promoter; psgCP: coat protein subgenomic promoter. (B) Schematic representation of pAp-His-$Gra4_{163\text{-}345}$. 35sCaMVx2: duplicated *CaMV* 35S promoter; T-nos: nopaline synthase terminator sequence; Ω: translational enhancer sequence. *bar*: gluphosinate-resistant gene; RB and LB: right and left borders of the T region, respectively.

2.6. Extraction of apoplastic washing fluid

Apoplastic washing fluid (AWF) was extracted by the vacuum infiltration/centrifugation technique (De Wit and Spikman, 1982). Leaves previously infiltrated with vector pAp-His-Gra4$_{163-345}$ were weighted and infiltrated with PBS. Subsequently, the pressure was reduced to 600 mm Hg for 1 min and then brought slowly to normal pressure. Leaves were removed from the PBS and dry blotted. AWF was recovered by centrifugation at 2000 rpm for 20 min at 4 °C.

2.7. Western blot analysis

The total protein content in leaf extracts and AFW was estimated according to the Bradford's assay, using bovine serum albumin as standard. Extracts were resuspended using cracking buffer (250 mM Tris–HCl, pH 6.8, 6% SDS, 5% glycerol, 0.05 mg/mg Bromophenol Blue), separated by 12% SDS–PAGE and then transferred onto an Immunoblot-ECL membrane (Amersham). The membrane was incubated with a mouse anti-Gra4 polyclonal antibody (1:500) followed by anti-mouse IgG peroxidase conjugate as secondary antibody (1:5000; NEN Life Science). In addition, human serum samples from *Toxoplasma*-infected individuals (Nigro et al., 2003) were assayed at 1:100 dilution. Horseradish peroxidase anti-human IgG polyclonal serum was used as secondary antibody (1:5000; Sigma). Peroxidase activity was measured using the ECL detection kit (Amersham). Prestained protein (Invitrogen) was included in Western blots as molecular weight markers. The quantification of Gra4$_{163-345}$ expressed in leaf extracts and EcGra4$_{163-345}$ was estimated using the program "Gel-Pro analyzer" (Media Cybernetics).

2.8. RT-PCR analysis

Total RNA was isolated from frozen material using Trizol (Gibco). RNA (3–5 μg) was reverse-transcribed using oligo (dT)$_{18}$ primer (Invitrogen) and the SuperScript™ Synthesis System for RT-PCR (Invitrogen). PCR was performed for 30 cycles for both His-Gra4$_{163-345}$ and actin using an initial denaturation step at 94 °C for 2 min, followed by 30 denaturation (30 s at 94 °C), annealing (30 s at 65 °C) and extension (1 min at 72 °C) cycles. After the last cycle, a final extension was carried out for 10 min at 72 °C. PCR products were visualized on 1.2% (w/v) agarose gels using a UV light transilluminator. Oligonucleotides 5′-actcctccaccaccagcatcttacggctac-3′ (Gra4F1), 5′-tctagattactcttttctcattctttc-3′ (Gra4XbaIR), 5′-ggattctggtgatggtgttag-3′ (ActF) and (5′-acttcctctcaggtggtgttag-3′ (ActR) were used as primers for amplification of the Gra4 and *actin* coding sequences, respectively.

3. Results and discussion

The C-terminal region of Gra4 (Gra4$_{163-345}$) had been previously cloned as recombinant His-tagged fusion in the pQE31 vector (pQE31-Gra4$_{163-345}$) and expressed in *E. coli* (Nigro et al., 2003). This region contains the B-cell and T-cell epitopes (Mevelec et al., 1998), displays antigenic properties (Nigro et al., 2003; Altcheh et al., 2006) and confers immune protection against Toxoplasmosis in mice when combined with alum (Martin et al., 2004). Because of these properties, Gra4$_{163-345}$ was chosen for expression in tobacco plants using two different constructs. One of them, the *Gra$_{163-345}$* coding sequence was ligated into the pZPVX vector to generate the amplicon pZPVXHis-Gra4$_{163-345}$ (Fig. 1A). The second construct, referred to as pAp-His-Gra4$_{163-345}$, was obtained by adding the tobacco AP24 osmotin apoplasmic signal peptide to the His-Gra4$_{163-345}$ sequence controlled by a duplicated CaMV 35 S constitutive promoter (Fig. 1B). The apoplasmic signal is cleaved when the protein is translocated to the apoplastic space (Wirth et al., 2004). The inclusion of a His tag in the recombinant protein may prove useful in future studies that require purified Gra4 protein.

Recombinant *Agrobacterium* carrying these constructs and the respective controls (pZPVX and pZP200) were used to agroinfiltrate leaves of the *Nicotiana tabacum cv. Xanthi* transgenic line X-27-8 (Mallory et al., 2002). This line expresses high levels of the *Tobacco etch virus* (TEV) P1/HC-Pro sequence. *Agrobacterium* mediated transient expression declines sharply as a result of post-transcriptional gene silencing (PTGS). Certain plant viruses encode silencing suppressors that may inhibit PTGS and are utilized to extend and enhance *Agrobacterium*-mediated transient expression (Voinnet et al., 2003). In previous works, the TEV P1/HC-Pro protein has been shown to suppress transgene-induced post-transcriptional and virus-induced gene silencing (Mallory et al., 2002; Brigneti et al., 1998). However, we cannot rule out the possibility that lines including wild type *N. tabacum cv. Xhanti* may also express the recombinant Gra4 after agroinfiltration.

His-Gra4$_{163-345}$ was detected in the soluble fraction of the total leaf extract (including proteins present in the apoplastic space) agroinfiltrated with both pZPVXHis-Gra4$_{163-345}$ and pAp-His-Gra4$_{163-345}$ vectors (Fig. 2A and B). Only one reactive band of ~25 kDa was detected in the pAp-His-Gra4$_{163-345}$-infiltred leaf extracts; whereas in the pZPVXHis-Gra4$_{163-345}$-infiltrated leaf extracts (cytosolic expression system) additional reactive bands were also observed. Using RT-PCR, His-Gra4$_{163-345}$ expression could be only detected in pZPVXHis-Gra4$_{163-345}$ and pAp-His-Gra4$_{163-345}$-infiltrated leaves (Fig. 2C). RT-PCR of *actin* mRNA was used as a control for total mRNA integrity (Fig. 2C). The apoplastic washing fluids (AWF) were extracted from pAp-His-Gra4$_{163-345}$-infiltrated leaves in order to assess whether the recombinant Gra4 had been efficiently targeted to the apoplasmic space. Fig. 2B shows that His-Gra4$_{163-345}$ was also detected in this fraction, while His-Gra4$_{163-345}$ was not detected in AWF from pZPVXHis-Gra4$_{163-345}$-infiltrated leaves (Fig. 2A).

Given that anti-rGra4 polyclonal serum cross react with other leaf-proteins, His-Gra4$_{163-345}$ accumulation in tobacco leaves was estimated by Western blot analysis (Fig. 2A and B). In leaves infiltrated with pZPVXHis-Gra4$_{163-345}$, His-Gra4$_{163-345}$ (~25 kDa) yields were in the order of ~0.01% of total soluble proteins (Fig. 2A), whereas in leaves infiltrated with pAp-His-Gra4$_{163-345}$, His-Gra4$_{163-345}$ accumulation yield were roughly 0.1% of AWF (Fig. 2B). Recombinant proteins usually accumulate in plants at cytosolic levels below 0.1% of the total soluble protein (Di Fiore et al., 2002). Our estimation indicates that recombinant His-Gra4$_{163-345}$ expression with pZPVXHis-Gra4$_{163-345}$ is low. This could be due to the choice of the amplicon used and/or the cytosolic expression. However, other proteins that were expressed with the pZPVX amplicon presented higher rates of expression of the recombinant proteins (Clemente et al., 2005; Zelada et al., 2006). In contrast, recombinant protein yields obtained by pAp-His-Gra4$_{163-345}$ resulted similar to those previously described by Wirth et al. (2004), who used the same apoplastic signal. It has been reported that higher accumulation could be obtained by diversion to the secretory pathway (Di Fiore et al., 2002; Fischer et al., 2004). This appears to be the case for His-Gra4$_{163-345}$, in which targeting to the extracellular space produced His-Gra4$_{163-345}$ nearly an order of magnitude higher expression than cytosolic expression. In a preliminary experiment, His-Gra4$_{163-345}$ was purified from tobacco leaf extracts expressing the recombinant antigen; however, purification was poor and His-Gra$_{163-345}$ could only be visualized by Western blot as a faint band (data not shown). Other protocols available in the literature could be assayed to improve the His-Gra4$_{163-345}$ purification.

To confirm that transiently expressed His-Gra4$_{163-345}$ is recognized by seropositive sera, recombinant protein was tested by Western blot assays using 6 samples from recently *Toxoplasma*-

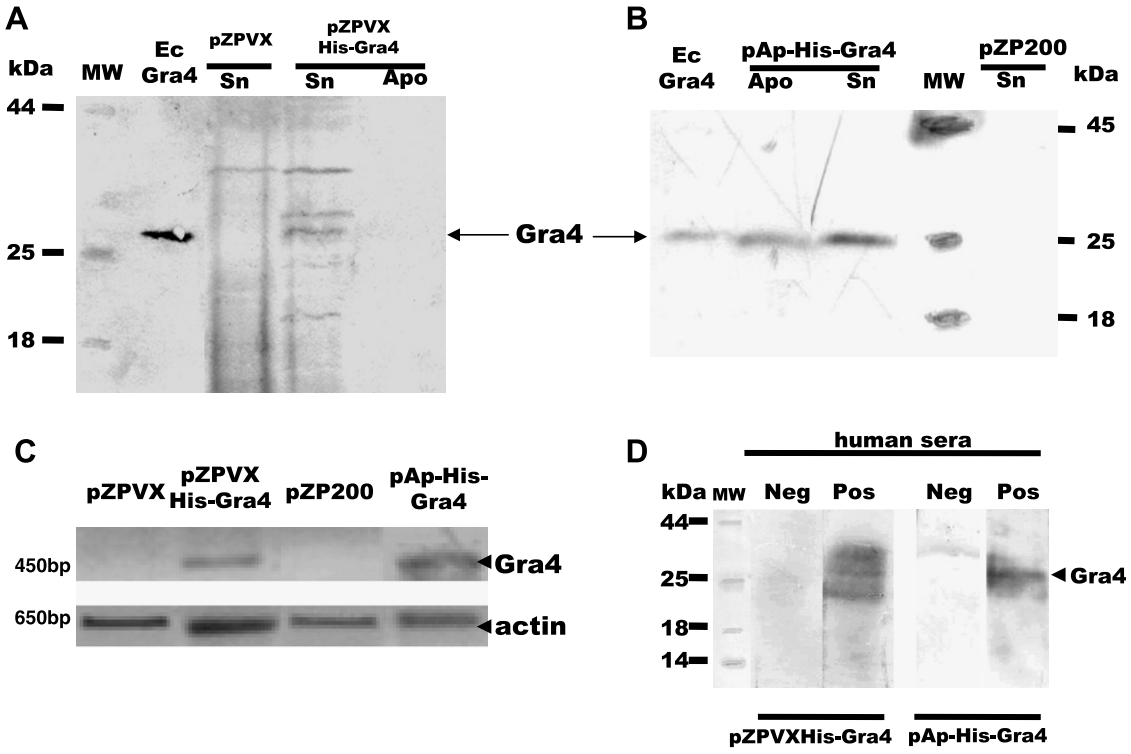

Fig. 2. Transient expression of His-Gra4$_{163\text{-}345}$ in tobacco leaves. (A) and (B): Western blot analysis. (A) Samples containing 90 μg of total soluble protein from pZPVXHis-Gra4$_{163\text{-}345}$- and pZPVX-agroinfiltrated leaf extracts (Sn) and 15 μg of total protein from AWF (Apo) from pZPVXHis-Gra4$_{163\text{-}345}$. (B) Samples containing 15 μg of total protein from AWF (Apo) and 90 μg of total soluble protein from pAp-His-Gra4$_{163\text{-}345}$-agroinfiltrated leaf extract (Sn). Ninety microgram of total soluble protein from leaves infiltrated with pZP200-recombinant *Agrobacterium* (pZP200). (A) and (B): Twenty nanogram of *E. coli*-expressed Gra4 (EcGra4$_{163\text{-}345}$) expressed and purified as described (Martin et al., 2004) were included. Accumulation in tobacco leaves was estimated by Western blot analysis using Gel-Pro analyzer program to build a quantitation calibration curve with amount standards of EcGra4$_{163\text{-}345}$ (data not shown). (C) RT-PCR of mRNA extracted from pZPVX- and pZPVXHis-Gra4$_{163\text{-}345}$-, pZP200- and pAp-His-Gra4$_{163\text{-}345}$-agroinfiltrated leaves. The RNA integrity was confirmed by amplification of *actin* mRNA. (D) Immunoblot profiles carried out with a human seropositive (Pos) and seronegative (Neg) serum samples. Fifthteen microgram of pAp-His-Gra4$_{163\text{-}345}$-agroinfiltrated leaf AWF were included in each lane. Positions of molecular weight markers are indicated in kDa. Gra4: band corresponding to His-Gra4$_{163\text{-}345}$. In all cases, data show representative results of three independent experiments.

infected individuals and 5 seronegative samples from control individuals (Nigro et al., 2003). The 6 seropositive serum samples recognized down to 1 μg of EcGra4 (Nigro et al., 2003) by Western blot analysis and one of them detected as little as 100 ng (data not shown). Owing to the low amounts of recombinant Gra4$_{163\text{-}345}$ present in transiently infiltrated leaves, this serum sample [serum 155, Hospital Alemán, Argentina] (Nigro et al., 2003) was used only in the Western blot assays. His-Gra4$_{163\text{-}345}$ expressed in both systems could be detected with this serum in agroinfiltrated leaf extracts (Fig. 2D). The antigenic reactivity of His-Gra4$_{163\text{-}345}$ was not unexpected since this sequence contains a linear epitope that is highly antigenic (Mevelec et al., 1998). In contrast, none of the seronegative samples reacted with the recombinant protein and none of the serum samples reacted with pZP200 infiltrated leaf-extracts (Fig. 2D and data not shown).

Here we showed that a truncated version of *Toxoplasma* Gra4 could be efficiently expressed in tobacco leaves using both a PVX-based amplicon and an apoplast-targeting system, both based on a transient expression method. Transient expression has several advantages over production of transgenic plants, particularly in terms of timesaving and higher expression levels (Hood et al., 2002; Twyman et al., 2003; Huang and Mason, 2004). Several transient expression techniques are available for rapid evaluation of genetic constructs and product quality (Fischer et al., 1999). In addition, one of these techniques, agroinfiltration of tobacco leaves, can be used to produce large amounts of recombinant proteins (Kapila et al., 1997; Vaquero et al., 2002). Transient expression is also well suited to analyze the feasibility of expressing an antigen of interest with different strategies before making a decision on the optimal system to generate the definitive transgenic plant.

In our case, the targeting to the secretory pathway seems a promising approach for increasing the production of His-Gra4$_{163\text{-}345}$. Like other recombinant proteins of economic importance, such as antibodies (Ma and Hein, 1995; Woodard et al., 2003), Gra4 was successfully accumulated into the apoplastic space. Extracellular targeting allows recovery of recombinant products from apoplastic washing fluids (in the case of leaf infiltration systems) or from the nutrient medium (in the case of hydroponically cultivated transgenic plants), thus simplifying the extraction of fractions enriched in the recombinant protein.

Expression of parasite antigens in plants has been poorly explored. In fact, only a few examples have been reported so far (Turpen et al., 1995; Ghosh et al., 2002; Clemente et al., 2005; Wang et al., 2008). As the expression of parasite proteins in transgenic plants is difficult and time consuming, methods should be optimized as regards to expression systems and vectors prior to up scaling to transgenic plants. In addition, the protein produced by transient expression could be used for additional experimental purposes. Plant expression of His-Gra4$_{163\text{-}345}$ is highly relevant for toxoplasmosis control. Both this antigen as well as SAG1 (Clemente et al., 2005), are good candidates for the development of a multiantigenic vaccine against *Toxoplasma*. With regard to this, their joint expression in plants may provide an excellent opportunity to explore the application of an oral vaccine against Toxoplasmosis based on edible plant tissues.

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