Experimental Parasitology 120 (2008) 118–122

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

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

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ARTICLE INFO

Article history:
Received 3 April 2008
Received in revised form 9 May 2008
Accepted 3 June 2008
Available online 10 June 2008

Index Descriptors and Abbreviations:
Vacuum infiltration
Plant transient expression
PVX amplicon
Secretory pathway
Toxoplasma gondii
Gra4

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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0014-4894/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2008.06.002

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.

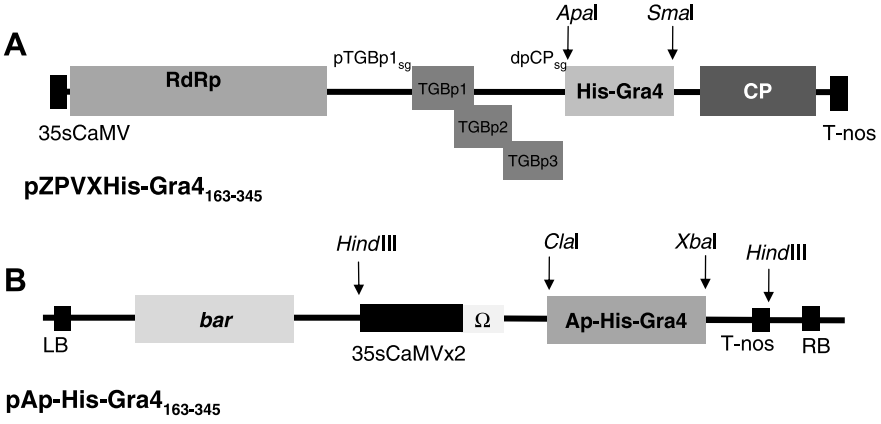

Fig. 1. Schematic representation of constructs used in the *Agrobacterium*-mediated transient expression systems. (A) pZPVXHis-$Gra4_{163\text{-}345}$. RdRp: RNA-dependent RNA-polymerase; TGBp1, TGBp2, TGBp3: triple gene block protein 1, protein 2 and protein 3, respectively; CP: coat protein gene; psgp1: 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.