

A chloroplast-derived *Toxoplasma gondii* GRA4 antigen used as an oral vaccine protects against toxoplasmosis in mice

María del L. Yácono^{1,†}, Inmaculada Farran^{2,†}, Melina L. Becher¹, Valeria Sander¹, Vanesa R. Sánchez³, Valentina Martín³, Jon Veramendi² and Marina Clemente^{1,*}

¹Laboratorio de Biotecnología Vegetal, IIB-INTECH, CONICET-UNSAM, Chascomús, Argentina

²Instituto de Agrobiotecnología, Universidad Pública de Navarra-CSIC-Gobierno de Navarra, Pamplona, Spain

³Centro de Estudios de Salud y Medio Ambiente (CESyMA), Escuela de Ciencia y Tecnología, UNSAM, San Martín, Argentina

Received 25 April 2012;

revised 16 August 2012;

accepted 25 August 2012.

*Correspondence (Tel +54 02241 430323; email mclemente@intech.gov.ar)

[†]These two authors contributed equally to the work.

Summary

The parasitic protozoan *Toxoplasma gondii*, the causal agent of toxoplasmosis, can infect most mammals and birds. In human medicine, *T. gondii* can cause complications in pregnant women and immunodeficient individuals, while in veterinary medicine, *T. gondii* infection has economic importance due to abortion and neonatal loss in livestock. Thus, the development of an effective anti-*Toxoplasma* vaccine would be of great value. In this study, we analysed the expression of *T. gondii* GRA4 antigen by chloroplast transformation (chlGRA4) in tobacco plants and evaluated the humoral and cellular responses and the grade of protection after oral administration of chlGRA4 in a murine model. The Western blot analysis revealed a specific 34-kDa band mainly present in the insoluble fractions. The chlGRA4 accumulation levels were approximately 6 µg/g of fresh weight (equivalent to 0.2% of total protein). Oral immunization with chlGRA4 resulted in a decrease of 59% in the brain cyst load of mice compared to control mice. ChlGRA4 immunization elicited both a mucosal immune response characterized by the production of specific IgA, and IFN-γ, IL-4 and IL-10 secretion by mesenteric lymph node cells, and a systemic response in terms of GRA4-specific serum antibodies and secretion of IFN-γ, IL-4 and IL-10 by splenocytes. Our results indicate that oral administration of chlGRA4 promotes the elicitation of both mucosal and systemic balanced Th1/Th2 responses that control *Toxoplasma* infection, reducing parasite loads.

Keywords: *Toxoplasma gondii*, GRA4, chloroplast transformation, plant vaccine, oral immunization, humoral and cellular response.

Introduction

The parasitic protozoan *Toxoplasma gondii*, the causal agent of toxoplasmosis, can infect most mammals and birds (Tenter *et al.*, 2000). *Toxoplasma gondii* is recognized as a category B priority pathogen by the National Institute of Health, Bethesda, USA. In humans, *T. gondii* infection is widespread, and for most people, the parasite causes an asymptomatic infection. However, *T. gondii* can cause complications in pregnant women and immunodeficient individuals (Mitchell *et al.*, 1990; Luft and Remington, 1992; Rorman *et al.*, 2006). Also, recent studies have shown that chronic *T. gondii* infections can induce neuropsychiatric disorders such as schizophrenia (Flegr, 2007; Pedersen *et al.*, 2011). Transmission to humans is either through consumption of food contaminated with tissue cysts and meat products from infected animals or by ingestion of oocysts released in the faeces of infected cats (Hill and Dubey, 2002). In veterinary medicine, *T. gondii* infection has economic importance due to abortion and neonatal loss in livestock (Dubey, 2004, 2009). Indeed, millions of productive lambs are lost through this infectious disease (Dubey, 2009). On the other hand, treatment of toxoplasmosis is difficult due to the toxic effects of available drugs, and re-infection also occurs rapidly. Thus, the development of either an effective vaccine or new anti-*Toxoplasma* drugs would be of great value to human and veterinary medicine (Henriquez *et al.*, 2010).

In recent years, significant advances have been made in identifying possible new vaccines that can induce a protective immune response against this parasite. For example, excretory/secretory antigens of *T. gondii* have been shown to play an important role in stimulating immune responses to *T. gondii* (Cesbron-Delauw, 1994). The main components of the excretory/secretory fraction are the dense granule (GRA) proteins (Cesbron-Delauw, 1994). In particular, GRA4 appears as an interesting component for the development of an anti-*Toxoplasma* multiantigenic vaccine, which could be based either on DNA or on recombinant proteins (Martin *et al.*, 2004; Mevelec *et al.*, 2005; Zhang *et al.*, 2007; Sánchez *et al.*, 2011). Its sequence contains a linear epitope that is highly antigenic (Mevelec *et al.*, 1998). Indeed, GRA4 is an effective promoter of the expression of IgA and IgG antibodies (Mevelec *et al.*, 1998; Nigro *et al.*, 2003; Altcheh *et al.*, 2006). Also, immunization with DNA or recombinant GRA4 is able to induce a cellular response with accumulation of IFN-γ (Desolme *et al.*, 2000; Mevelec *et al.*, 2005; Sánchez *et al.*, 2011). On the other hand, oral immunization of C57BL/6 (H-2d) mice with truncated soluble forms of GRA4 in association with cholera toxin induces a significant Th2-like mucosal response and partial resistance to oral infection with *T. gondii* (Mevelec *et al.*, 1998). These results indicate that GRA4 is a potential candidate for oral vaccination against *T. gondii*, able to induce both mucosal and systemic immune responses.

The production of plant-derived vaccines has been widely assayed in the last 20 years. Several antigens from human and animal pathogens have been correctly expressed and shown to produce a specific humoral response, and in some cases, a protective response against infection in murine models (Kong *et al.*, 2001; Clemente *et al.*, 2005; Gómez *et al.*, 2008; Santi *et al.*, 2008; Kostrzak *et al.*, 2009; Zhang *et al.*, 2009; Lagüa Becher *et al.*, 2010; Gonzalez-Rabade *et al.*, 2011). One of the main advantages of plants is that their tissues provide a natural environment for antigen encapsulation, which protects the antigen from degradation (Limaye *et al.*, 2006; Hayden *et al.*, 2012). In this way, when the plant tissue is digested, a sufficient quantity of antigen can be captured from the mucous membranes and stimulate an immune response (Berinstein *et al.*, 2005; Kapusta *et al.*, 2010). For this reason, plants are an ideal vehicle for oral vaccine administration.

Previously, a His-tagged truncated version of the *T. gondii* GRA4 antigen (residues 163–345), transiently expressed in tobacco leaves using vacuum agroinfiltration, has been found to be able to react with seropositive human sera (Ferraro *et al.*, 2008). Compared to nuclear transformation, chloroplast transformation offers many advantages, including high levels of transgene expression likely due to a high copy number, absence of epigenetic effects, transgene containment via maternal inheritance and multigene expression in a single transformation event (Lössl and Waheed, 2011). In addition, tobacco chloroplast-derived vaccines are able to induce neutralizing antibodies against human or animal infections (Molina *et al.*, 2005; Fernández-San Millán *et al.*, 2008). More importantly, proteins expressed in plastids have been found to be stable in the gut and efficiently transported to the circulatory system (Limaye *et al.*, 2006). For these reasons, the main goal of this study was to explore the expression of *T. gondii* GRA4 antigen by chloroplast transformation in tobacco plants, and to determine the ability of this antigen to induce protection in a *T. gondii* murine infection model when administered orally without any adjuvant.

Results

Integration of the *GRA4* sequence into the tobacco plastid genome

The *GRA4* sequence (corresponding to amino acids 183–345; Mevelec *et al.*, 1998) was expressed from the plastid constitutive promoter and 5'UTR of the *psbA* gene and translationally fused to the 3'-end of the first 45 nucleotides of the *GFP* sequence, which has been shown to be a desirable downstream box (DB) to improve protein accumulation in tobacco chloroplasts (Farran *et al.*, 2010). The chimeric gene was cloned into the chloroplast transformation vector pAF-TDHIS for insertion between the *trnI* and *trnA* genes in the tobacco plastome (Figure 1a). This vector contains a chimeric *aadA* gene as the selectable marker, which provides spectinomycin resistance for selecting stable transformants. Additionally, the pAF-TDHIS vector allows the translational fusion of the transgene to the 42-amino acid tetramerization domain (TD) from the human transcription factor p53 (Ortigosa *et al.*, 2010) and introduces a C-terminal 7xHis tag.

The pAF-TDHIS-*GRA4* construct was introduced into tobacco (cv. Petite Havana) plastids by biolistic transformation (Daniell, 1997). The stable integration of the foreign gene into the chloroplast genome and the homoplasmy of the transformed plants were confirmed by Southern blot analysis. After two rounds of selection, total leaf DNA from the regenerated plants

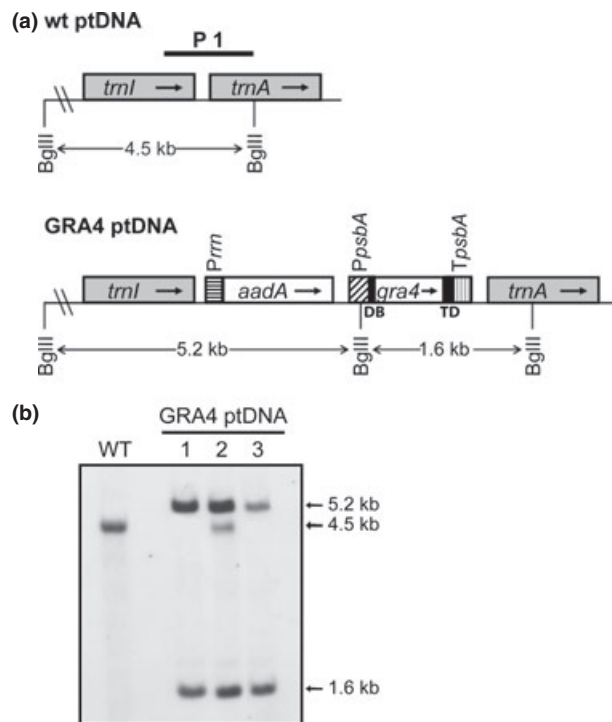


Figure 1 Integration of chimeric *GRA4* gene into the plastid genome and homoplasmy verification. (a) Map of the wild-type and *GRA4*-transformed plastid genomes. The transgenes are targeted to the intergenic region between *trnI* and *trnA*. The *Gra4* expression cassette includes the *psbA* promoter and 5'-untranslated region (*PpsbA*) and the *psbA* terminator (*TpsbA*) from tobacco. A downstream box (DB) with the first 15 amino acids of the soluble modified GFP protein was used. The *GRA4* sequence was translationally fused to the 42 amino acid tetramerization domain (TD) from the human transcription factor p53. The selectable marker gene *aadA* is driven by the ribosomal RNA operon promoter (*Prrn*). The expected sizes of DNA fragments in Southern blot analysis with the restriction enzyme *BglII* are indicated. The 0.8-kb fragment (P1) of the targeting region for homologous recombination was used as probe. Arrows indicate the direction of transcription. (b) Southern blot analysis of three independent transplastomic tobacco lines is shown. Total DNA was digested with *BglII* and probed with P1.

was digested with the *BglII* restriction enzyme, which cuts in two positions flanking the insertion site and in one position in the *psbA* promoter (Figure 1a). The 0.8-kb probe P1, which is homologous to the flanking regions *trnI* and *trnA* (Figure 1a), was used. Two of the three transplastomic lines analysed showed the expected hybridization pattern and revealed the absence of residual copies of the wild-type plastome (Figure 1b, lanes 1 and 3). To ultimately confirm homoplasmy, seeds from the T_0 generation were germinated *in vitro* on spectinomycin-selective medium. Lack of segregation for spectinomycin resistance in the T_1 generation demonstrated homoplasmy (data not shown). Line 1 was selected for further studies.

Expression and quantification of the *GRA4*₁₈₃₋₃₄₅ protein

The recombinant protein *GRA4* in transformed chloroplasts (chl*GRA4*) was detected by Western blot with an anti-*GRA4* polyclonal antibody. Leaves from four plants were analysed (Figure 2a). Each sample showed a specific 34-kDa band (the *GRA4* antigen fused to the DB and the TD) (Figure 2a). This band

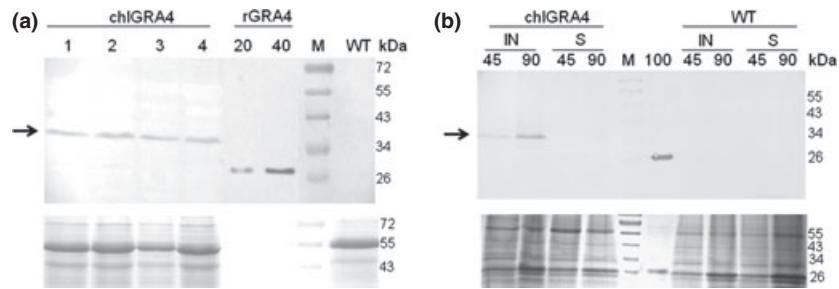


Figure 2 Chloroplast-derived GRA4 protein expression. (a) Western blot indicating the expression of chlGRA4 from four independent transplastomic plants. GRA4 polyclonal antibody specific for rGRA4 protein was used. Wild-type (WT) protein served as the control. Lanes 1, 2, 3 and 4: Protein samples from four independent transplastomic plants (transgenic plants 1, 2, 3 and 4, respectively) extracted from 100 mg of leaf fresh weight. 20 and 40 : 20 and 40 ng rGRA4, respectively. (b) Western blot analysis of chlGRA4 protein expression in soluble (S) and insoluble (IN) fractions. Proteins extracted from 100 mg of leaf fresh weight of homogenous stocks of five transplastomic (chlGRA4) plants and wild-type (WT) plants 45 and 90 days after transplanting were loaded in lanes. 100 : 100 µg of rGRA4. Coomassie blue-stained gels were used as controls of the protein loading. M: prestain molecular weight protein marker (Fermentas). The arrow indicates the chlGRA4 protein.

showed an electrophoretic mobility higher than the *E. coli*-derived and purified GRA4 (rGRA4) as a consequence of the translational fusions described above (Figure 2a). ChlGRA4 accumulation in these plants was approximately 6 µg/g of fresh weight (FW) (equivalent to 0.2% of total protein) (Figure 2a), as estimated by Western blot analysis using rGRA4 (Martin *et al.*, 2004), previously quantified. No protein was detected in samples extracted from wild-type control plants.

The effect of plant age on chlGRA4 protein expression was analysed in plants growing in the glasshouse at 45 and 90 days. Soluble and insoluble proteins were extracted from fully developed leaves, and chlGRA4 expression was analysed by Western blot (Figure 2b). The specific 34-kDa band was observed in 45 and 90-day-old plants mainly in the insoluble fraction, and no degradation products were detected (Figure 2b). The higher expression level after 90 days of culture in the glasshouse in relation to that after 45 days indicates that chlGRA4 is stable in the stroma of the chloroplast.

Leaves from chlGRA4 over-expressing tobacco plants were excised 120 days after transplanting, lyophilized, ground to powder and stored at 4 °C. ChlGRA4 accumulation in lyophilized plant leaf stocks was estimated by Western blot analysis using a dilution series (10, 20, 40, 60 and 80 ng) of rGRA4 (Martin *et al.*, 2004), previously quantified as reference (Figure S1). The amount of chlGRA4 protein accumulated in the chloroplast was estimated to be approximately 12 µg/g of lyophilized weight. Lyophilized material was chosen for oral administration to mice.

Immunogenicity of chloroplast-derived GRA4

Considering that *T. gondii* infects the host through the gut mucosa, the efficacy of oral immunization with transplastomic plants expressing chlGRA4 was analysed. C57BL/6(H-2d) mice were immunized orally with leaf extracts from chlGRA4 plants (GRA4 group), leaf extracts from wild-type plants (control group) or PBS buffer (PBS group). To determine whether this vaccination strategy was able to promote protection against *T. gondii* infection, 2 weeks after the last boost, mice were challenged by intragastric gavage with a nonlethal dose of Me49 tissue cysts and individual parasitemia levels were assessed 1 month later. An important decrease in the brain cyst burden (59%) was observed as compared to the control and PBS groups (Figure 3). No differences in the parasitemia levels were observed between these two control groups (Figure 3).

Systemic immune response generated by chlGRA4

To examine whether leaf extracts from chlGRA4 plants administered orally were able to elicit a systemic immune response, blood samples were obtained 14 days after the immunization schedule was completed and the specific IgG antibodies against rGRA4 were determined by direct ELISA (Figure 4). As shown in Figure 4, GRA4-specific serum IgG antibodies were detected in all mice receiving chlGRA4 with titres of 1000 (Figure S2), while mice vaccinated with leaf extracts from wild-type plants or PBS did not display significant titres. To investigate the dominant Th pattern stimulated following immunization, GRA4-specific IgG isotypes were determined by direct ELISA. Significant levels of IgG2b and IgG1 were observed only in chlGRA4-vaccinated mice with titres of 500 for IgG2b and 250 for IgG1 (Figure 4b,

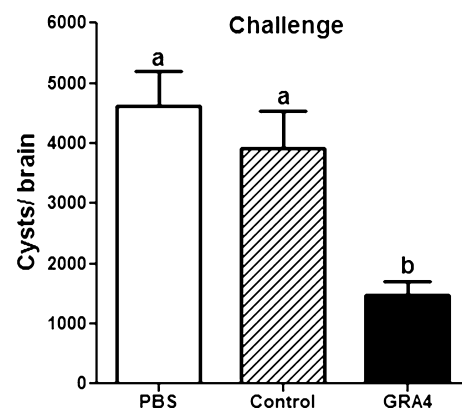


Figure 3 Protection of C57BL/6(H-2d) mice against *Toxoplasma* infection. Eight- to ten-week-old mice (eight mice per group) were immunized on days 0, 7, 14, 21 and 28 by oral administration. Two weeks after the last boost, mice were challenged by gavages with 20 cysts of the Me49 strain (LD50). Thirty days after the challenge, the number of brain cysts in mice was determined. Control: mice vaccinated with leaf extracts from wild-type plants; GRA4: mice vaccinated with leaf extracts from chlGRA4 plants; PBS: mice vaccinated with buffer PBS. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's multiple comparison test. Results are expressed as the means ± SEM and represent one of two similar experiments. Different letters indicate statistically significant differences ($P < 0.001$ GRA4 vs. control and PBS).

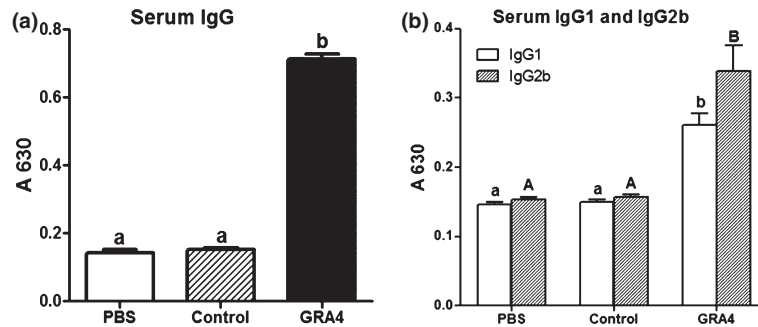


Figure 4 Determination of specific anti-*chlGRA4* humoral response in orally vaccinated C57BL/6(H-2d) mice. Two weeks after the immunization schedule was completed, blood samples (eight mice per group) were obtained and anti-*GRA4*-specific antibodies were determined by ELISA. (a) Specific total IgG and (b) IgG isotype in sera from mice vaccinated with leaf extracts from wild-type plants (control), leaf extracts from *chlGRA4* plants (GRA4) or with buffer PBS (PBS) were determined in duplicate. Mouse sera were diluted 1 : 100 for *GRA4*-specific antibodies determination (nonsaturating dilutions). Results are expressed as the mean of the OD630 ± SEM and are representative of two experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's multiple comparison test. Different letters indicate statistically significant differences ($P < 0.001$ GRA4 vs. control and PBS). Capital letters correspond to statistical analysis of IgG2b isotype. Lower case letters correspond to statistical analysis of IgG and IgG1 isotype.

Figure S2). Then, the ability of the vaccination protocol to promote antigen-specific cellular response was assessed in the spleen. The concentrations of Th1- or Th2-associated cytokines were quantified in supernatants of spleen cells restimulated *in vitro* with excretory-secretory antigens (ESA). Spleen cells of mice immunized with *chlGRA4* produced significant levels of IFN- γ , IL10 and IL-4 when compared to the control and PBS groups (Figure 5). These results show that oral immunization with leaf extracts from *chlGRA4* plants induces a mixed Th1/Th2 response at the systemic level.

Mucosal immune response generated by *chlGRA4*

To examine whether oral administration of leaf extracts from *chlGRA4* plants is able to induce antibody-specific response at mucosal levels, intestinal lavages were collected 45 days after the last immunization, and the presence of *GRA4*-specific secretory immunoglobulin A (sIgA) was evaluated by ELISA. *chlGRA4*-vaccinated mice elicited significant levels of *GRA4*-specific sIgA compared to the control and PBS groups (Figure 6). Then, the ability of this oral vaccination protocol to promote antigen-specific cellular response was assessed in gut-associated lymphoid tissue (GALT). Forty-five days after the immunization schedule was completed, we estimated the concentration of Th1- and Th2-associated cytokines by capture ELISA in supernatants of mesenteric lymph node cells stimulated *in vitro* with ESA. A significant increase in the amount of secreted IFN- γ , IL10 and IL-4 was observed in mice immunized with *chlGRA4* when compared to the control and PBS groups (Figure 7). These results show that a vaccination protocol based on oral administration of leaf extracts from *chlGRA4* plants triggers a strong mucosal response characterized by the presence of *GRA4*-specific sIgA and secretion of Th1- and Th2-associated cytokines in the GALT.

Discussion

A live *Toxoplasma* vaccine based on the attenuated S48 strain of the parasite has been registered and is currently used in veterinary medicine (Hiszczynska-Sawicka *et al.*, 2011). However, it is not suitable for humans due to the risks for breakthrough infection (Hiszczynska-Sawicka *et al.*, 2011). Therefore, new approaches to

vaccine development using subunit vaccines based on *T. gondii* antigens have been widely initiated (Jongert *et al.*, 2009).

Plant expression systems have been intensively studied to provide an attractive alternative for oral vaccine production (Paul and Ma, 2010). In the present work, the *GRA4* antigen of *T. gondii* was expressed in tobacco plants. Chloroplast transformation was carried out to improve the expression level of the heterologous protein. Previously, the yields of *GRA4* in tobacco infiltrated leaves were in the order of 0.01% of total soluble protein, which represents around 0.2 $\mu\text{g/g}$ of fresh weight (Ferraro *et al.*, 2008). Here, the *chlGRA4* expression levels in the transplastomic plants were up to 6 $\mu\text{g/g}$ of fresh weight (or 0.2% of total protein). As expected, chloroplast transformation allowed a significant 30-fold increase in *GRA4* protein accumulation in the plant. In addition, recombinant protein levels were stable over the whole life cycle of the plants. However, *chlGRA4* levels were not as high as the usual figures in plastid transformation. Given that *chlGRA4* seems to be stable in the chloroplast, low expression levels could be due to an inefficient translation initiation, despite the use of a DB that has been proven to be useful for protein accumulation (Farran *et al.*, 2010). The combination of a DB with a particular ORF may alter mRNA folding and thus affect translation mediated by the DB, suggesting that empirical optimization is required for each ORF. Gray *et al.* (2011) demonstrated the specific effects of three DBs in the expression level of beta-glucuronidase in tobacco chloroplasts.

GRA4 is a target antigen for both mucosal and systemic immune responses, and therefore, it is a valuable candidate to be used in oral vaccination strategies (Bout *et al.*, 1995; Mevelec *et al.*, 1998). To our knowledge, this is the first report that tested the immunogenicity of *GRA4* antigen in an oral vaccination approach without the use of any adjuvant. It was previously shown that oral administration of chloroplast-derived vaccine antigens produced in tobacco and lettuce conferred combined protection against malaria and cholera (Davoodi-Semiromi *et al.*, 2010).

Oral administration of adjuvant-free *chlGRA4* was enough to elicit an immune response capable of inducing partial protection against *T. gondii* infection measured as a marked reduction in brain cyst loads. Similar results were obtained with the r*GRA4* protein administered intramuscularly in combination with adju-

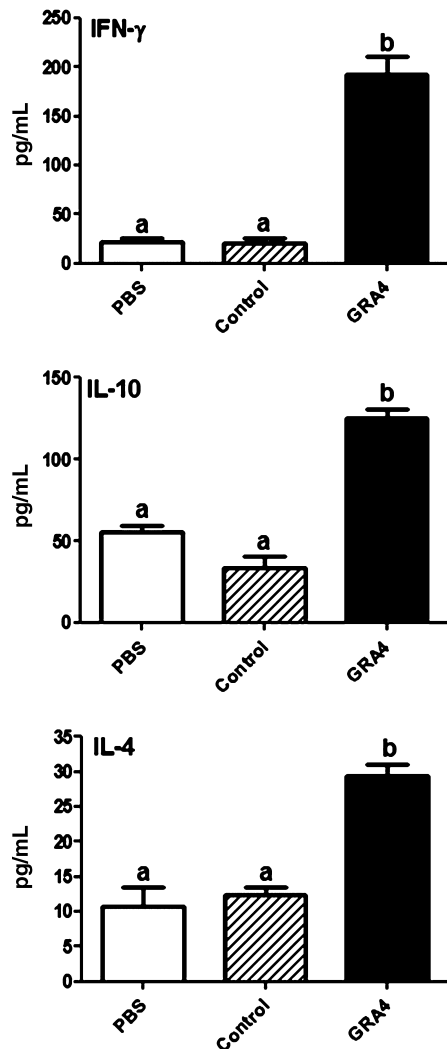


Figure 5 Cytokine productions by spleen cells in orally vaccinated C57BL/6(H-2d) mice. Forty-five days after the immunization schedule was completed, spleen and lymph node cells were isolated from mice (five mice per group) and stimulated *in vitro* with excretory-secretory antigens (ESA) (10 μ g/mL). Values for IFN- γ , IL-4 and IL-10 were measured at 72 h of culture. Results are mean cytokine concentrations in spleen cells from the five mice of each experimental group. Control: mice vaccinated with leaf extracts from wild-type plants; GRA4: mice vaccinated with leaf extracts from chlGRA4 plants; PBS: mice vaccinated with buffer PBS. Results from one of two similar experiments are shown and are expressed as the mean \pm SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's multiple comparison test. Different letters indicate statistically significant differences ($P < 0.001$ GRA4 vs. control and PBS).

vant-like alum (Martin *et al.*, 2004) or CpG (Sánchez *et al.*, 2011). On the other hand, a protocol based on priming with plant-derived *T. gondii* SAG1 protein by oral administration and intradermal (i.d.) boosting with rSAG1 expressed in bacteria (Laguía Becher *et al.*, 2010) elicited a protection (50%) similar to that obtained with chlGRA4 (59%). Therefore, the inclusion of exogenous adjuvants or a rGRA4 i.d. boost or GRA4-SAG1 mixed plant materials could be implemented to achieve a higher level of protection against *Toxoplasma* infection.

Our results also showed that the partial protection against *T. gondii* infection was a consequence of the development of

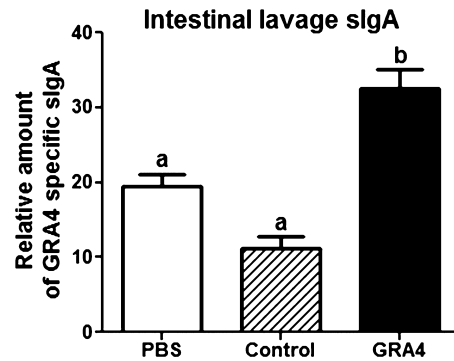


Figure 6 Determination of specific anti-chlGRA4 sIgA in intestinal lavages of orally vaccinated mice. Forty-five days after the immunization schedule was completed, intestinal lavage samples (five mice per group) were obtained and antibody concentration was determined by ELISA. Results are expressed as the percentage of GRA4-specific IgA with respect to the total IgA present in the sample \pm SEM and are representative of two experiments. The total mean IgA values \pm SEM for the different groups were as follows: GRA4, 32.58 \pm 2.52 ng/mL; control group, 11.22 \pm 1.60 ng/mL; and PBS group, 19.54 \pm 1.58 ng/mL. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's multiple comparison test. Different letters indicate statistically significant differences ($P < 0.001$ GRA4 vs. control; $P < 0.01$ GRA4 vs. PBS).

both an effective mucosal and systemic immune response. Specific mucosal sIgA and serum IgG were produced in mice immunized with chlGRA4. The high levels of serum IgG would be indicating a mucosal origin of these antibodies, which was confirmed by an effective activation of mucosal sIgA. Particularly, sIgA provide immune protection against *Toxoplasma* in mucosal tissues. However, mucosal defences can also be mediated by cytokines (Dimier-Poisson *et al.*, 2003). Given that the initial activation of T cells probably occurs in Peyer's patches and mesenteric lymph nodes, we investigated the induction of Th1- and Th2 cytokines in the local lymph nodes. The Th1/Th2 cytokine levels in chlGRA4-vaccinated mice were higher than in the other two control groups. These results suggest that the antigen expressed in the chloroplasts would have been recognized by the antigen-presenting cells and presented in the gut-associated lymphoid tissues, resulting in the activation of T helper cells.

chlGRA4 vaccination showed to elicit a mixed IgG2b/IgG1 systemic response with predominance of IgG2b. However, protection against *T. gondii* infection is mainly attributed to a cell-mediated systemic immunity. IFN- γ has been shown to be a major mediator of resistance against toxoplasmosis (Suzuki *et al.*, 1988; Gazzinelli *et al.*, 1991). Nevertheless, the absence of some type 2 cytokines such as IL-4 or IL-10 can result in an increase in the immunopathology or mortality in mice (Gazzinelli *et al.*, 1996; Henriquez *et al.*, 2010). In this context, restimulation of spleen cells from the chlGRA4-vaccinated group led to the production of IFN- γ , IL-4 and IL-10. The higher levels of production of IFN- γ compared with those of IL-4 and the higher levels of serum IgG2b over IgG1 indicate that a Th1-mediated predominant T-cell response was generated. The presence of large amounts of IL-10 in restimulated splenocytes from chlGRA4-immunized mice suggests that this vaccination protocol also elicits a feedback modulation of this predominant Th1 response. Finally, oral chlGRA4 vaccination also induced the production of IL-4, a Th2 cytokine, suggesting a B-cell contribution to the protection obtained after *T. gondii* infection in vaccinated mice.

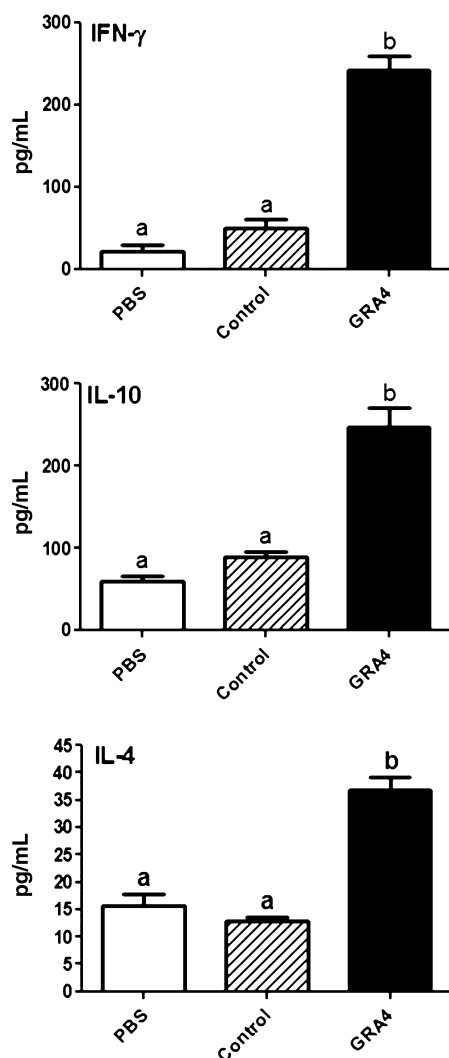


Figure 7 Cytokine productions by lymph node cells in orally vaccinated C57BL/6(H-2d) mice. Forty-five days after the immunization schedule was completed, spleen and lymph node cells were isolated and stimulated *in vitro* with excretory-secretory antigens (ESA) (10 μ g/mL). Values for IFN- γ , IL-4 and IL-10 were measured at 72 h of culture. Results are mean cytokine concentrations in lymph node cells from five mice per experimental group. Control: mice vaccinated with leaf extracts from wild-type plants; GRA4: mice vaccinated with leaf extracts from chlGRA4 plants; PBS: mice vaccinated with buffer PBS. Results from one of two similar experiments are shown and are expressed as the mean \pm SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) using the Bonferroni's multiple comparison test. Different letters indicate statistically significant differences ($P < 0.001$ GRA4 vs. control and PBS).

These results are supported with the T and B epitopes mapped in the GRA4 sequence (Mevelec *et al.*, 1998). However, additional adjuvants present in the plant material could be contributing with the modulation of the immune response observed in mice vaccinated with chlGRA4 (Gonzalez-Rabade *et al.*, 2011; Corigliano *et al.*, 2011).

In conclusion, our findings suggest that chloroplast-derived GRA4 is able to induce a protective immune response against *Toxoplasma* infection in mice that correlates with a mucosal and systemic balanced Th1/Th2 response. This indicates that chlGRA4 displays a good potential for toxoplasmosis control by oral

vaccination. Moreover, plant expression of GRA4 as well as SAG1 (Clemente *et al.*, 2005; Laguía Becher *et al.*, 2010) provides an excellent possibility for the development of a multicomponent vaccine against *Toxoplasma*.

Experimental procedures

Genetic engineering of the chloroplast expression vectors

The GRA4 sequence was amplified by PCR from the pQ-GRA4 vector (Martin *et al.*, 2004) using the primers 5'-cccgggCTGCAGTTGAGACAACAGTC-3' (upstream) and 5'-ctcgagCTCTTTGCGCATTCTTCC-3' (downstream), which introduces the *Sma*I and *Xho*I sites, respectively. The *Sma*I-*Xho*I GRA4 fragment was cloned into the pSK-GFP:EDA plasmid (Farran *et al.*, 2010), which allows the translational fusion of GRA4 to the first 15 amino acids of the soluble modified GFP protein (GenBank accession no. U_70495). The resulting pSK-GFP:GRA4 vector was digested by *Sal*I and *Xho*I, and the generated fragment, which also contains the promoter and 5'UTR of the tobacco *psbA* gene, was introduced into the tobacco chloroplast transformation vector pAF-TDHIS (Ortigosa *et al.*, 2010) previously digested by *Sal*I, to produce pAF-GRA4.

Bombardment and regeneration of chloroplast transgenic plants

Gold microprojectiles coated with pAF-GRA4 plasmid DNA were bombarded into *in vitro* grown tobacco (*Nicotiana tabacum* var. Petite Havana) leaves using the PDS1000/He (Bio-Rad, Hercules, California) biolistic device, as previously described (Daniell, 1997). After bombardment, leaves were incubated in the dark for 2 days at 28 °C. Leaves were then cut into small pieces (approximately 5 \times 5 mm) and placed adaxial side up on regeneration medium (RMOP) in Magenta vessels (Sigma, St. Louis, MO) containing 500 mg/L spectinomycin dihydrochloride as the selecting agent. The regenerated spectinomycin-resistant shoots were subjected to a second round of selection under the same conditions. These regenerated plants were then transplanted and grown in a greenhouse for homoplasmy confirmation and seed production.

Southern blot analysis

Total plant DNA (10 μ g) was digested with *Bgl*II, separated on a 0.8% (w/v) agarose gel and transferred to a nylon membrane. The digestion by *Bgl*II and *Bam*HI of the chloroplast vector generated a 0.8-kb probe (P1) homologous to the flanking sequences (Figure 1a). Probe labelling and hybridization were performed using the chemiluminescent AlkPhos direct labelling and detection system (GE Healthcare, Buckinghamshire, UK). After homoplasmy confirmation by Southern analysis, seeds were harvested and germinated *in vitro* on spectinomycin-selective medium. The T1 seedlings were isolated and cultured for 4 weeks in MS medium (Sigma). Finally, plants were transferred to pots and grown in a greenhouse for further analysis.

Protein extraction and Western blot analysis

Leaf samples (100 mg) were ground in liquid nitrogen, homogenized in Laemmli buffer 2 \times (Laemmli, 1970) and the extract heated at 100 °C for 5 min. To separate soluble and insoluble protein fractions, the pulverized samples (100 mg) were homogenized in a soluble protein extraction buffer (20 mM NaPO₄, 250 mM NaCl, 0.1% Triton X-100, Fernández-San Millán *et al.*,

2007). After centrifugation at 10 000 **g**, the soluble fraction (supernatant) and insoluble fraction (pellet) were homogenized in Laemmli buffer 2×. Proteins were separated by SDS-PAGE (15% gel) and transferred onto polyvinylidene fluoride membranes (PVDF; GE Healthcare) using an Electro-transfer unit (Bio-Rad). The membranes were sequentially incubated with rabbit polyclonal anti-GRA4 antibody (1 : 500) and alkaline phosphatase conjugated goat anti-rabbit IgG (1 : 5000; Sigma). After washing, the reaction was developed by the addition of nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) substrate (Promega, Fitchburg, Wisconsin). Prestained protein (Fermentas, Burlington, ON, Canada) was included in Western blots as molecular weight markers. To estimate the GRA4 accumulation in tobacco leaves, *E. coli*-derived and purified GRA4 (rGRA4) of known concentration was also separated by SDS-PAGE (15% gel), detected by Western blot and used as a standard to build a calibration curve. Amounts of GRA4 in leaf extracts were determined by comparison with the rGRA4 calibration curve using the 'Gel-Pro analyzer' software (Media Cybernetics, Rockville, MD).

Animals

Female C57BL/6(H-2d) mice were bred and housed at the animal facilities of the Biotechnology Research Institute (IIB), National University of General San Martín (UNSAM), Buenos Aires, Argentina, and used at 8–10 weeks of age. All procedures requiring animals were performed in agreement with institutional guidelines and approved by the Independent Ethics Committee for the Care and Use of Experimental Animals of the National University of General San Martín (C.I.C.U.A.E., IIB-UNSAM, 09/2011).

Oral immunization of mice

In all cases, mice were deprived of food and water for 2 h prior to each immunization and were gavaged with 83 mg of lyophilized powder resuspended in a final volume of 200 µL of PBS 1× buffer. Mice from GRA4 group were orally immunized with five doses of transplastomic leaf extracts homogenized in PBS 1× buffer, without enrichment or purification, containing the equivalent of approximately 1 µg of chlGRA4 at 1-week intervals. The control group received five doses of wild-type leaf extracts homogenized in PBS 1× buffer, while the PBS group was immunized with five doses of PBS 1× buffer. Eight and five mice per experimental group were used in the challenge infection experiments and cytokine analysis, respectively.

Measurement of antibody responses

Two weeks after the last immunization, blood was collected from the tail vein and sera were stored at –20 °C until analysed for the presence of specific antibodies. Pre-immune serum samples were used as negative controls.

Antigen-specific antibodies were analysed by enzyme-linked immunosorbent assay (ELISA) as previously described (Clemente *et al.*, 2005; Laguía Becher *et al.*, 2010). Briefly, each well of 96-well microtitre plates (Immuno Plate Maxisorp; Nunc, Rochester, NY) was coated overnight at 4 °C with 5 µg/mL of rGRA4 diluted in 0.05 M carbonate buffer (pH 9.6). Rat anti-mouse immunoglobulin G (IgG)–horseradish peroxidase conjugate (1 : 5000) was used as a secondary antibody (Sigma), and rat anti-mouse IgG1- or IgG2a-horseradish peroxidase conjugates (1 : 3000) (Pharmingen) were used for isotype analysis. Immune complexes were revealed with trimethylbenzidine substrate (TMB, One-Step;

Invitrogen, Carlsbad, CA), and optical density was read at 630 nm with an automatic ELISA reader (Dynatech MR4000). Sera were used at 1 : 50–1 : 8000 dilutions. To attain a comparative analysis of IgG profiles, serum samples were used at a 1 : 100 dilution. The negative control serum samples included in the assay displayed values lower than the cut-off.

On the other hand, 45 days after the last immunization, five mice were sacrificed and intestinal lavages were obtained by flushing the organs with several aliquots of PBS supplemented with 50 mM EDTA, 5% fetal bovine serum (FBS, GIBCO, Carlsbad, CA) and 10 mM PMSF with a final volume of 1 mL. Lavages were then centrifuged to remove debris (10 min at 3000 **g**), and supernatant fluids were stored at –80 °C. The amount of total and GRA4-specific IgA present in intestinal lavages was determined by ELISA as previously described by Rharbaoui *et al.* (2002). To establish the IgA standard curve, plates coated with goat anti-mouse IgA (Pharmingen) as capture antibody were further incubated with serial dilutions of purified mouse IgA (Pharmingen). As secondary antibody, biotinylated goat anti-mouse IgA (Pharmingen) was used and plates were developed with trimethylbenzidine substrate (TMB, One-Step; Invitrogen) and optical density was read at 630 nm with an automatic ELISA reader (Dynatech MR4000). GRA4-specific IgA was analysed by a direct ELISA with GRA4 as the coating antigen, as described above, and specific amounts were calculated using the IgA standard curve described before. To compensate for variations in the efficiency of recovery of secretory antibodies between animals, the results were normalized and expressed as percentage of GRA4-specific IgA with respect to the total amount of IgA present in the sample.

Cytokine analysis

Spleens and mesenteric lymph nodes were aseptically removed from five mice per group 45 days after the last immunization.

Single-cell preparations were obtained by crushing spleens through stainless steel meshes followed by suspension in erythrocyte lyses buffer (0.15 M NH₄Cl, 1.0 M KHCO₃, 0.1 mM EDTA, pH 7.2). The viability of the cells used in the experiments was always higher than 80%, as measured by trypan blue exclusion (Sigma). The cells were then suspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (GIBCO), 2β-mercaptoethanol (final concentration, 5 × 10^{–5} M), penicillin (100 U/mL) and streptomycin (100 µg/mL). Supernatants from cultured splenocytes and lymph cells (10 × 10⁶/mL) were collected after 72 h of stimulation with excretory-secretory antigens (ESA) of *T. gondii* (10 µg/mL) and stored at –70 °C. Cytokine production was measured in supernatants at 72 h (IFN-γ, IL-4 and IL-10) by capture ELISA commercial kits (Pharmingen; BD Biosciences, Sandiego, CA). The sensitivity limits for the assays were 20 pg/mL for IFN-γ, 50 pg/mL for IL-10 and 10 pg/mL for IL-4. At least two independent ELISAs were performed for each sample.

Challenge infection

Mice (eight per experimental group) were orally infected with 20 Me49 tissue cysts (sublethal dose) 2 weeks after the last immunization. Mice were observed daily for mortality. One month after the challenge, mice were sacrificed and their brains removed. Each brain was homogenized in 2 mL of PBS with a Dounce tissue grinder. The mean number of cysts per brain was determined by observation under an optical microscope, by counting four samples of 20 µL aliquots of each homogenized brain.

Statistical analysis

Statistical analysis was carried out with the Prism 5.0 Software (GraphPad, San Diego, CA) using one-way analysis of variance (ANOVA). A Bonferroni's test was carried out to compare means. Values of $P < 0.05$ were considered significant.

Acknowledgements

This work was supported by PIP 168/2010 of the National Research Council (CONICET, Argentina), PICT 691/2007 of the National Agency for Promotion of Science and Technology (ANPCyT, Argentina) and by Grant BIO2005-00155 (Ministerio de Educación y Ciencia, España).

References

- Altcheh, J., Diaz, N.S., Pepe, C.M., Martin, V., Nigro, M., Freilij, H. and Angel, S.O. (2006) Kinetic analysis of the humoral immune response against 3 *Toxoplasma gondii*-recombinant proteins in infants with suspected congenital toxoplasmosis. *Diagn. Microbiol. Infect. Dis.* **56**, 161–165.
- Berinstein, A., Vazquez-Rovere, C., Asurmendi, S., Gómez, E., Zanetti, F., Zabal, O., Tozzini, A., Conte Grand, D., Taboga, O., Calamante, G., Barrios, H., Hopp, E. and Carrillo, E. (2005) Mucosal and systemic immunization elicited by Newcastle disease virus (NDV) transgenic plants as antigens. *Vaccine*, **23**, 5583–5589.
- Bout, D., Buzoni-Gatel, D., Chardès, T., Debard, N. and Mevelec, M.N. (1995) Mucosal vaccination against toxoplasmosis. *Vet. Res.* **26**, 214–215.
- Cesbron-Delauw, M.F. (1994) Dense-granule organelles of *Toxoplasma gondii*: their role in the host-parasite relationship. *Parasitol. Today*, **10**, 293–296.
- Clemente, M., Curilovic, R., Sassone, A., Zelada, A., Angel, S.O. and Mentaberry, A.N. (2005) Production of the main surface antigen of *Toxoplasma gondii* in tobacco leaves and analysis of its antigenicity and immunogenicity. *Mol. Biotechnol.* **30**, 41–50.
- Corigliano, M.G., Maglioco, A., Laguía Becher, M., Goldman, A., Martín, V., Angel, S.O. and Clemente, M. (2011) Plant Hsp90 proteins interact with B-cells and stimulate their proliferation. *PLoS ONE*, **6**, e21231.
- Daniell, H. (1997) Transformation and foreign gene expression in plants by microprojectile bombardment. *Methods Mol. Biol.* **62**, 463–489.
- Davoodi-Semiromi, A., Schreiber, M., Nalapalli, S., Verma, D., Singh, N.D., Banks, R.K., Chakrabarti, D., et al. (2010) Chloroplast-derived vaccine antigens confer dual immunity against cholera and malaria by oral or injectable delivery. *Plant Biotechnol. J.* **8**, 223–242.
- Desolme, B., Mévélec, M.N., Buzoni-Gatel, D. and Bout, D. (2000) Induction of protective immunity against toxoplasmosis in mice by DNA immunization with a plasmid encoding *Toxoplasma gondii* GRA4 gene. *Vaccine*, **18**, 2512–2521.
- Dimier-Poisson, I., Aline, F., Mévélec, M.N., Beauvillain, C., Buzoni-Gatel, D. and Bout, D. (2003) Protective mucosal Th2 immune response against *Toxoplasma gondii* by murine mesenteric lymph node dendritic cells. *Infect. Immun.* **71**, 5254–5265.
- Dubey, J.P. (2004) Toxoplasmosis - a waterborne zoonosis. *Vet. Parasitol.* **126**, 57–72.
- Dubey, J.P. (2009) Toxoplasmosis in sheep-the last 20 years. *Vet. Parasitol.* **163**, 1–14.
- Farran, I., McCarthy-Suárez, I., Río-Manterola, F., Mansilla, C., Lasarte, J.J. and Mingo-Castel, A.M. (2010) The vaccine adjuvant extra domain A from fibronectin retains its proinflammatory properties when expressed in tobacco chloroplasts. *Planta*, **231**, 977–990.
- Fernández-San Millán, A., Farrán, I., Molina, A., Mingo-Castel, A.M. and Veramendi, J. (2007) Expression of recombinant proteins lacking methionine as N-terminal amino acid in plastids: human serum albumin as a case study. *J. Biotechnol.* **127**, 593–604.
- Fernández-San Millán, A., Ortigosa, S.M., Hervás-Stubbs, S., Corral-Martínez, P., Seguí-Simarro, J.M., Gaétan, J., Coursaget, P. and Veramendi, J. (2008) Human papillomavirus L1 protein expressed in tobacco chloroplasts self-assembles into virus-like-particles that are highly immunogenic. *Plant Biotechnol. J.* **6**, 427–441.
- Ferraro, G., Laguía Becher, M.L., Angel, S.O., Zelada, A., Mentaberry, A.N. and Clemente, M. (2008) Efficient expression of a *Toxoplasma gondii* dense granule Gra4 antigen in tobacco leaves. *Exp. Parasitol.* **120**, 118–122.
- Flegr, J. (2007) Effects of toxoplasma on human behavior. *Schizophr. Bull.* **33**, 757–760.
- Gazzinelli, R.T., Hakim, F.T., Hieny, S., Cheever, A. and Sher, A. (1991) Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J. Immunol.* **146**, 286–292.
- Gazzinelli, R.T., Wysocka, M., Hieny, S., Schariton-Kersten, T., Cheever, A., Kühn, R., Müller, W., Trinchieri, G. and Sher, A. (1996) In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J. Immunol.* **157**, 798–805.
- Gómez, E., Chimenoz, S., Carrillo, E., Estela Roux, M. and Berinstein, A. (2008) Mucosal immunity induced by orally administered transgenic plants. *Immunobiology*, **213**, 671–675.
- Gonzalez-Rabade, N., McGowan, E.G., Zhou, F., McCabe, M.S., Bock, R., Dix, P.J., Gray, J.C. and Ma, J.K. (2011) Immunogenicity of chloroplast-derived HIV-1 p24 and a p24-Nef fusion protein following subcutaneous and oral administration in mice. *Plant Biotechnol. J.* **9**, 629–638.
- Gray, B.N., Yang, H., Ahner, B.A. and Hanson, M.R. (2011) An efficient downstream box fusion allows high-level accumulation of active bacterial beta-glucosidase in tobacco chloroplasts. *Plant Mol. Biol.* **76**, 345–355.
- Hayden, C.A., Streatfield, S.J., Lamphear, B.J., Fake, G.M., Keener, T.K., Walker, J.H., Clements, J.D., Turner, D.D., Tizard, I.R. and Howard, J.A. (2012) Bioencapsulation of the hepatitis B surface antigen and its use as an effective oral immunogen. *Vaccine*, **30**, 2937–2942.
- Henriquez, F.L., Woods, S., Cong, H., McLeod, R. and Roberts, C.W. (2010) Immunogenetics of *Toxoplasma gondii* informs vaccine design. *Trends Parasitol.* **26**, 550–555.
- Hill, D. and Dubey, J.P. (2002) *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin. Microbiol. Infect.* **8**, 634–640.
- Hiszczynska-Sawicka, E., Ołędzka, G., Holec-Gąsior, L., Li, H., Xu, J.B., Sedcole, R., Kur, J., Bickerstaffe, R. and Stankiewicz, M. (2011) Evaluation of immune responses in sheep induced by DNA immunization with genes encoding GRA1, GRA4, GRA6 and GRA7 antigens of *Toxoplasma gondii*. *Vet. Parasitol.* **177**, 281–289.
- Jongert, E., Roberts, C.W., Gargano, N., Förster-Waldl, E. and Petersen, E. (2009) Vaccines against *Toxoplasma gondii*: challenges and opportunities. *Mem. Inst. Oswaldo Cruz*, **104**, 252–266.
- Kapusta, J., Pniewski, T., Wojciechowicz, J., Bociag, P. and Plucienniczak, A. (2010) Nanogram doses of alum-adsorbed HBs antigen induce humoral immune response in mice when orally administered. *Arch. Immunol. Ther. Exp. (Warsz)*, **58**, 143–151.
- Kong, Q., Richter, L., Yang, Y.F., Arntzen, C.J., Mason, H.S. and Thanavala, Y. (2001) Oral immunization with hepatitis B surface antigen expressed in transgenic plants. *Proc Natl. Acad. Sci. USA*, **98**, 11539–11544.
- Kostrzak, A., Cervantes Gonzalez, M., Guetard, D., Nagaraju, D.B., Wain-Hobson, S., Tepfer, D., Pniewski, T. and Sala, M. (2009) Oral administration of low doses of plant-based HBsAg induced antigen-specific IgAs and IgGs in mice, without increasing levels of regulatory T cells. *Vaccine*, **27**, 4798–4807.
- Laemmli, U.K. (1970) Cleavage of structured proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.
- Laguía Becher, M., Martín, V., Kraemer, M., Corigliano, M., Yacono, M.L., Goldman, A. and Clemente, M. (2010) Effect of codon optimization and subcellular targeting on *Toxoplasma gondii* antigen SAG1 expression in tobacco leaves to use in subcutaneous and oral immunization in mice. *BMC Biotechnol.* **10**, 52.
- Limaye, A., Koya, V., Samsam, M. and Daniell, H. (2006) Receptor-mediated oral delivery of a bioencapsulated green fluorescent protein expressed in transgenic chloroplasts into the mouse circulatory system. *FASEB J.* **20**, 959–961.
- Lössl, A.G. and Waheed, M.T. (2011) Chloroplast-derived vaccines against human diseases: achievements, challenges and scopes. *Plant Biotechnol. J.* **9**, 527–539.

- Luft, B.J. and Remington, J.S. (1992) Toxoplasmic encephalitis in AIDS. *Clin. Infect. Dis.* **15**, 211–222.
- Martin, V., Supanitsky, A., Echeverría, P.C., Litwin, S., Tanos, T., De Roodt, A.R., Guarnera, E.A. and Angel, S.O. (2004) Recombinant GRA4 or ROP2 protein combined with alum or the *gra4* gene provides partial protection in chronic murine models of toxoplasmosis. *Clin. Diagn. Lab. Immunol.* **11**, 704–710.
- Mevelec, M.N., Mercereau-Pujalon, O., Buzoni-Gatel, D., Bourguin, I., Chardès, T., Dubremetz, J.F. and Bout, D. (1998) Mapping of B epitopes in GRA4, a dense granule antigen of *Toxoplasma gondii* and protection studies using recombinant proteins administered by the oral route. *Parasite Immunol.* **20**, 183–195.
- Mevelec, M.N., Bout, D., Desolme, B., Marchand, H., Magné, R., Bruneel, O. and Buzoni-Gatel, D. (2005) Evaluation of protective effect of DNA vaccination with genes encoding antigens GRA4 and SAG1 associated with GM-CSF plasmid, against acute, chronic and congenital toxoplasmosis in mice. *Vaccine*, **23**, 4489–4499.
- Mitchell, C.D., Erlich, S.S., Mastrucci, M.T., Hutto, S.C., Parks, W.P. and Scott, G.B. (1990) Congenital toxoplasmosis occurring in infants perinatally infected with human immunodeficiency virus 1. *Pediatr. Infect. Dis. J.* **9**, 512–518.
- Molina, A., Veramendi, J. and Hervás-Stubbs, S. (2005) Induction of neutralizing antibodies by a tobacco chloroplast-derived vaccine based on a B cell epitope from canine parvovirus. *Virology*, **342**, 266–275.
- Nigro, M., Gutierrez, A., Hoffer, A.M., Clemente, M., Käufer, F., Carral, L., Martin, V., Guarnera, E.A. and Angel, S.O. (2003) Evaluation of *Toxoplasma gondii* recombinant proteins for the diagnosis of recently acquired toxoplasmosis by an immunoglobulin G analysis. *Diagn. Microbiol. Infect. Dis.* **47**, 609–613.
- Ortigosa, S.M., Fernández-San Millán, A. and Veramendi, J. (2010) Stable production of peptide antigens in transgenic tobacco chloroplasts by fusion to the p53 tetramerisation domain. *Transgenic Res.* **19**, 703–709.
- Paul, M. and Ma, J.K. (2010) Plant-made immunogens and effective delivery strategies. *Expert Rev. Vaccines*, **9**, 821–833.
- Pedersen, M.G., Stevens, H., Pedersen, C.B., Nørgaard-Pedersen, B. and Mortensen, P.B. (2011) *Toxoplasma* infection and later development of schizophrenia in mothers. *Am. J. Psychiatry*, **168**, 814–821.
- Rharbaoui, F., Drabner, B., Borsutzky, S., Winckler, U., Morr, M., Ensoll, B., Mühlradt, P. and Guzmán, C. (2002) The *Mycoplasma*-derived lipopeptide MALP-2 is a potent mucosal adjuvant. *Eur. J. Immunol.* **32**, 2857–2865.
- Rorman, E., Zamir, C.S., Rilks, I. and Ben-David, H. (2006) Congenital toxoplasmosis-prenatal aspects of *Toxoplasma gondii* infection. *Reprod. Toxicol.* **21**, 458–472.
- Sánchez, V.R., Pitkowski, M.N., Fernández Cuppari, A.V., Rodríguez, F.M., Fenoy, I.M., Frank, F.M., Goldman, A., Corral, R.S. and Martin, V. (2011) Combination of CpG-oligodeoxynucleotides with recombinant ROP2 or GRA4 proteins induces protective immunity against *Toxoplasma gondii* infection. *Exp. Parasitol.* **128**, 448–453.
- Santi, L., Batchelor, L., Huang, Z., Hjelm, B., Kilbourne, J., Arntzen, C.J., Chen, Q. and Mason, H.S. (2008) An efficient plant viral expression system generating orally immunogenic Norwalk virus-like particles. *Vaccine*, **26**, 1846–1854.
- Suzuki, Y., Orellana, M.A., Schreiber, R.D. and Remington, J.S. (1988) Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. *Science*, **240**, 516–518.
- Tenter, A.M., Heckeroth, A.R. and Weiss, L.M. (2000) *Toxoplasma gondii*: from animals to humans. *Int. J. Parasitol.* **30**, 1217–1258.
- Zhang, G., Huang, V.T., Battur, B., Zhou, J., Zhang, H., Liao, M., Kawase, O., Lee, E.G., Dautu, G., Igarashi, M., Nishikawa, Y. and Xuan, X. (2007) A heterologous prime-boost vaccination regime using DNA and a vaccinia virus, both expressing GRA4, induced protective immunity against *Toxoplasma gondii* infection in mice. *Parasitology*, **134**, 1339–1346.
- Zhang, X., Yuan, Z., Duan, Q., Zhu, H., Yu, H. and Wang, Q. (2009) Mucosal immunity in mice induced by orally administered transgenic rice. *Vaccine*, **27**, 1596–1600.

Supporting information

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

Figure S1 chGRA4 quantification by western blot analysis based on a dilution series of rGRA4 as reference (10, 20, 40, 60 and 80 ng).

Figure S2 GRA4-specific IgGt, IgG1 and IgG2b antibody titres of serum samples.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.