# Transgenic tomato plants expressing the antigen gene *PfCP-2.9* of *Plasmodium falciparum*

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Abstract – The objective of this work was to obtain transgenic tomato plants expressing the PfCP-2.9 protein (a chimera of the antigens *MSP1* and *AMA1* of *Plasmodium falciparum*). Cotyledons of seven-day-old tomatoes, cultivar Summers, were transformed via *Agrobacterium tumefaciens*. Transgenic expression in the T0 plants was verified in the DNA extracted from fruits. PCR analysis was used to test the presence of the gene of interest in the T1 generation. Reverse transcriptase PCR provided evidence of gene expression at the RNA level, and Western blot analysis confirmed the presence of the protein of interest in the T1 plants. This is the first report of successful transformation with the expression of a malaria antigen (*PfCP-2.9*) in transgenic tomato plants from the T0 and T1 generations.

Index terms: gene expression, genetic transformation, malaria, plant-made vaccines.

# Plantas transgênicas de tomate expressando o gene do antígeno *PfCP-2.9* de *Plasmodium falciparum*

Resumo – O objetivo deste trabalho foi obter plantas transgênicas de tomate que expressem a proteína PfCP-2.9 (uma quimera dos antígenos *MSP1* e *AMA1* de *Plasmodium falciparum*). Cotilédones de tomate, cultivar Summers, com sete dias de idade, foram transformados via *Agrobacterium tumefaciens*. A expressão transgênica nas plantas T0 foi verificada no DNA extraído dos frutos. A análise por PCR foi utilizada para testar a presença do gene de interesse na geração T1. A evidência da expressão do gene no RNA foi constatada por meio da PCR de transcriptase reversa, e a análise "Western blot" confirmou a presença da proteína de interesse nas plantas T1. Este é o primeiro relato de transformação bem sucedida com a expressão de um antígeno da malária (*PfCP-2,9*) em plantas transgênicas de tomate da geração T0 e T1.

Termos para indexação: expressão gênica, transformação genética, malária, vacina a partir de plantas.

## Introduction

Malaria, a disease caused by protozoan parasites of the genus *Plasmodium*, is one of the world's current biggest challenges (Chowdhury & Bagasra, 2007). Over 2 billion individuals reside in the malaria endemic areas, especially in the tropical countries, where this disease affects 300–500 million people annually (Patz et al., 2003). In fact, in 2004, *Plasmodium falciparum* was considered one of the leading causes of death worldwide, being induced by a single infectious agent (World Health Organization, 2009). Vaccines are a cost-effective way of eradicating infectious diseases of this magnitude. However, the available malaria vaccines produced by conventional methods are still unaffordable for most people due to their high price, attributed to significant costs of fermentation and purification systems and other additional expenses associated with adjuvant, cold storage, transportation, and sterile delivery (Daniell et al., 2009). Therefore, there is an urgent need to find less expensive alternative methods to develop vaccines to combat malaria.

Pan et al. (2004) reported a potential malaria vaccine candidate, the PfCP-2.9 protein produced through the fusion of the antigen *MSP1-19* with *AMA-1* (III). This vaccine candidate induces high production of antibodies in rabbits and monkeys, inhibiting the parasite development in the blood, as shown through in vitro studies. Peng et al. (2010) observed that the components of this chimeric protein did not change their structure after fusion, which indicates that this protein is a strong vaccine candidate in combating this disease.

There have been several reports of plant-made vaccines or pharmaceuticals (Huang et al., 2005; Alvarez et al., 2006; Zhang et al., 2006). Arntzen et al.

(1996), for example, developed an edible vaccine, using transgenic tomatoes, against plague (*Yersinia pestis*). The immunogenicity of these transgenic tomatoes was confirmed in mice, which were primed subcutaneously with bacterially-produced F1–V and boosted orally using transgenic tomato fruit. In another experiment, tomatoes expressed antigen genes encoding HBsAg, HIV gag, and Rabies capsid proteins (Sala et al., 2003). Additionally, the Norwalk virus capsid protein produced in transgenic tomatoes showed immunogenicity in mice when the transgenic freeze-dried tomato was orally delivered (Huang et al., 2005).

Turpen et al. (1995) were the first to report the expression of a malaria antigen in the tobacco plant. Recently published works indicate the expression of antigens against malaria in lettuce and tobacco (Davoodi-semiromi et al., 2010), *Arabidopsis thaliana* seeds (Lau et al., 2010), and green algae (Dauvillée et al., 2010). The malaria antigens used were *AMA1*, *MSP1* (Dauvillee et al., 2010; Davoodi-semiromi et al., 2010), and $MSP1_{42}$ (Lau et al., 2010).

The production of an edible vaccine in tomato plants could prevent the development of malaria parasites by activating the entire immune system mechanism, which, consequently, would interfere with the malaria parasite from one life stage to another (Chowdhury et al., 2009). Since the tomato is a very popular vegetable in malaria endemic areas, such as Africa and Asia, edible vaccines expressed in tomatoes would represent an effective, safe, and inexpensive way of vaccination (Chowdhury & Kantor, 2008; Chowdhury et al., 2009).

The objective of this work was to obtain transgenic tomato plants expressing the PfCP-2.9 protein (a chimera of the antigens *MSP1* and *AMA1* of *Plasmodium falciparum*).

## Materials and Methods

The Summers tomato variety was used for the transformation experiment via *Agrobacterium* (Wang, 2006). The *Plasmodium* gene of interest (*PfCP-2.9*=1036bp) was codon optimized and synthesized by GenScript (GenScript – Transforming Biology Research, Piscataway, NJ, USA) for expression in tomato. The *PfCP-2.9* DNA was then cloned in the pPS1 plant transformation vector, and the plasmid was introduced in the *Agrobacterium* strain LBA 4404 through electroporation (Seidman et al., 2001). Two *Agrobacterium* colonies were obtained after the process of electroporation. After performing PCR, only one colony presented both genes of interest, *PfCP-2.9* and *nptII* (kanamycin resistance determinant). This colony was resuspended in liquid Murashige & Skoog medium (Murashige & Skoog, 1962), and the optical density was adjusted to 0.4 cells $mL^{-1}$. Seven-day-old tomato cotyledons were transferred to the *Agrobacterium* suspension containing 100 µmol $L^{-1}$ acetosyringone and incubated at room temperature for 20 min.

The cotyledons were then blotted dry using sterile filter paper, transferred to solid MS medium as described by Velcheva et al. (2005), without acetosyringone, and incubated in a dark growth chamber at 23°C. After co-cultivation for 72 hours, the explants were transferred to the MS selection medium containing 300 mg $L^{-1}$ ticarcillin (timentin) and 50 mg $L^{-1}$ kanamycin, for four weeks, followed by transfer to the same selection medium containing 400 mg $L^{-1}$ timentin and 100 mg $L^{-1}$ kanamycin, for another eight weeks. The DNAs of putative transformants were analyzed by the PCR method. A total number of three transformed plants (plant 1, 2, and 3) with the *PfCP-2.9* gene reached maturity and produced fruit. Ripe tomato fruits were harvested as they reached maturity. Seven fruits were harvested from plant 1, 36 fruits from plant 2, and nine fruits from plant 3.

DNA was extracted from the ripe fruits of transformed (T0) plants. The seeds harvested from the tomato transgenic plants were germinated, and the DNA was isolated from the cotyledons of each plant source by the CTAB method (Ausubel et al., 1994). The DNA samples were tested for the presence of the gene of interest using the PCR method. Additionally, DNA was extracted from the hypocotyledons of plant 2 and tested. After DNA amplification using the PCR method, samples were analyzed by agarose gel electrophoresis.

The following primers were chosen for the detection of the *nptII* gene: Forward: 5'-GAGGCTATTCGGCTATGACTG-3' and Reverse: 5'-ATCGGGAGCGGCGATACGTA-3'. The primers used for the detection of the *PfCP-2.9* malaria gene were: Forward: 5'-ATACAAAACAAACGAATCTC-3' and Reverse: 5'-GGAGTGTCTTAGGTGACTTA-3'. Both sets of primers were synthesized by Eurofins (Eurofins MWG Operon, Huntsville, AL, USA). The PCR program steps were: initialization at 94°C for

4 min, followed by denaturation at 94°C for 1 min, then by annealing temperature at 50°C for 1 min and elongation temperature at 72°C for 1 min. The final extension was performed at 72°C for 10 min. The cycles were repeated 30 times.

After identifying the presence of the gene of interest in the DNA extracted from the first generation of transformed plants, the next step was to demonstrate that the gene of interest was successfully integrated into the plant genome. Since DNA testing is not enough to determine the functionality and the successful integration of *PfCP-2.9* into the plant genome, reverse transcriptase PCR (RT-PCR) was performed. The RT-PCR method is commonly used for a rapid identification and expression of the gene of interest in the transgenic plants genome (Huang et al., 2011). For this experiment, seeds from plants 1, 2, and 3 of the T0 generation were germinated and RNA was extracted from the leaves of one young plantlet representing each plant. The Aqua Pure RNA isolation kit (Bio-Rad, Hercules, CA, USA) was used for this procedure. For cDNA synthesis, the following program steps were used: 5 min at 15°C, followed by 30 min at 42°C and another 5 min at 85°C. The PCR amplification program was the same as the one used for DNA amplification.

The Bradford method (Bradford, 1976) and the plant protein extraction kit (Sigma-Aldrich, St. Louis, MO, USA) were used to quantify the protein extracted from fresh cotyledons of the T1 plants. Different dilutions of 2 mg $mL^{-1}$ BSA were used to develop the standard curve, and the concentrations of unknown protein samples were determined by comparison to the standard curve. The device used for reading the values was a Multiskan Ex ELISA reader (Thermo Fisher Scientific, Hudson, NH, USA), and the data was interpreted with the Ascent software, version 2.6 (Thermo Fisher Scientific, Hudson, NH, USA). The amounts of proteins extracted from tomato fruits were much lower than the quantities obtained from leaves. As a consequence, the protein extracted from leaves was used for the Western blot experiment.

The polyacrylamide gel was analyzed with the Western blot method. Due to the absence of purified antibodies against the PfCP-2.9, the antibody against AMA1 (which is part of PfCP-2.9) was used to test the total soluble protein (TSP) extracted from leaves. The Western blot experiment was performed using the protocol described in the Mini Trans-Blot electrophoretic transfer cell instruction manual (Bio-Rad, Hercules, CA, USA) with a few changes, including current intensity or concentration of antibodies. Modifications to the protocol regarding antibody concentrations were performed according to Alvarez et al. (2006). Samples from leaves of the T1 transformed plants with the *PfCP-2.9* gene, representing all four plants of the T0 generation, were analyzed in duplicate and subjected to migration in two acrylamide gels, one for protein identification and the other for use in the Western blot experiment. The proteins were transferred from the acrylamide gels to polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot apparatus electrophoretic transfer cell catalog #170-3930, (Bio-Rad, Hercules, CA, USA).

Primary antibodies were diluted (1:2000) in TTBS (Tris-buffered saline with 0.05% Tween 20), and a total volume of 60 mL was prepared for each membrane. The next step consisted of the dilution of secondary antibodies goat anti-rabbit HRP conjugate, (Bio-Rad, Hercules, CA, USA) in TTBS using a 1:10000 dilution. The membranes were then washed with TTBS six times after removing the antibody solution. After using the electrophoretic method to transfer proteins from the gel on the PVDF membrane, the presence of the protein of interest was recorded with a Kodak Gel Logic 1500 imaging system camera. No positive controls were observed due to the absence of the purified PfCP-2.9 protein.

## Results and Discussion

Three transgenic (T0) tomato plants from a total number of 336 explants (0.89% regeneration rate) reached maturity and produced fruit and seeds. The obtained regeneration rate is lower than the one reported by Velcheva et al. (2005), who found a rate between 1.4 and 34%.

All plants transformed with the gene of interest *PfCP-2.9* showed DNA bands when tested for the presence of the malaria antigen gene (Figure 1 A). The size of the observed DNA bands was 1,036 bp for the *PfCP-2.9 g*ene and 700 bp for the *nptII* gene (Van Den Broeck et al., 1985), whereas the negative control showed no band (Figure 1 A and B). The successful expression of malaria antigens, such as *AMA1* and *MSP1*, was reported in lettuce and tobacco (Davoodi-semiromi et al., 2010). However, these genes

were individually introduced. In the present study, the *PfCP-2.9* gene introduced in tomatoes is a fused product of part of the previously mentioned genes, which is far superior as to immunogenicity (Pan et al., 2004). For example, in rabbits and monkeys, *PfCP-2.9* produces a chimeric protein that initially appeared to be highly immunogenic. Since the anti-AMA-1(III) antibody titer induced by *PfCP-2.9* was 18-fold higher than that induced by AMA-1(III), and the anti-MSP1-19 antibody titer was 11-fold higher than that induced by MSP1-19 (Pan et al., 2004), it was suggested that *PfCP-2.9* in combination with these recombinant pre-erythrocytic antigens induces the production of antibodies that inhibit growth of blood-stage malarial parasites.

All samples tested for DNA extracted from the first generation of transformed plants (T1) showed the bands corresponding to both genes of interest (*nptII* and *PfCP-2.9*) (Figure 2). This is the first report of the successful expression of the malaria antigen gene in the T1 generation of transgenic tomato plants, indicating the stable integration of the *PfCP-2.9* gene in the tomato genome and its transmission to the next generation through seed production. Lau & Korban (2009) found similar results of integration, stability, and transmission of the respiratory syncytial virus antigen in the T3 generation of transgenic tomato plants.

RNA extracted from the three transformed plants was tested, and all plants were confirmed positive with the detection of an expected 1,036 bp fragment, whereas the negative control had no target band (Figure 3). The presence of the *PfCP-2.9 g*ene was confirmed in the DNA extracted from the transgenic plants, as well in the plant RNA, illustrating the successful integration and expression of the target gene in transformed plants. The integration of foreign DNA in a plant genome is the first step of transgene expression. However, to confirm gene expression, it is important to determine RNA production for the gene of interest in the transgenic plant. In the present study, all three plants showed RNA synthesis. Similar results of RNA expression were found in transgenic tomato plants producing chimeric protein T and B cell epitope, containing immunogenic (TBI) hepatitis B surface antigen (HBSAg), as reported by Salyaev et al. (2005).

So far, current studies have permitted the quantification of total proteins extracted from leaves of

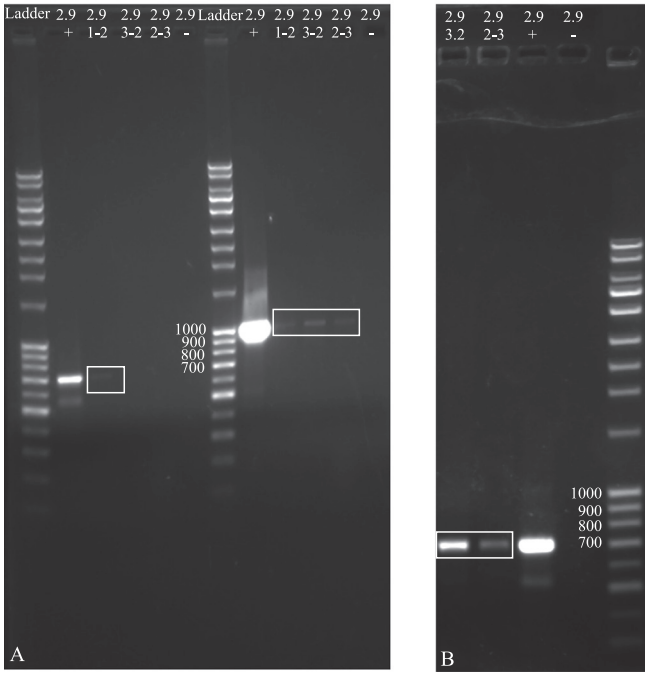


**Figure 1.** DNA from ripened tomato fruits tested for the presence of the *nptII* gene and the *PfCP-2.9* malaria gene. A, 1,036 bp band corresponding to the *PfCP-2.9* gene (right side) in all plants tested, and band corresponding to the *nptII* gene (left side) only in plant 1; B, 700 bp band corresponding to the *nptII* gene found when the DNA extracted from plants 2 and 3 was retested.

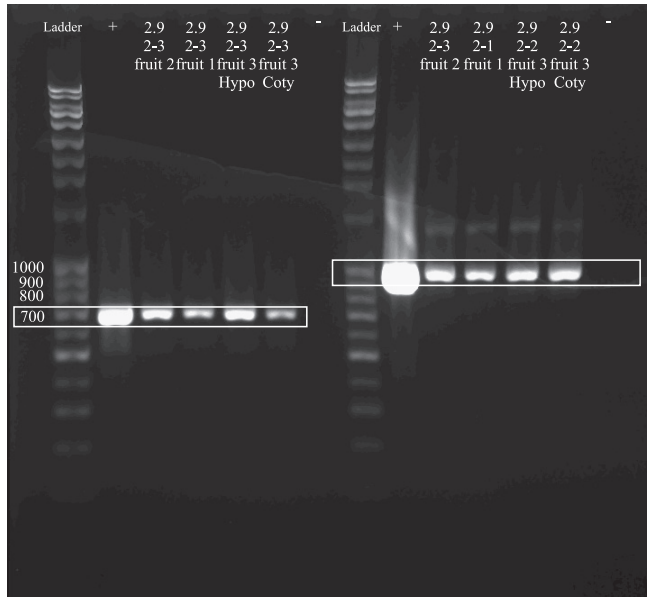


**Figure 2.** Gel showing the 700 bp band corresponding to the *nptII* gene (left side) and the 1,036 bp band corresponding to the *PfCP-2.9* malaria gene (right side), in first generation of transgenic tomato.

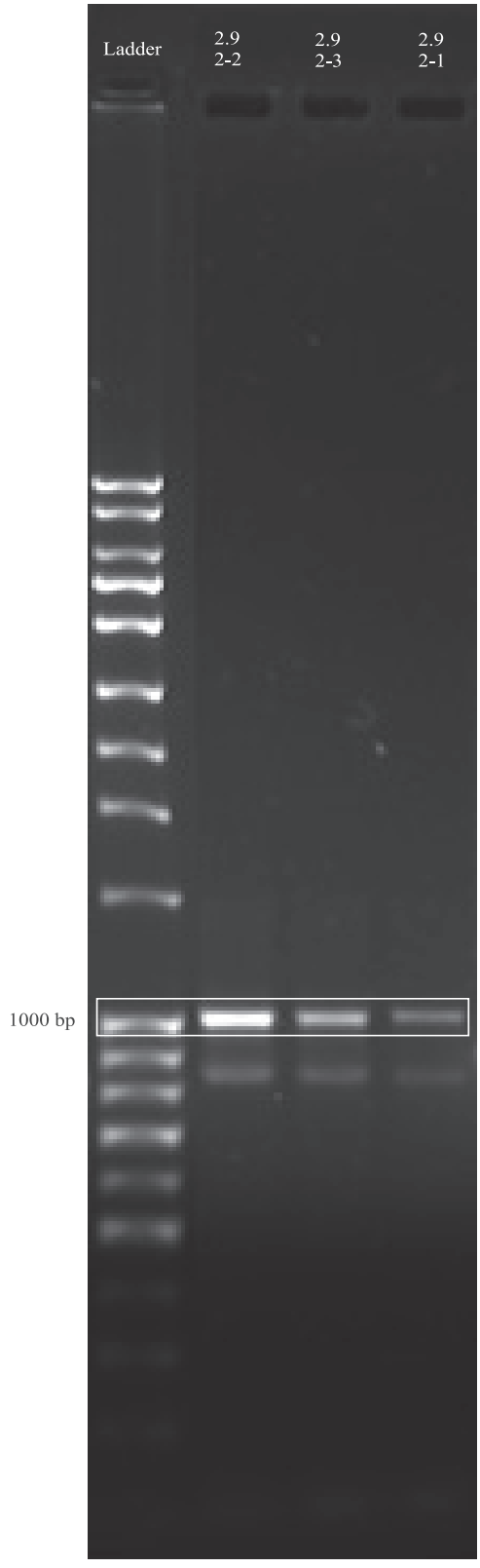


**Figure 3.** Gel showing the 1,036 bp cDNA synthesized from mRNA isolated from the first generation of tomato plants transformed with the *PfCP-2.9* gene.

T1 plants. The largest amount of total soluble protein (17.5 µg $mL^{-1}$ of TSP) was extracted from plant 3 of the Summers tomato variety, transformed with the *PfCP-2.9* gene (Table 1). The obtained TSP amount corresponds to 35 mg of TSP per gram of fresh leaf. The TSP extracted from the tomato leaves is similar to that obtained by other researchers (Biswas et al., 2012). When TSP was analyzed using the Western blot method, only one (plant 1) out of the three transformed plants with the *PfCP-2.9* gene showed a protein band of 32.5 kDa (Figure 4), which is the same size protein as that reported by Zhang et al. (2007) when the *PfCP-2.9* gene was expressed in *Pichia pastoris.*

**Table 1.** Protein concentrations extracted from fresh leaves of transformed 'Summers' tomato plants(1).

| Transformed plants with the PfCP-2.9 gene | O.D.600 values (cells $mL^{-1}$) | TSP (µg $mL^{-1}$) | Leaf weight (mg) | TSP content (mg $g^{-1}$) |
|---|---|---|---|---|
| Plant 1, sample 1 | 0.134/0.133 | 11.1605 | 26 | 22.321 |
| Plant 1, sample 2 | 0.109/0.1 | 5.6935 | 32 | 11.387 |
| Plant 2, sample 1 | 0.122/0.124 | 9.1815 | 55 | 18.363 |
| Plant 2, sample 2 | 0.138/0.133 | 11.5375 | 15 | 23.075 |
| Plant 3, sample 1 | 0.166/0.104 | 11.443 | 27 | 22.886 |
| Plant 3, sample 2 | 0.154/0.181 | 17.5 | 29 | 35 |

(1)O.D., optical density; TSP, total soluble protein.

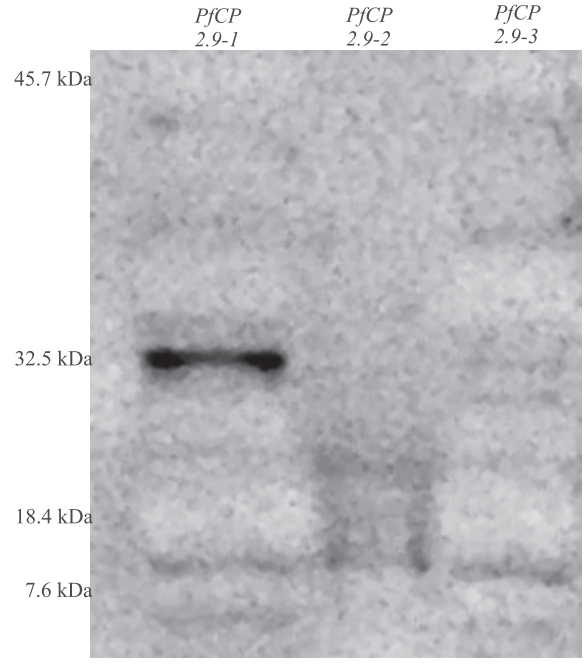


**Figure 4.** Protein gel showing the chimeric *PfCP-2.9* protein identified in total protein extracted from first the generation of transgenic tomato leaf hybridized with *AMA-1* monoclonal antibody.

## Conclusions

1. Tomato plants transformed with the *PfCP-2.9* gene express the malaria antigen gene in tomato fruit.

2. The gene *PfCP-2.9* is transferred to the next generation of tomato plants.

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