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Summary of PhD thesis

Tomato transgenic plants with malaria antigens

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CONTENT

Introduction

Malaria, a disease caused by protozoan parasites of genus *Plasmodium*, is one of the medical world's biggest challenges in the 21st century (CHOWDHURY and BAGASRA 2007). Over 2 billion individuals reside in malaria endemic areas, especially in the tropical countries, the disease affecting 300-500 million people annually. As a result of malarial infection, more than one million lives are lost annually, a large majority being represented by children less than 5 years of age. Therefore, in order to eradicate the infectious disease in a cost-effective way it is necessary to make vaccines easily available to the infected population, process that currently involves huge investments and the use of a lot of resources. On the one hand, because the malaria parasite has a complex life cycle, it is a real challenge to develop vaccines that would inhibit its development. On the other hand, this complex life cycle that takes place in different organisms allows us to intervene at various stages of the development of the parasite. Hence, at this time there is no clinically proven malaria vaccine on the market to combat malaria. The majority of the people living in the malaria endemic areas is poor and cannot afford expensive medication. Due to the fact that several hosts and different development stages are needed to transmit the parasite to humans, it takes approximately10-15 antigens to confer a full protection against all the development stages of the parasite (CHOWDHURY and BAGASRA, 2007). Currently used standard protocols for conventional vaccination do not allow this large number of antigens to be injected into the body with one single vaccination (CHOWDHURY and KANTOR, 2008). Even if the malaria vaccines were available and were produced through conventional methods, they would still be unaffordable to these people given that the conventional method of vaccine production is too expensive. Some of the reasons for its high prices include: significant costs of fermentation and purification systems, as well as additional expenses associated with adjuvant, cold storage, transportation and sterile delivery. Therefore there is an urgent need for developing less expensive alternative methods of vaccine production for combating malaria. The main goal of the present thesis was to create an edible vaccine against malaria expressed in tomato

plants. The motivation for this research came from the need to obtain an edible vaccine at a low cost, and to make it affordable for the people affected by malaria.

Stages of the malaria parasite development

Various researchers have described in their papers different vaccine candidates for malaria, candidates that could stop the development of the parasite in various life stages of development (pre-and post-erytrocytic). In this regard, protozoan parasites of genus *Plasmodium* have a complex life cycle. To complete the life cycle of *Plasmodium*, two hosts are required, namely the human and female anopheles mosquito. In humans, the mosquito infects both the liver and the red blood cells. The process begins when a female mosquito injects a person with sporozoites when it feeds (LODISH et al., 2008). After injection with sporozoites, in less than 30 minutes the hepatocytes are invaded. Then, a period of 6-16 days is required for sporozoites to mature (OKITSU, 2006). Sporozoites' maturation occurs in the liver of the infected person. The development stage of the parasite, which occurs in the liver, ends through the releasing of thousands of merozoites in the blood. These will invade the red blood cells and will begin to multiply and mature. The next stages of the parasite development are the trophozoite stage, followed by the schizont development stage (CDC, 2010). The schizont will release a new generation of merozoites, which will invade red blood cells, the cycle being repeated (CDC, 2010). Potential candidate malaria vaccine antigen genes of all three stages of *Plasmodium* life cycle have been studied for a long time. As a consequence, several vaccines are currently in the developmental pipeline and at different stages of clinical trials.

It was shown that *Plasmodium* sp. manifests differently from one geographical area to another, which becomes another challenge that must be taken into consideration while developing an effective vaccine against malaria. As a result of diversity caused by a specific area, such a vaccine could cure people in the areas from where samples were collected. However, even if the vaccine works on the same antigen, it does not mean that it will be effective when used in a different environment.

The current stage of knowledge

Today, the most advanced vaccine against malaria is RTS, S vaccine created in 1987 by researchers from GlaxoSmithKline Biologicals company. RTS, S vaccine is currently in the phase 3 of trials since May 2009, and it was tested on 15,461 children in several African countries (WHO, 2011). As far as the effectiveness of this vaccine, by the second phase of testing it was able to induce immunity in 35% of patients that showed clinical signs of disease and in 42% of patients severely affected by malaria (ALONSO et al., 2004 ; ALONSO et al., 2005). In a more recent study a 53% efficacy was observed when the vaccine was administrated to children with ages between 5 and 17 months (BEJON et al., 2008).

A vaccine able to prevent the development of the parasite and interact with it when passing from one stage to another stage of life, if successful, would be the ideal candidate for vaccine development against malaria (CHOWDHURY et al., 2009). PfCSP and PfCSP-RC-C are two recombinant antigens derived from *Plasmodium falciparum* CSP. When tested on rabbits and mice in combination, they induced a high body immunization (PAN et al., 2004, PAN et al., 2007). The results showed that the antigens can be useful to develop a polyvalent vaccine against malaria.

PAN *et al.* (2004) have published results on potential malaria vaccine candidates in which they analyzed the PfCP-2.9 protein created through the fusion of the antigen MSP1-19 with AMA-1 (III). This vaccine candidate induces high production of antibodies that inhibit parasite development in the blood shown through in vitro studies (PAN *et al.,* 2004). In the experiments conducted by PAN *et al.* (2004) the PfCP-2.9 chimeric protein seemed to elicit high immune response when tested on rabbits and monkeys. For anti-AMA-1 (III) Ab antibody titers induced by PfCP-2.9 was 18 times higher than that induced by AMA-1 (III) alone, while anti-MSP1-19 Ab titer was 11 times greater than that induced by MSP1-19 alone (PAN *et al.,* 2004). It has been therefore suggested that PfCP-2.9 induces the production of antibodies that inhibit parasite development in the life cycle that occurs in blood. Although when tested on different animals PfCP-2.9 the vaccine induced a high level

of immunity against the malaria parasite, after testing the vaccine in humans a high level of antibodies was observed, without parasite development inhibition (MALKIN *et al.* , 2008). Also, when tested on humans, the chimeric protein components they have created did not change their structure after fusing the two proteins, suggesting that the chimeric protein is a strong candidate and that it could be a potential candidate vaccine in combating malaria (PAN *et al.* 2010).

The current situation of edible vaccines

The successful production of a vaccine against hepatitis B virus in potato by ARNTZEN *et al.* (1996) has been followed by numerous published articles on plant-made vaccines or pharmaceuticals. These articles reported different plants used for vaccine production (ALVAREZ *et al.* , 2006; CHEN *et al.* , 2006; COKU, 2007; DONG *et al.* , 2005; HUANG *et al.* , 2005; MASON *et al.* , 2006; MEI *et al.* , 2006; ROJASS-ANAYA *et al.* , 2009; RYBICKY, 2008; STREATFIELD, 2006; THANAVALA *et al.* , 2005;), or used as production factories for different proteins (HARTWELL *et al.* , 2008).

Regarding the current situation of edible vaccines against malaria, recently published works indicate the expression of antigens against malaria in lettuce and tobacco (DANIELL *et al.*, 2010), Arabidopsis thaliana seeds (Sun *et al.* , 2010), and green algae (DAUVILLEE *et al.* , 2010). In the above mentioned works the utilized malaria antigens were AMA1, MSP 1 (DANIELL *et al.*, 2010; DAUVILLEE *et al.*, 2010), and MSP142 (SUN *et al.*, 2010). The protein of interest's levels of expression in transformed plants were 10.11% for tobacco plants transformed with the gene AMA1, 13.17% in those transformed with MSP1, and in between 6.1% (AMA1) and 7.3% (MSP1) in plants of lettuce (DANIELL *et al.* , 2010). About 5% of the protein of interest was successfully extracted from the total soluble protein from the Arabidopsis seeds transformed with the gene $MSP1_{42}$ (SUN *et al.*, 2010).

By creating an edible vaccine in tomato one can prevent the development of malaria parasite by activating the entire immune system mechanism, which consequently leads to the development of a vaccine that interferes with the malaria parasite when it passes from one

stage of life to another (CHOWDHURY *et al.*, 2009). Since tomato is a very popular vegetable in the malaria endemic areas (Africa and Asia), creating an edible vaccine expressed in tomato would be an effective, safe and inexpensive way of vaccination (CHOWDHURY and KANTOR, 2008; CHOWDHURY *et al.*, 2009).

The process of obtaining transgenic plants

Different working techniques needed for both tissue culture and plant transformation had been optimized in order to create a transgenic tomato that can be used in vaccination. Our objective was to develop an effective working protocol by which to achieve a maximum level of protein for malaria vaccine candidates.

Consequently, it was necessary to optimize the production of transgenic tomato objective achieved by meeting the following requirements:

1) testing several tomato varieties and identifying the varieties with the highest regenerative capacity;
2) analyzing and selecting the most favorable tissue culture media for the development of small regenerants;
3) optimization of the most efficient way of tomato genetic transformation using *Agrobacterium*;
4) identification of transgenic plants by using various molecular techniques and transgenic plant regeneration;
5) testing the protein level of expression in plants /transgenic fruits.

Genetic transformation

Plant genetic information, as in most of the eukaryotes, is stored as polymers, also called DNA. Each gene encodes for a protein of the plant, which makes the total number of genes from one plant to be very high. The difference between prokaryotes and eukaryotes is that the DNA composition of bacterial chromosome is circular.

Chromosome numbers and sizes may vary from one species to another. Transformation is the step in the process of genetic engineering in which nine genes

(transgenes) are inserted into a cell chromosome. Gene transfer can be achieved by two methods of transformation: the direct method and indirect method. Indirect transformation method was the first to be considered, this method involving the transformation of explants by using *Agrobacterium tumefaciens* bacteria.

In order to achieve a successful plant transformation, the first step was to transform the *Agrobacterium* bacteria. Then, the resulting transformed colonies tested positively for the presence of the gene of interest could be used in plant transformation experiments. Following the plant transformation step, the transformed plants can gain valuable characteristics such as: resistance to herbicides, different viruses or plants pathogens, obtain edible vaccines. Plants genetically modified (GMO) are examples of plants obtained by genetic transformation. In our case, the plants used for genetic modifications and occupying the largest areas were cotton and corn.

Agrobacterium tumefaciens is a bacteria that grows in soil and infects the roots or stem of a wounded plant. It is able to transfer a part of its DNA into plant cells (infects various different ornamental plants, fruit trees and vegetables), causing tumors (TRIGIANO and GRAY, 1999).

Agrobacterium tumefaciens is one of the most convenient methods of genetic transformation. T-DNA is a segment of Ti plasmid of *Agrobacterium* which is transferred to the plant cells when the bacteria infect the plant. The T-DNA region is framed by the left and right side. Everything between these two sides is transferred to the infected plant. T-DNA region of plasmid belonging to the Ti's can be amended to introduce the gene of interest after the removal of the *vir* region which induces the tumor formation. This step represented the beginning of the development of procedures for transfer of genes or DNA in plants.

Results and discussion

The first step of this study included the selection of two antigens (PfCP-2.9 and PfCSP-RC) from a large pool of antigens reported by different researchers (BEJON *et al.*, 2008, CHENET *et al.*, 2008, WEEDALL *et al.*, 2007 BOJANG *et al.*, 2005). pPS1 binary vector was chosen to introduce the genes of interest into *E. coli*, the cloning of the genes being performed by the GenScript company. The pPS1 vector, the vector map, and the instructions were received as a gift from HUGH MASON. *Agrobacterium bacterium* was transformed through the electroporation method, (HUANG and MASON, 2004).

The studies of the two colonies (resulted after electroporation with PfCP2.9) and the 18 colonies (resulted after electroporation with PfCSP-RC) had the main goal of identifying the transformed *Agrobacterium* colonies with the gene of interest. Several colonies transformed with PfCSP-RC gene tested positive for the presence of the gene of interest after PCR amplification. Colonies 3, 5, 10, 12, 15, 16, and 18 tested positive for both genes of interest (kanamycin gene and malaria gene). Colony number two that resulted from the transformation with the gene PfCP-2.9 presented both genes of interest after DNA migration in agarose gel.

Tomato varieties were selected based on a vegetation period between 58 days (Summer Sweet) and 95 days (Pineapple). They had different shapes [egg-shape (Roma), round-oblate (Pineapple, Brandywine), round (Rutgers, Pink-Girl, Summer Sweet)] and tomato fruit weight ranged from 57 g (Summer) to 900 g (Pineapple).

The great diversity of tomato varieties used in the present study was chosen with the purpose to obtain edible vaccines expressed in tomato fruits of different shapes, sizes and colors (BAGASRA and CHOWDHURY, 2007). Results obtained from the tissue culture experiment were analyzed with the SPSS software. As a result, the best culture medium out of six culture media tested was MS+1Z +0.05 IAA.

After analyzing the data from the tomato transformation experiment a greater number of regenerates were obtained when the *Agrobacterium* concentration chosen in the transformation experiment was OD_{600} = 0.4nm. This concentration was used in most of the

tomato transformation experiments. Although the ideal values of *Agrobacterium* cells density was reported by various researchers to be 0.8 nm (BETCHTOLD *et al.*, 1993, YONG *et al.*, 2006), 1 nm (VAIN *et al.* 2007, PARK *et al.* , 2003) or 0.2 nm (QIU *et al.*, 2007) for the current transformation experiments the above mentioned concentration did not give us the best results. The *Agrobacterium* concentration used in the current research was initially 1 nm, and later on was reduced to 0.4 nm.

The results obtained from the transformation experiments depended on the concentration of antibiotics in the selection media. For example, the concentration used by VELCHEVAA *et al.* (2005), was 350mg/l^{-} of timentin and 100 mg/l^{-} kanamycinin, while the concentration used for the current research was 300mg/l^{-} of timentin and 50 mg/l^{-} kanamycin. Lower concentrations were used to allow the regeneration of as many regenerants possible, although lower concentrations can lead to a greater number to explants untransformed or escapes (McCORMICK, 1991).

In order to avoid the regeneration of non-transformed plants, all regenerants were transferred to elongation media containing 400mg/l^{-} timentin and 100 mg/l of kanamycin. The percentage of regenerants resulted after the transformation experiment ranged between 0 and 24.1%.The maximum percentage was recorded when the tomato variety Pink-Girl was transformed with with gene PfCSP-RC colony 18. This percentage corresponds to 20 initial regenerants that were produced from 83 explants.

When the ration between the number of initial explants and the number of initial regenerants was calculated for the explants transformed with the Agrobacterium colony PfCSP-RC-15, the highest percentage of regenerants was 3.96%, achieved by the Pineapple variety, followed by 2.41% of the Summers variety. These percentages correspond to a total number of 12 regenerants obtained from 497 explants used in the transformation.

When data obtained from the plant transformation with PfCP-2.9 was analyzed, the number of regenerants obtained was also lower than the number obtained when the plant transformation was performed with PfCSP-RC colony 18. The highest percentage of regenerants was recorded in variety Roma with 4%, percentage which corresponds to 4

regenerants obtained from 100 explants. The second variety with the highest percentage of regenerants was the Pineapple variety with 2.9%, this percentage corresponding to 25 regenerants obtained from 854 explants.

The final number of transformed plants that have reached maturity was 13, of which five plants were obtained after the transformation with PfCP-2.9 gene and 8 plants after the transformation with PfCSP-RC gene. DNA was extracted from the leaves and green fruits of transformed plants and tested with the PCR method. All the plants resulting after the transformation with PfCSP-RC gene were tested positive for the presence of both genes of interest, with the exception of the plant number 3 (which was tested positive only for the presence of *npt*II gene). DNA was extracted from ripe fruits of the T0 plants transformed with PfCP-2.9 gene and tested for the presence of malaria genes and *npt*II gene. All transformed plants presented a DNA band that corresponded to the expected DNA size of the gene of interest.

The second generation of transformed plants with PfCP-2.9 (plant 1, 2 and 3) were also analyzed for the presence of the genes of interest in the DNA extracted from plantlets cotyledons. All analyzed samples were tested positively for the presence of both genes of interest. Plants were germinated from the seeds of transformed plants (number 1, 2 and 3) with PfCSP-RC, DNA was extracted and tested for the presence of genes of interest. All DNA samples analyzed tested positive for the presence of the gene of interest, including plant 3, which has previously tested negatively for the presence of malaria gene. Second generation of transformed plants with PfCSP-RC-15 belonging to variety Pineapple were also tested using PCR method, all the samples that were analyzed presenting the *npt*II, though none of them showing the malaria gene in the agarose gel.

As it can be seen from the previously presented data, out of the 13 transformed plants that reached maturity only 11 plants presented both genes of interest. After identifying the presence of DNA in transformed plants, the next step was to demonstrate the integration of DNA in the plant genome. Therefore, ARN was extracted and later on analyzed using Real

Time PCR and Reverse transcriptase PCR, both plants 1 and 2 from the Summers variety and transformed with PfCP-2.9 tested positive for both the malaria genes of interest.

After using the Real time PCR method the following plants transformed with PfCSP-RC were tested positive for the malaria gene: plant 1,4,5,6 and 7. All 5 transformed plants were from the Summers variety. ARN was extracted from the second generation of plants 4,5,6 and 7, while the ARN sample tested positive when analyzed in plant 1 was extracted from ripped fruit. Plants 1,2 and 3 belonging to Summers variety transformed with PfCP-2.9 gene were also tested using Real Time PCR method, all of them presenting the malaria gene of interest.

Due to the high costs of the Real time PCR reagents, 16 ARN samples extracted from leaves were analyzed using Reverse transcriptase method. Plants 2, 5 and 7 belonging to Summers variety and transformed with PfCSP-RC15 gene, as well as plant number 4 transformed with PfCP-2.9 gene were tested positive for the presence of both genes of interest. Plant number 1, 4, and 6 belonging to Summers variety and transformed with PfCSP-RC15 gene also tested positive for the presence of malaria gene. RNA from the second generation of transformed plants (plant 1,2 and 3) belonging to Summers variety and transformed with PfCSP-RC gene were then tested for the presence of genes of interest. However, plant 3 did not show the presence of the gene of interest.

The Real time PCR and Reverse Transcriptase experimental results confirmed the previous results obtained from DNA experiments. Therefore, based on the data presented from both ARN experiments the current research demonstrated the successful transmission of the gene of interest to the second generation of plants. The uniqueness of the present study lays on the fact that the current research is the only one to mention a successful transmission of the gene to the second generation of plants.

The extraction of proteins from tomato fruit is a difficult process given that tomatoes are included in the recalcitrant plants category (SARAVANAN *et al.*, 2004), the amounts of protein extracted from the fruit plants being often too low. So far, the present studies have permitted the quantification of total proteins with the help of the

Bradford method, a method of analysis consistent with the extraction kit used. As a result, when data were examined, from the total analyzed samples only one showed negative values, the remaining ones showing smaller or larger protein concentrations.

The largest amount of protein (17.57μg/μl) was extracted from plant number 3 of Summer tomato variety, transformed with the PfCP-2.9 gene. Because it would have been very difficult and time consuming to obtain antibodies and purified proteins for any of the genes used in plant transformation, and also because the genes of interest are chimeras, it has been decided to identify instead the genes of interest of the plants transformed with the help of antibodies against one of the two genes that make up the genes of interest.

Studies of proteins extracted from plants transformed with the PfCSP-RC gene allowed the identification of protein of interest in transformed plants, protein identification being performed with the sandwich ELISA method. The reagents used in this experiment were purchased from CDC (Atlanta, GA, USA) and included a kit utilized for the identification of the CS proteins (circumsporozoite proteins) from mosquitoes infected with *Plasmodium falciparum*. Considering that the kit was designed to test for the presence of the CS protein in mosquitoes infected with *Plasmodium*, its testing for the presence of protein in plants transformed with CSP was a critical step, but it was the only available option. Unfortunately, not all the tested plants showed positive values, values that would have indicated the presence of proteins of interest. Plants 1, 2 and 3 did not register positive values of protein in the analyzed samples. The highest concentration of the protein of interest has been expressed in plant number 4 (31.59pg/μl), followed by plant number 5. Positive values were also recorded in plants 6 and 7, but the concentrations of protein of interest were lower than protein concentrations from the other transformed plants. As expected, the non-transformed plant which was tested for the presence of protein of interest has been tested negative in all dilutions. ELISA method was reported by several researchers (CLARK and ADAMS, 1976, ALVAREZ *et al.*, 2006) as an efficient method to detect very small quantities of protein used in the detection of viruses found in plants (CLARK and ADAMS, 1976), and to detect the proteins of

interest in transformed plants (ALVAREZ *et al.* ,2006, LI *et al.* , 2005, SUN *et al.* , 2010, MARTINEZ-GONZALEZ *et al.* , 2011, KOYA *et al.* , 2005, QIU *et al.* , 2006, MASON *et al.* , 2006, MASON *et al.* , 2005, HUANG and MASON, 2004).

Conclusions and recommendations

(Conclusion)

Data from the tissue culture experiment were consistent with those of other researchers (SHEEJA *et al.*, 2004; ICHIMIURA and ODA, 1995; BHATIA *et al.*,2004; CORTINA and CULIANEZ, 2004; CHAUDHRY *et al.* ,2007; HAMZA and CHUPEAU, 1993), reconfirming that the most suitable tissue culture media for tomato regeneration must have in its composition the IAA auxin (the most frequently used auxin for tomato tissue culture, most commonly used concentration being 0.05 mg/l^{-}). The number of explants developed on media containg IAA was signicantly higher than the number of explants obtained on NAA media.

(Conclusion)

The results from the tomato tissue culture experiment are similar to those reported in the literature, the percentages ranging between 6 and 40% (QIU *et al.* , 2006; PARK *et al.* , 2003); between 5and 32.9% (CORTINA and CULIANEZ, 2004), and between 1.4 and 34 % (VELCHEVA *et al.* ,2004). Although after the first transformation experiments a relatively high number of regenerants were obtained, after transferring them on selection media with a higher antibiotic concentration a few regenerants failed to develop roots, most of them dying after a couple of weeks.

(Conclusion)

The relatively small amounts of protein of interest detected by ELISA method may be due to the optimization of ELISA kit for the detection of protein CS in mosquitoes, not in plants.

(Recommendation)

An important approach that can be taken into consideration for future studies concerns the introduction of malaria genes in the chloroplast genome. This way unwanted pollination of transgenic tomatoes with other related species can be avoided. Although tomatoes are self-pollinated plants, in order to increase protein production and to avoid potential problems given that the crop is genetically modified, malaria genes can be expressed in the tomato chloroplast genome. Chloroplast transformation has the advantage of a high level of gene expression caused by the presence of a large number of plastids per cell (10000)

In conclusion, the current study is the first to report the successful expression of the malaria antigens (PfCP -2.9 and PfCSP-RC) in the tomato fruit and also to confirm the transfer of gene of interest to the next generation tomato plants. The obtained results represent a major step in the area of plant transformation, offering not only an economical solution to the problem currently faced by countries affected by malaria, but also a significant step in the eradication of this disease.

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