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Molecules of Interest

# New advances in the production of edible plant vaccines: chloroplast expression of a tetanus vaccine antigen, TetC

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**Abstract**

Vaccines are a proven method of controlling disease. However there are issues with the delivery and administration of vaccines. A particular problem is that the majority of vaccines currently used are injected, which can be unsafe if needles are reused in areas where blood-borne diseases are prevalent. Vaccines targeting the mucosal immune system avoid many of the problems associated with injections. One potential form of mucosal vaccine is based on the expression of vaccine antigens in plants. Current research in this area has focused on the expression of immunogens from the plant's nuclear genome but low expression levels generally achieved using this system have limited progress. In recent work we have used the model antigen, TetC, which confers resistance to Tetanus infection, to demonstrate the feasibility of expressing vaccine antigens at high levels in the plant chloroplast.


*Keywords:* *Nicotiana tabacum*; *Solanaceae*; Tobacco; Edible vaccines; Vaccine; TetC

## 1. Introduction

Plant transgenics and biotechnology has been used to produce proteins with potential human and animal pharmaceutical value for more than 13 years (Ma et al., 2003; Sala et al., 2003). This role is especially important given the increasing prevalence of these molecules and the limitations and cost of conventional fermentors. One example of this that has excited attention recently has been the use of plants for the production of vaccines. Vaccination programs are a proven measure to control disease (Makela, 2000; Nossal, 1999) and are becoming especially relevant with the emergence of new diseases, for example HIV and Ebola, the re-emergence of 'old' diseases, for example tuberculosis, and the continuing spread of multi-antibiotic resistant bacteria. These issues are such a problem that in 2000, approximately 55.7 million people died of a communicable disease according to the World Health Organisation (WHO) world health report 2001. Here we review the current status of plant vaccines and highlight our recent work which has confirmed the feasibility of using chloroplast transformation technology as a means of generating a plant-based vaccine, in our case against tetanus, which still represents a major world-wide disease (Tregoning et al., 2003).

## 2. Tetanus toxin

The symptoms of tetanus are caused by tetanus toxin (tetanospasmin), a powerful protein toxin produced by the bacterium *Clostridium tetani*. Tetanus usually occurs after an acute injury, such as a puncture wound or laceration. Generalized tetanus, the most common form, is characterized by tetanic muscular contractions and an over-activity of the autonomic nervous system, which leads to spasms and a narrowing of the blood vessels, causing a rise in the blood pressure. Localized tetanus can occur in muscles near the wound, but it may progress to the generalized form. Tetanus can be prevented by immunisation, which causes the production of anti-tetanus toxin antibodies, which block its action.

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Tetanus toxin is composed of two subunits (H and L chains) and has three functional domains. Domain $H_C$, or the TetC fragment (Fig. 1A), consists of the 50-kDa carboxyl-terminal portion of the H chain. Crystal structures of TetC have revealed that it consists of two subdomains, a lentil lectin-like N-terminal jelly roll domain and a C-terminal β-trefoil domain (Umland et al., 1997) (Fig. 1B). TetC and in particular its C-terminal region (Bizzini et al., 1977; Helting et al., 1977; Morris et al., 1980) is mainly responsible for binding of tetanus toxin to the GD1b and GT1b gangliosides (Van Heyningen, 1968), found on the surface of neuronal cells.

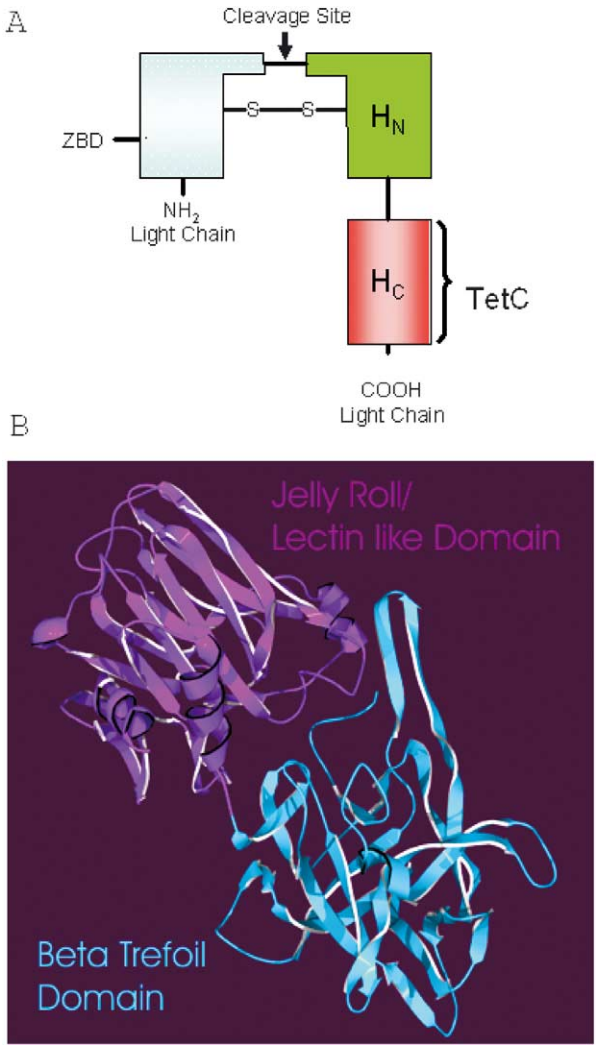


Fig. 1. Structure of tetanus toxin and the TetC fragment. (A) Schematic diagram of the three functional domains of tetanus toxin: the catalytic light chain (the L domain) in blue including zinc-binding motif (ZBD); the $H_N$ membrane translocation domain in green and the $H_C$ ganglioside binding domain in red. TetC is the $H_C$ domain. The diagram shows the putative site for the cleavage of the active L domain from the binding and translocation regions, which most likely happens in acidic cellular compartments. (B) Crystal structure of TetC. The purple region is the lectin-like jelly roll domain, for which several roles have been proposed, including retrograde transport up the axon. The blue region is the ganglioside-binding beta trefoil C-terminus domain, which is responsible for the binding to cells.

Once the toxin is bound to the surface of cells, it is internalised into acidic vesicles, which move retroaxonally to the intersynaptic space, and enter the inhibitory interneurons (Dumas et al., 1979; Schwab et al., 1979). The second domain of the protein, $H_N$, causes the translocation of the L catalytic domain into the cytosol after cleavage of the protein following acidification of the vesicle. The L subunit has been identified as a zinc-dependent protease of components of the neuroexocytosis apparatus (Schiavo et al., 1992). This action prevents vesicle docking and fusion, and causes the sustained blockage of neurotransmitter release. TetC has been widely used as a model vaccine antigen because of its non-toxicity and the potency of the response in both mucosal and parenteral systems (for example Anderson et al., 1996; Stratford et al., 2001; Tregoning et al., 2003; Villarreal-Ramos et al., 1998). Current licensed tetanus vaccines are, however, based on chemically inactivated tetanus toxin mixed with an alum-based adjuvant delivered by injection.

Despite the availability of a parenteral (introduced by injection) vaccine, tetanus is still a common disease in many parts of the world, particularly in the African Region, where neonatal tetanus (NT) remains an important public health problem, being responsible for 10–30% of all infant deaths in many countries. The regional NT mortality rate is estimated at 5–10 per 1000 live births resulting in 120,000 deaths annually (WHO website). It is also a problem in pregnant mothers where *Clostridium tetani* may enter the uterine cavity on unclean instruments or hands, particularly during non-professional abortions or non-institutional deliveries. The newborn is usually infected from unclean instruments used in cutting the cord or from contaminated substances applied as traditional cord dressings. Globally 300,000 people die from tetanus each year.

## 3. The case for edible vaccines

There are a number of limitations with the present generation of vaccines especially in developing countries where immunisation programs are often problematical (Fooks, 2000; Jacobs, 2001). The reasons for this are often generic and include: a lack of investment and logistical support in countries that are unstable politically and economically, the requirement for an effective cold-chain from the producer to the end-user and the need for trained medical staff and sterile needles to deliver the vaccine (UNICEF–WHO, 2002). Delivery by needle is a particular problem in developing countries, where safe injection practices cannot be guaranteed, especially when needles are reused (Hutin and Chen,

1999; Simonsen et al., 1999). A vaccination scheme without needles would avoid these problems. Such a vaccine could be produced in a plant and delivered orally or via an intra nasal spray (Carter and Langridge, 2002). A vaccine delivered this way could overcome these problems, making for cheaper, safer more efficacious vaccines.

The mucous membrane lining the gut, lung and urogenital tract, is collectively referred to as the mucosa and vaccines delivered this way, for example orally or intranasally are described as mucosal. Mucosal vaccines do produce their own challenges. A balance needs to be struck at these surfaces between tolerance of benign antigens and organisms and rapid defence against pathogenic ones. These difficulties can be overcome, for example in the poliomyelitis vaccine, and the challenge is to exploit the immunoregulation of the mucosal immune system to develop effective vaccines (Olszewska and Openshaw, 2004).

## 4. Development of plant vaccines

Plant vaccines have several advantages: they are cheap; the cost of production being limited to the cost of growing the specific plant, and yield is easily increased by increasing the acreage (Ma, 2000). Plants can be grown on site reducing the need for costly refrigerated transport and storage (Chargelegue et al., 2001; Streatfield et al., 2001). A plant vaccine may also be easier to administer.

Some of the initial work in the field of plant vaccines was performed by Charles Arntzen's group at Texas A & M in the USA. Initially they worked on the expression of Hepatitis B surface antigen (HbsAg) in tobacco (Mason et al., 1992). They found that if expressed as a transgene from the tobacco nuclear genome, the plant HbsAg was similar to yeast-derived HbsAg when used in parenteral immunisation studies in mice. Their data showed that a plant-derived vaccine could be as effective as one produced by the conventional cell culture methods. Haq et al. (1995) expressed the *E. coli* heat-labile enterotoxin B subunit (LT-B) at low levels in transgenic tobacco and potato. Purified plant-derived LT-B was compared with bacterial LT-B for immunogenicity. The tobacco derived LT-B was introduced into the mice by oral gavage. When the same amount of purified LT-B from bacteria and plants was administered, specific anti-LT-B serum antibody levels were similar after both administrations. In a subsequent study, LT-B derived from potatoes was tested by feeding mice raw transgenic potato tuber. However the low levels of expression meant that relatively large amounts of potato needed to be consumed to elicit a response. This immunisation strategy induced both local IgA and serum IgG anti Lt-B responses. As the authors state, this work: 'demonstrates the feasibility of using transgenic plants as expression and delivery systems for oral vaccines' (Haq et al., 1995). Nevertheless the authors acknowledged that the low amount of recombinant protein as a percentage of total plant protein was a limiting factor.

Thus far oral vaccination has been observed to produce specific immune responses in experimental models with several antigens including: LT-B expressed in tobacco and potato (Haq et al., 1995; Mason et al., 1998; Tacket et al., 1998); the expression of the B subunit of cholera toxin (CT-B) (Arakawa et al., 1998); HbsAg expressed in lettuce and potato (Kapusta et al., 1999; Richter et al., 2000) and Norwalk virus capsid protein in tobacco and potato (Mason et al., 1996) (For specific details see Table 1). However, until our studies on TetC, it had not been demonstrated that plant vaccines were actually protective against disease.

## 5. Chloroplast expression of TetC

One method of increasing transgene expression level in plants is to express the transgene from the chloroplast genome. This has other advantages including maternal inheritance, specific gene targeting, high copy number, removal of selectable markers, the potential use of operons and retention of the gene product in the plastid (Maliga, 2002).

In recent work we tested the feasibility of expressing TetC in the tobacco chloroplast (Tregoning et al., 2003). Varying levels of expression were achieved by altering two different factors whilst keeping the promoter (that of the 16S rRNA operon) constant: the codon usage of the gene and the 5′ untranslated region of the gene. Both factors had an effect on the level of gene expression. Altering the gene to being more AT-rich doubled the expression from 10 to 20% total soluble protein (TSP). The reason for this is possibly the prokaryotic root of the chloroplast genome. This effect has also been seen in the expression of the bar gene (Lutz et al., 2001). Changing the 5′ UTR from the *rbcL* UTR to the T7 gene 10 5′ UTR also doubled the expression level of TetC; this has also been seen in the expression of the *EPSPS* gene (Ye et al., 2001). The reason that this has an effect is possibly more efficient loading of mRNAs onto the ribosomes (Maliga, 2003). The alteration of gene expression levels is important for optimising the characteristics of the transformed plants since too high an expression level of transgene can be detrimental to the plant.

Importantly we were able to show that TetC expressed in tobacco chloroplasts was able to induce an immune response in mice (Tregoning et al., 2003). Both oral and nasal immunisation were performed. Oral vaccination with TetC produced a high titre of anti-TetC antibodies, which was of particular note given the

Table 1
Expression of potential vaccine antigens in plants. The table shows the source of the antigen and the target plant as well as the total amount of protein expressed. It also briefly summarises whether any immunological data was generated using the transgenic plant

| Source of protein | Protein/peptide | Plant | Maximum expression as a % of total soluble protein (TSP) | Immunology | Reference |
|---|---|---|---|---|---|
| Enterotoxigenic *Escherichia coli* | Heat labile enterotoxin B subunit (LT-B) | Tobacco | <0.01% TSP | Low level systemic and local antibody production following oral gavage. | (Haq et al., 1995) |
| Enterotoxigenic *E. coli* | LT-B | Potato | 0.19% TSP | Low level systemic and local antibody following feeding. | (Haq et al., 1995) |
| Enterotoxigenic *E. coli* | LT-B | Tobacco Chloroplast | 2.5% TSP | None tested. | (Kang et al., 2003) |
| Enterotoxigenic *E. coli* | LT-B | Maize kernels | 8.7% endosperm | None tested. | (Chikwamba et al., 2003) |
| *Vibrio cholerae* | Cholera toxin B-subunit (CT-B) | Potato | 0.30% TSP | Local and systemic antibodies developed following feeding. | (Arakawa et al., 1998) |
| *Vibrio cholerae* | CT-B | Tobacco Chloroplast | 4.1% TSP | None tested. | (Daniell et al., 2001) |
| Hepatitis B virus | Hepatitis-B surface antigen | Tobacco | <0.01% TSP | None tested. | (Mason et al., 1992) |
| Hepatitis B virus | Hepatitis-B surface antigen | Potato | <0.01% fresh weight | Antibodies produced following feeding of potatoes to mice. | (Richter et al., 2000) |
| Norwalk virus | Capsid protein (NVCP) | Tobacco | 0.23% TSP | Low titre serum IgG in mice following feeding. | (Mason et al., 1996) |
| Norwalk virus | NVCP | Potato | <0.001% TSP | Human trial, 19 of 20 volunteers developed an immune response of some kind, although the level of serum antibody increases was modest. | (Tacket et al., 2000) |
| Rabies virus | Glycoprotein | Tomato | 1.00% TSP | None tested. | (McGarvey et al., 1995) |
| Foot and mouth disease virus | VP1 | Alfalfa | Not Given | Induction of a protective systemic antibody response. | (Wigdorovitz et al., 1999) |
| *Clostridium tetani* | TetC | Tobacco Chloroplast | 25% TSP | Protective antibodies produced following intranasal immunisation, antibodies raised by feeding. | (Tregoning et al., 2003) |

general difficulties of getting any immune response with oral vaccination in the past. However, compared to oral administration of the antigen, nasal immunisation was more potent, for a smaller dose administered. The specific anti-TetC antibody response was high enough in the nasally immunised mice to provide protection against a lethal tetanus toxin challenge introduced subcutaneously. This protection is mediated by CD4+ T cell driven B cell antibody production. More importantly intranasal immunisation provides a more beneficial response than oral vaccination, requiring less immunogen, no adjuvant, no booster and inducing protection at multiple mucosal sites. Nasal vaccination of plant-derived TetC therefore represents an important step towards a suitable vaccine for the developing world.

## 6. Future work

Our studies have demonstrated unambiguously that a chloroplast expressed vaccine antigen can be an effective immunogen and protect against a lethal toxin challenge (Tregoning et al., 2003). In essence the initial phase of development of plant vaccines is over. It has been shown that they are a viable option in experimental plants. A number of other issues now need to be addressed.

The next important step in their development will be the selection and development of a vaccine in an appropriate plant for human use. For an effective oral plant vaccine, expressed from the chloroplast, the plant would need to be edible, plastid rich, be easy to manipulate genetically, have a sequenced chloroplast genome, be easy to grow where the vaccine is required, be edible raw and be non perishable. Crop plants that seem to fit these requirements are tomatoes, spinach and maize. Tomato has the advantage that chloroplast transgenics have been achieved using it (Ruf et al., 2001). However, a plant vaccine, especially if it were delivered nasally does not need to be expressed in an edible plant; a non-toxic plant such as alfalfa could be used. In either case, work needs to be done in order to express vaccine antigens in a plant system that could then be used in a full-scale human trial.

Further work also needs to be done to find more antigens that can be used to protect against other diseases when administered mucosally. One potential problem with the development of plant vaccines is whether there will be the development of an adverse reaction to plant proteins that are normally not immunogenic when they are introduced at the same time as vaccine antigens. Another area that has yet to be investigated is whether the plant itself has an effect on the immune response. There is data that suggests that some plant chemicals have an effect, for example nicotine has been shown to decrease inflammatory cytokines (Matsunaga et al., 2001) and plant lectins can have a mucosal adjuvant effect (Lavelle et al., 2001).

Plant vaccines have now been developed past an idea into a working possibility. What is now required is further research into moving them from the lab to the hospital.

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