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Immunogenicity of a recombinant coronavirus spike glycoprotein expressed in transgenic plants

Summary

Recently, it has been demonstrated that plants offer the possibility of producing low cost subunit vaccines that can be either parenterally or orally administered. Here we review data we obtained on the immunological response elicited by two recombinant versions of the glycoprotein S from the swine transmissible gastroenteritis coronavirus (TGEV) expressed in transgenic plants. Arabidopsis or potato plants were genetically transformed with cDNAs constructs encoding the N-terminal domain (aa residues 1–750) or the full-length glycoprotein S of TGEV, responsible for the neutralizing antibody induction against the virus, under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. Genomic DNA and mRNA analysis of leaf extracts from transformed plants demonstrated the incorporation of the foreign cDNA into the plant genomes as well as their transcription. Expression of recombinant polypeptides was observed in most transgenic plants by ELISA using specific antibodies. Mice immunized either parenterally with leaf extracts from transgenic Arabidopsis plants or, more interestingly, fed with potato tubers, developed antibodies that specifically reacted with TGEV in ELISA, immunoprecipitated the glycoprotein S and in some cases neutralized the virus infectivity. From the above results, we conclude that transgenic plants expressing glycoprotein S polypeptides may be potentially used as a source of recombinant antigen for vaccine production.

Key words

Genetically modified plant · Vaccine · Coronavirus · TGEV

Swine transmissible gastroenteritis virus (TGEV) is the causative agent of acute diarrhea of newborn piglets that provokes high rate mortalities in affected farms. Protective immunity against this disease has to be developed in pregnant sows in order to confer passive protection to the piglets through colostrum and milk. Neutralizing antibodies against the virus are directed mainly to glycoprotein S [1, 2], and relevant epitopes in neutralization have been mapped into the N-terminal domain of this protein [3]. Four major antigenic sites have been described in glycoprotein S, of which site A is the immunodominant [4, 5, 6]. Only vectors expressing the glycoprotein S with tropism that favored antigenic presentation in the mucosal surfaces have conferred protection against TGEV in suckling piglets [7].

Durch Expression immunogener Proteine von Krankheitserregern in Pflanzen könnten essbare Impfstoffe in grosser Menge und zu geringen Kosten hergestellt werden.

The concept of vaccine production in transgenic plants was first introduced by Mason and co-workers in 1992 [8]. Proteins involved in protective immune response can be produced at a low cost and easily purified from plant extracts for parenteral inoculation. In addition, oral immunization by edible vaccines produced in transgenic plants could stimulate immune responses at the portal of entrance for many pathogens, facilitating the design of large-scale immunization programs. The presence of specific antigens in plants, even at low levels, can raise immune reactions by the oral route comparable to conventional vaccines [9, 10, 11].

Today, hepatitis B surface antigen [12], *E. coli* heat-labile enterotoxin (LT-B) antigen [9], Norwalk virus capsid protein [8], VP1 antigen from foot and mouth disease virus [11, 13], Cholera toxin B subunit [14], glycoprotein S from swine transmissible gastroenteritis virus [15], and VP60 from rabbit hemorrhagic disease virus [16] are vaccine antigens expressed in transgenic plants and tested for the immune response elicited in immunized animals. Here, we review the data obtained about the feasibility of expressing the glycoprotein S

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Immunisierende Wirkung eines rekombinanten Coronavirus-Proteins aus transgenen Pflanzen

Zusammenfassung

Kürzlich wurde gezeigt, daß es mit Hilfe von Pflanzen möglich ist, kostengünstig Unter-einheiten von Vakzinen zu produzieren, die entweder parenteral oder oral angewendet werden können. Hier berichten wir über die immunologische Reaktion, die durch zwei in Pflanzen exprimierte, rekombinante Versionen des Glycoproteins S des Coronavirus der transmissiblen Gastroenteritis des Schweines (TGEV) hervorgerufen worden ist. Arabidopsis- oder Kartoffel-Pflanzen wurde gentechnisch verändert mit cDNA-Konstrukten, die für die N-terminale Domäne (Aminosäuren 1 bis 750) oder für die gesamte Länge des Glycoprotein S aus TGEV kodieren und unter der Kontrolle des 35S Promoters des Cauliflower Mosaic Virus (CaMV) stehen; das Glycoprotein S bewirkt die Antikörper-Induktion gegen TGEV. Die Analyse von genomischer DNA und von mRNA aus Blatt-extrakten der transformierten Pflanzen be- weisen sowohl die Integration der Fremd-DNA in das Pflanzen-Genom als auch ihre Transkription. Die Expression der rekombin- anten Polypeptide wurde in den meisten transgenen Pflanzen im ELISA-Test durch spezifische Antikörper nachgewiesen. Mäu- se, die parenteral mit Blattextrakten von transgenen Arabidopsis-Pflanzen oder, weit interessanter, oral mittels transgener Kartof- felknollen immunisiert worden waren, ent- wickelten Antikörper, die im ELISA-Test spezifisch mit TGEV reagierten, das durch den Virus induzierte Protein immunpräzipi- tierten und in einigen Fällen die Virusinfek- tion aufhoben. Aufgrund dieser Ergebnisse sind wir der Meinung, daß transgene Pflan- zen, welche Glycoprotein-S-Polypeptide ex- primieren, möglicherweise benutzt werden können, um rekombinantes Antigen zur Her- stellung entsprechender Vakzine zu produ- zieren.

Schlüsselwörter

Gentechnisch veränderte Pflanze · Vakzine · Coronavirus · TGEV

from TGEV in two different transgenic plants, as well as the antigenicity and immunogenicity of the plant-derived protein administered either parenteral- ly or orally to mice. The protein S is an excellent model for developing oral vac- cines against enteric pathogens of mam- mals because of its immunogenicity and resistance to degradation in the gut.

Results

Arabidopsis and potato plant transformation

The binary pRoK I and pRoK II recom- binant plasmids (Fig. 1), carrying a cDNA coding for the N-terminal region or the full-length glycoprotein S respec- tively, were obtained by subcloning the corresponding sequences from previ- ously obtained constructs into pRoK 2 plasmid [17]. Recombinant pRoK de- rived plasmids allow selection of trans- formants on media containing kanamy- cin and stable integration into nuclear chromosomal DNA from the plant. PRoK 2 uses the CaMV 35S promoter to drive nominally constitutive transcrip- tion of the cloned genes.

Plant transformation with pRoK I and II was carried out as described [16, 18] by *A. tumefaciens*-mediated trans- formation. Potato plants were trans- formed only with pRoK I plasmid. The transgenic plants resistant to the selec- tive medium appeared similar in mor- phology to non-transgenic plants. More than 20 different lines of arabidopsis transformants containing each con- struct were self-pollinated to obtain F₂ lines. Fifteen different transformed po- tato clones were also obtained with pRoK I. All lines from arabidopsis and potato were positive when screened for the presence of the recombinant genes by PCR analysis (Fig. 1, A1 and B1). Most plants harboring recombinant genes showed specific transcription of foreign genes by RT-PCR analysis (Fig. 1, A2).

Recombinant glycoprotein S expression in transgenic plants

The presence of the recombinant poly- peptides in the plants harboring and ex- pressing the foreign genes was investi- gated by ELISA and Western blot using an anti-TGEV polyclonal serum. Results demonstrated that leave or potato ex-

tracts from all analyzed plants resulted positive in ELISA (Fig. 1, A3 and B2). However, no specific reaction in Western blot was detected in any of the plant ex- tracts analyzed (data not shown), prob- ably due to the low levels of recombinant protein expression and to the conforma- tional nature of most of the immunodo- minant epitopes present in this protein.

From a titration ELISA using differ- ent virus dilutions and a monospecific anti-glycoprotein S antibody, we have found that about 30–60 µg of soluble leave or tuber protein contain a glyco- protein S antigenic mass equivalent to that contained in 0.02 µg of purified TGEV. The percentage of the total solu- ble protein corresponding to recombi- nant glycoprotein S polypeptides accu- mulated in the tissues of plant transfor- mants could represent between 0.06 to 0.03% of the total soluble protein.

Immunogenicity of the plant-derived glycoprotein S polypeptides

Leave extracts from transgenic ara- bidopsis plants expressing the N-termi- nal or full-length glycoprotein S, were used to immunize mice. A control mouse was immunized with a leave extract from a plant transformed with pRoK 2 plasmid. After three immunization dos- es (40 µg of total protein per animal per injection), the specificity of mice sera was tested by an ELISA using purified TGEV as antigen. Figure 2 (A1) shows that all sera reacted with the virus show- ing, as expected, different titers. Mice de- veloped specific antibodies against gly- coprotein S after 2 immunization doses (Fig. 2, A2).

Durch Injektion von Pflanzenextrakt oder Verfütterung transgener Pflanzen konnte in Mäusen eine spezifische Antikörperbildung gegen das TGEV-Glykoprotein S induziert werden.

Sera from all immunized mice were test- ed in a TGEV neutralization assay. Both glycoprotein S polypeptides produced in transgenic plants elicited virus neutral- izing antibodies (neutralization indexes between 2.2 and 3.5; Fig. 2, A3). Serum from a nonimmunized mouse or from the mouse immunized with the plant transformed with pRoK 2 plasmid did not show virus neutralization activity (Fig. 2, A3). Transgenic potato tuber ex-

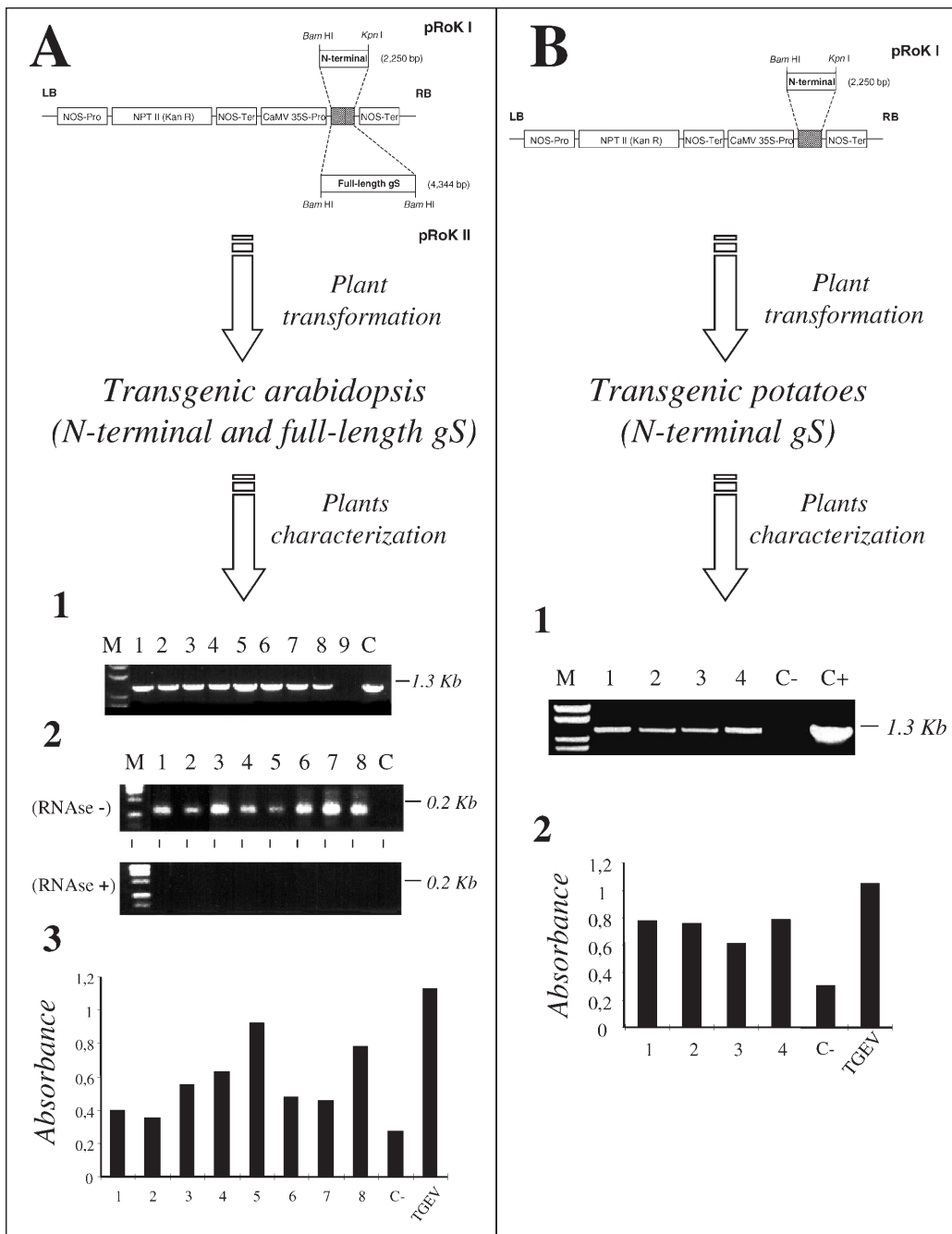


Fig. 1 ▲ Characterization of transgenic arabidopsis (A) and potato plants (B). In this figure the schematic structure of the binary plasmids pRoK I and II used for *Agrobacterium*-mediated plant transformation is represented.

The DNA sequence encoding for the full-length or N-terminal domain of glycoprotein S from TGEV was cloned downstream of the CaMV 35S promoter in recombinant pRoK plasmids, followed by the nopaline synthase (NOS) terminator. These plasmids contain the left (LB) and right (RB) borders of transferred DNA that demarcates the sequences that are incorporated into the plant genome. Panel A. (1) Presence of the recombinant genes in representative transgenic arabidopsis plants detected by PCR. Plants 1 to 4 transformed with pRoK I, 5 to 8 with pRoK II, 9 with pRoK 2. C represents the control amplification of the same DNA fragment from pRoK 2. (2) Foreign gene transcription in representative transformed plants analyzed by RT-PCR, in presence or absence of RNase. (3) Detection of glycoprotein S polypeptides in protein extracts from transgenic plants by ELISA. Absorbance readings were obtained from arabidopsis leaves extracts transformed with pRoK I (1 to 4) and pRoK II (5 to 8). Positive control is purified virus (TGEV) and negative control is a plant transformed with pRoK 2 plasmid (C-). Panel B. (1) Presence of the recombinant gene (encoding the globular part of the glycoprotein S) in representative transgenic potatoes (1 to 4) detected by PCR. Plants transformed with pRoK 2 (C-) and amplification of the same DNA fragment from pRoK I (C+) is also shown. (2) Detection of N-terminal domain of glycoprotein S in protein extracts from transgenic plants by ELISA. Absorbance readings were obtained from potato tuber extracts transformed with pRoK I. Positive control is purified virus (TGEV) and negative control is a plant transformed with pRoK 2 plasmid (C-).

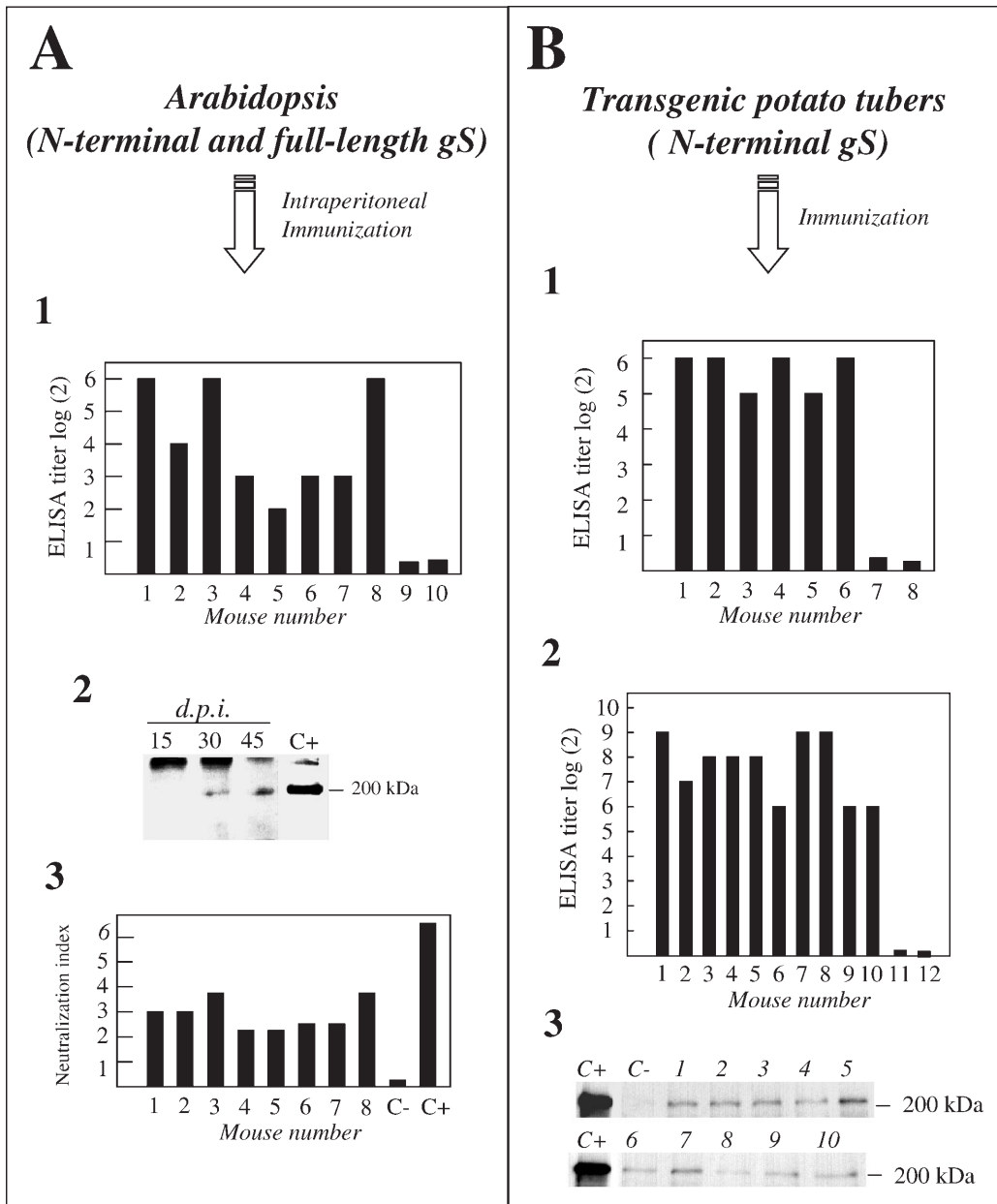


Fig. 2 ▲ Antibody responses to the plant-derived glycoprotein S polypeptides in immunized mice. **Panel A.** (1) ELISA titers of sera from mice inoculated with arabidopsis plant extracts expressing the N-terminal (mice 1 to 4) or the full-length glycoprotein S (mice 5 to 8), using purified TGEV as antigen.

Mice 9 and 10 represent ELISA titers of two mice immunized with a pRoK 2 transformed plant extract. (2) Immunoprecipitation of glycoprotein S induced by TGEV by sera from an immunized mouse after one (15), two (30) or three (45) immunization doses with transgenic plant extracts. A pool of monoclonal antibodies against glycoprotein S was used as control (C+). (3) Neutralization indexes of sera from immunized mice with plant extracts expressing the N-terminal (mice 1 to 4) or full-length glycoprotein S (mice 4 to 8). The neutralization index is defined as the ratio between the log of virus titer in the presence of a control mouse serum and sera from mice immunized with transgenic plants expressing the antigens (1 to 8), or transformed with pRoK 2 (C-). A rabbit anti-TGEV serum showing high neutralization titer was also used as positive control (C+). ELISA and neutralization index values are the mean of three independent experiments. **Panel B.** (1) ELISA titers of sera from mice inoculated intraperitoneally with potato tuber extracts expressing the N-terminal domain of glycoprotein S (mice 1 to 6), using purified TGEV as antigen. Mice 7 and 8 represent ELISA titers of two mice immunized with a pRoK 2 transformed plant extract. (2) ELISA titers of sera from orally immunized mice with transgenic potato tubers expressing the N-terminal domain of glycoprotein S (mice 1 to 10). Mice 11 and 12 represent ELISA titers of two mice immunized orally with a pRoK 2 transformed potato. (3) Immunoprecipitation of glycoprotein S expressed by a recombinant vaccinia virus by antibodies present in sera from orally immunized mice with potato tubers expressing the N-terminal domain of the glycoprotein. A pool of specific monoclonal antibodies was used as a control positive serum (C+) and a serum from a mouse immunized with potato tubers transformed with pRoK 2 was used as a negative control (C-)

tracts also induced specific antibodies in immunized mice using the same immunization protocol (Fig. 2, B1).

Finally, to analyze the oral immunogenicity of glycoprotein S expressed in potato plants, a group of mice were fed with transgenic potato tubers expressing the N-terminal domain of glycoprotein S. The animals were fed three times per week with two gram of transgenic potatoes during a two months period. Sera from these animals were analyzed by ELISA showing a specific antibody response against the plant-derived glycoprotein S, indicating the oral immunogenicity of this protein when expressed in plants (Fig. 2, B2). As expected, no antibody response was detected in mice fed with potato tubers not expressing the glycoprotein S. The specificity of sera reactivity in ELISA with the virus-induced protein was confirmed by immunoprecipitation of glycoprotein S expressed by a recombinant vaccinia virus (Fig. 2, B3).

Discussion

In this report we show that full-length or the globular part (N-terminal domain) of TGEV spike protein (glycoprotein S) expressed in transgenic plants retained the antigenic properties and elicited neutralizing antibodies when used to immunize animals. Expression in eukaryotic hosts is required for antigenic determinants that are dependent on glycosylation. Of the three major antigenic sites defined on glycoprotein S involved in the induction of TGEV-neutralizing antibodies, sites A and B are complex, conformational, and glycosylation dependent. Site D can be represented by synthetic peptides, although glycosylation has a minor effect on its conformation [19]. Several genetically engineered vaccines using prokaryotic vectors have failed against TGEV. Glycoprotein S expressed at high levels in *Escherichia coli* and used to inoculate animals did not induce neutralizing antibodies or confer protection in vivo [20].

Plant cells present differences in protein glycosylation with respect to animal cells that could determine the loss of antigenic determinants in antigens expressed in transgenic plants. Glycosylation in plants may differ in the extent of glycosylation, processing, or both of

N-linked oligosaccharide side chains [21]. Furthermore, the complex glycans of plants are often smaller than those of animals in part due to the absence of sialic acid [21]. The only precedent of a glycoprotein expressed in plants for vaccine development is the glycoprotein G of rabies virus [22]. This protein expressed in tomato plants showed a molecular mass about four and six kDa less than that obtained from virus-infected cells but still larger than the unglycosylated polypeptide chain [22]. The molecular mass of glycoprotein S expressed in *Arabidopsis thaliana* could not be determined because we were not able to detect the recombinant protein in Western blotting. However, antigenic determinants with strong dependence of glycosylation seem to be preserved since the plant-derived antigens induced neutralizing antibodies in immunized animals, indicating that critical antigenic sites are at least in part correctly glycosylated in plants.

Die beschriebenen Versuchsergebnisse belegen die prinzipielle Machbarkeit einer Expression viraler Glykoprotein-Polypeptide in Pflanzen und deren Verwendung zur Immunisierung.

The above described work demonstrates the feasibility of expressing glycoprotein S polypeptides in plants. Because the site of insertion of the transferred DNA into the cellular chromosomal DNA is random, different levels of protein expression in independent transformants are expected. We obtained expression levels similar to that described with equivalent constructs expressing hepatitis B surface antigen or rabies virus glycoprotein [8, 22]. More recently, expression levels of Norwalk virus capsid protein in tobacco have been shown to be higher than the above mentioned antigens (up to 0.23% of total soluble protein) [10]. We have not found significant differences in foreign antigen plant expression between the two forms of glycoprotein S studied or among the different plants transformed. The use of different promoters, the use of plant-derived leader sequences and signal peptides, and mainly the modification of the codon usage of this protein could improve expression levels in plants.

Outlook

The demonstration that many proteins from pathogens, including some expressed in transgenic plants [9, 10, 11], are immunogenic when administered orally, encourage the study of other antigens expressed in plants to develop edible vaccines. Glycoprotein S from TGEV is an interesting model since this protein is resistant, at least when incorporated into the viral particle, to the gut degradation. In addition, the protective immune responses against TGEV have to be stimulated at the mucosal surfaces in order to induce secretory and lactogenic immunity [4, 23, 24, 25]. In the above described work, we have demonstrated that the N-terminal domain of glycoprotein S expressed in potato tubers is immunogenic when mice were fed with the transgenic potatoes [26]. In conclusion, these results make reasonable to extend similar immunological studies in pregnant sows, in order to test the lactogenic immunity conferred to suckling piglets by the plant-derived glycoprotein S.

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