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Expression of Immunogenic Glycoprotein S Polypeptides from Transmissible Gastroenteritis Coronavirus in Transgenic Plants

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The use of transgenic plants as vaccine production systems was described recently. We report 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 plants were genetically transformed with cDNAs constructs encoding either the N-terminal domain (amino acid 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 analyses of leaf extracts from transformed plants demonstrated the incorporation of the foreign cDNA into the arabidopsis genome, as well as their transcription. Expression of recombinant polypeptides were observed in most transgenic plants by ELISA using specific antibodies. Mice immunized with leaf extracts from transgenic plants developed antibodies that reacted specifically with TGEV in ELISA, immunoprecipitated the virus-induced protein, and neutralized the virus infectivity. From these results, we conclude that transgenic plants expressing glycoprotein S polypeptides may possibly be used as a source of recombinant antigen for vaccine production.

INTRODUCTION

Swine-transmissible gastroenteritis virus (TGEV) is the causative agent of acute diarrhea of newborn piglets that provokes high mortality rates in affected farms. Protective immunity against this disease must be developed in pregnant sows to confer passive protection to the piglets through colostrum and milk. Neutralizing antibodies against the virus are directed mainly to glycoprotein S (Garwes *et al.,* 1978; Jimenez *et al.,* 1986), and relevant epitopes in neutralization have been mapped into the N-terminal domain of this protein (Correa *et al.,* 1988). Four major antigenic sites have been described in glycoprotein S, of which site A is the immunodominant (De Diego *et al.,* 1992; Delmas *et al.,* 1990; Sánchez *et al*., 1990). Glycoprotein S from TGEV has been expressed using different vectors with tropism that favored antigenic presentation in the mucosal surfaces (Smerdou *et al.,* 1996; Torres *et al.,* 1995). These vaccination approaches promoted systemic and mucosal antibody induction and, in the case of adenovirus vector, conferred protection to suckling piglets (Torres *et al.,* 1996).

The development of genetic transformation technology in plants has made possible the expression of foreign genes in different plant species, making reasonable the idea of using plants as bioreactors to produce recombinant proteins. The concept of vaccine production in transgenic plants was first introduced by Mason *et al.* in 1992. Proteins involved in protective immune response can be produced at a low cost and easily purified from plant extracts for parental inoculation. In addition, oral immunization by edible vaccines produced in transgenic plants could stimulate immune responses at the portal entrance of many pathogens, facilitating the design of large-scale immunization programs. The presence of specific antigens into plants, even at low levels, can raise by the oral route immune reactions comparable to those raised by conventional vaccines (Haq *et al.,* 1995; Mason *et al.,* 1996).

Hepatitis B surface antigen (Thanavala *et al.,* 1995), *Escherichia coli* heat-labile enterotoxin (LT-B) antigen (Haq *et al.,* 1995; Tacket *et al.,* 1998), Norwalk virus capsid protein (Mason *et al.,* 1996), VP1 antigen from foot and mouth disease virus (Carrillo *et al.,* 1998), and cholera toxin B subunit (Arakawa *et al.,* 1998) are the vaccine antigens expressed in transgenic plants and tested for the immune response elicited in immunized animals. Additionally, rabies virus glycoprotein was expressed in transgenic tomatoes, but the immune response induced by administration of these plants to animals was not tested (McGarvey *et al.,* 1995). In the present study, we investigated the feasibility of expressing the glycoprotein S from TGEV in transgenic plants, as well as the antigenicity and immunogenicity of the plant-derived protein. The S protein is an excellent model for developing oral

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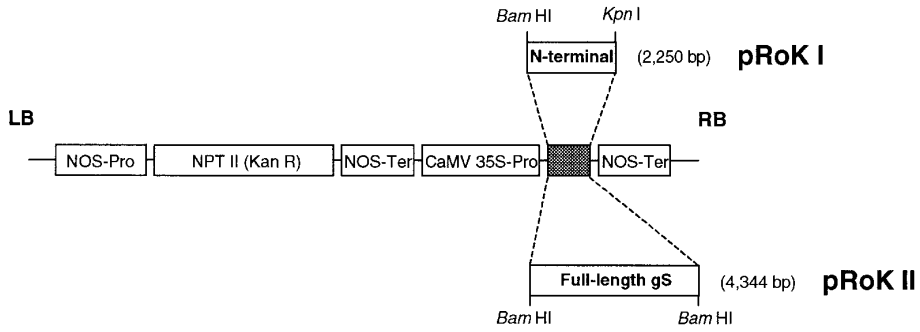

FIG. 1. Schematic structure of the binary plasmids pRoK I and II used for *Agrobacterium*-mediated plant transformation. The DNA sequences encoding for the full-length or N-terminal domain of glycoprotein S from TGEV are cloned downstream of the CaMV 35S promoter in recombinant pRoK plasmids, followed by the nopaline synthase (NOS) terminator. These plasmids contain the left (LB) and right (RB) borders of transferred DNA that demarcate the sequences that are incorporated into the plant genome.

vaccines against enteric pathogens of mammals because of its immunogenicity and resistance to degradation in the gut.

RESULTS

Plasmid construction and selection of transgenic plants

The binary pRoK I and pRoK II recombinant plasmids (Fig. 1), carrying a cDNA coding for the N-terminal region or the full-length glycoprotein S respectively, were obtained by subcloning the corresponding sequences from previously obtained constructs. Recombinant pRoK plasmids allow selection of transformants on media containing kanamycin and stable integration into nuclear chromosomal DNA from the plant. pRok uses the cauliflower mosaic virus 35S (CaMW 35S) promoter for nominally constitutive transcription of the cloned genes.

Plant transformation with pRoK I and II was carried out as described in Materials and Methods by *Agrobacterium tumefaciens*-mediated transformation. The transgenic plants resistant to the selective medium appeared similar in morphology to the nontransgenic arabidopsis plants. More than 20 different lines of transformants containing each construct were obtained and self-pollinated to obtain F2 lines. All lines were positive when screened for the presence of the recombinant genes by polymerase chain reaction (PCR) analysis (Fig. 2A).

Most plants harboring recombinant genes showed specific transcription of foreign genes by reverse transcription (RT)-PCR analysis (Fig. 2B). To rule out the possibility of amplification of contaminant DNA sequences present in the RNA preparations, we treated the purified RNA with ribonuclease before foreign gene amplification by using *Taq* polymerase. No amplified DNA fragments were detectable under those conditions, assessing the RNA dependence of the reaction (Fig. 2B).

Recombinant protein expression in transgenic plants

The presence of the recombinant polypeptides in the plants harboring and expressing the foreign genes was investigated in four plants of each construct, selected to be analyzed by ELISA and Western blotting using an anti-TGEV polyclonal serum. Results demonstrated that leaf extracts from all selected plants were positive on ELISA (Fig. 3). However, no specific reaction on Western blotting was detected in any of the plant extracts analyzed (data not shown), probably due to the low levels of recombinant protein expression and to the conformational nature of most of the immunodominant epitopes present in this protein.

From a titration ELISA using different virus dilutions and a monospecific anti-glycoprotein S antibody, we found that ~30–60 μg of soluble leaf protein contains a glycoprotein S antigenic mass equivalent to that contained in 0.02 μg of purified TGEV. The percentage of the total soluble protein corresponding to recombinant glycoprotein S polypeptides accumulated in the leaves of arabidopsis transformants could represent 0.06–0.03% of the total soluble leaf protein.

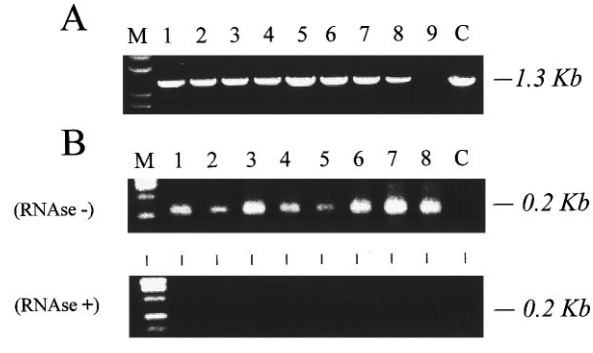

FIG. 2. Characterization of transgenic plants transformed with pRoK I (1–4), pRoK II (5–8), or pRoK 2 (9) plasmids. (A) Presence of the recombinant genes in representative transgenic arabidopsis plants detected by PCR. C, control amplification of the same DNA fragment from pRoK II. (B) Foreign gene transcription in representative transformed plants analyzed by RT-PCR. Samples were treated or not with RNase to assess the DNA specificity of the reactions. C, same analyses in arabidopsis plants transformed with pRoK 2 plasmid.

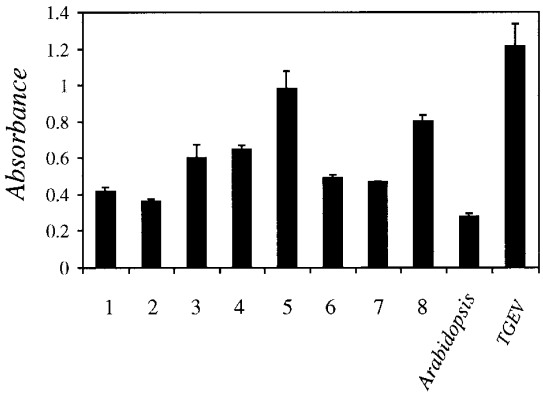

FIG. 3. Detection of N-terminal (1–4) and full-length (5–8) glycoprotein S polypeptides in protein extracts from transgenic plant leaves by ELISA. The figure shows the mean ± SE of the absorbance readings obtained in three independent assays. Positive control is purified virus (TGEV), and negative control is a plant transformed with pRoK 2 plasmid (*Arabidopsis*).

Antibody induction by plant-derived recombinant proteins

Leaf extracts from transgenic plants expressing the N-terminal (plants 1–4) or full-length glycoprotein S (plants 5–8) were used to immunize mice. A control mouse was immunized with a leaf extract from a plant transformed with pRok2 plasmid. After three immunization doses, the specificity of mice sera was tested by an ELISA using purified TGEV as antigen. Figure 4A shows that all sera reacted with the virus showing, as expected, different titers. A kinetic of antibody induction in an immunized mouse (number 3) was studied by immunoprecipitation of glycoprotein S induced by TGEV in infected ST cells. This mouse serum immunoprecipitated specifically the virus protein after two immunizations (Fig. 4B).

Finally, sera from all immunized mice were tested in a TGEV neutralization assay. Both glycoprotein S polypeptides produced in transgenic plants elicited virus-neutralizing antibodies (neutralization indexes of 2.2–3.5; Fig. 4C). Serum from a nonimmunized mouse (not shown) or from the mouse immunized with the plant transformed with pRoK 2 plasmid did not show virus neutralization activity (Fig. 4C).

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 (Gebauer *et al.,* 1991). 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* (Hu *et al.,* 1987).

Plant cells present differences in protein glycosylation with respect to animal cells that could determine the lose 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 (Faye *et al.,* 1993). Furthermore, the complex glycans of plants are often smaller than those of animals, in part due to the absence of sialic acid (Faye *et al.,* 1993). The only precedent of a glycoprotein expressed in plants for vaccine development is the glycoprotein G of rabies virus (McGarvey *et al.,* 1995). This protein expressed in tomato plants showed a molecular mass ~4 and ~6 kDa less than that obtained from virus-infected cells but still larger than the protein size predicted for the unglycosylated polypeptide chain (McGarvey *et al.,* 1995). The molecular mass of glycoprotein S expressed in *Arabidopsis thaliana* could not be determined because we were not able to detect the recombinant protein on Western blotting. However, antigenic determinants with strong dependence of glycosylation seem to be preserved because the plant-derived antigens induced neutralizing antibodies in immunized animals, indicating that critical antigenic sites are at least in part correctly glycosylated in plants.

This 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 (Mason *et al.,* 1992; McGarvey *et al.,* 1995). 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; Mason *et al.,* 1996). We have not found significant differences in foreign antigen plant expression between the two forms of glycoprotein S studied. 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.

The demonstration that many proteins from pathogens, including some expressed in transgenic plants (Haq *et al.,* 1995; Mason *et al.,* 1996), are immunogenic when administered orally, encourages the study of other antigens expressed in plants to develop edible vaccines.

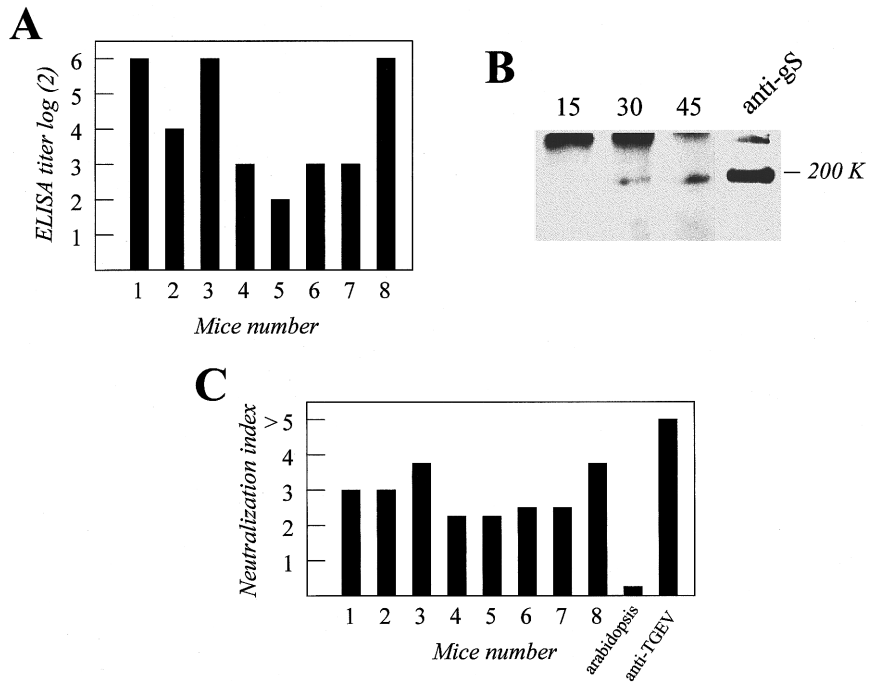

FIG. 4. Antibody responses to the plant-derived glycoprotein S polypeptides in parenterally inoculated mice. (A) ELISA titers of sera from mice inoculated with plant extracts expressing the N-terminal (mice 1–4) or the full-length glycoprotein S (mice 5–8). Titers are referred to the ELISA values obtained with the serum from a mouse immunized with a pRoK 2 transformed plant extract. (B) Kinetic of antibody induction against glycoprotein S in a mouse (mouse 3) after one, two, and three immunization doses (15, 30, and 45 days postinoculation, respectively) analyzed by immunoprecipitation of the glycoprotein S induced by TGEV in infected cells. An anti-glycoprotein S (anti-gS) serum was used as immunoprecipitation control. (C) Neutralization indexes of sera from immunized mice with plant extracts expressing the N-terminal (mice 1–4) or full-length glycoprotein S (mice 4–8). 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 (1–8) or transformed with pRoK 2 (*Arabidopsis*). A rabbit anti-TGEV serum (anti-TGEV) showing high neutralization titer was also used as positive control. ELISA and neutralization index values are the mean of three independent experiments.

Glycoprotein S from TGEV is an interesting model because this protein is resistant, at least when incorporated into the viral particle, to gut degradation. In addition, the protective immune responses against TGEV have to be stimulated at the mucosal surfaces to induce secretory and lactogenic immunity (De Diego *et al.,* 1992, 1993; Saif and Bohl, 1979; Wesley *et al.,* 1988). Once we have determined the feasibility of expressing immunological active polypeptides from TEGV glycoprotein S in plants, studies on the immune response of plant-derived glycoprotein S polypeptides in pigs are necessary.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (Heynh, ecotype Columbia) were sown in pots containing a mixture of universal substrate and vermiculite (3:1). To synchronize germination, pots were placed at 4°C for 48 h in darkness and then transferred to a growth chamber at 20°C with a 16-h photoperiod. Irrigation was carried out with distilled water and, occasionally, with a mineral nutrient solution (Haughn *et al.,* 1986).

Production of transgenic *Arabidopsis*

A 2250-pb cDNA fragment (nucleotides 1–2250; fragment I) and a 4344-pb cDNA fragment (nucleotides 1–4344; fragment II) encoding for the N-terminal and full-length glycoprotein S from TGEV Purdue strain, respectively, were amplified by RT-PCR from viral RNA and cloned into pBacPAK9 plasmid (Clontech). The RT primers used were 5′-CCCAACTATGGTACCATCAAT AACAGC-3′ (complementary primer to nucleotides 2225–2250) and 5′-CGCGGGATCCTTAATGGACGTG-CACTTTTTC-3′ (complementary primer to nucleotides 4313–4344). Then, the cDNA was synthesized by using the primer 5′-GCGCGGATCCATGAAAAACTATTTGT-GG-3′. Subsequently, DNA fragments I and II were subcloned in the binary pRoK2 plasmid (Baulcombe *et al*., 1986) under the control of the CaMW 35S promoter, yielding the recombinant plasmids pRoK I and pRoK II, respectively (Fig. 1).

Plasmids pRoK I and pRoK II were used for arabidopsis plant transformation as described elsewhere (Bechtold *et al*., 1993) with slight modifications. *A. tumefaciens* (C58C1 strain) containing pRoK I or pRoK II plasmids was grown in 600 ml of LB medium con-

taining 50 μg/ml kanamycin until an OD_{600} value of 2 was reached. After centrifugation, bacteria were resuspended in 200 ml of 2.35 g/l Murashige and Skoog medium containing 10 g/l 6-benzilaminopurine and 5% sucrose. The 6–7-week-old plants were immersed in the *A. tumefaciens* suspension by inversion of the pots, and vacuum infiltration was performed in a vacuum chamber at 50 mb for 15 min. Infiltrated plants were rinsed with water and placed in the greenhouse until attaining maturity. Transgenic T1 seeds were selected by germination in Petri dishes containing GM [4.7 g/l Murashige and Skoog, 1% sucrose, 0.5 g/l 2-(*N*-morpholino)ethanesulfonic acid (MES), 8 g/l agar, pH 5.7] and 50 μg/ml kanamycin. Two-week-old transgenic plants were transplanted into soil and allowed to attain maturity. The plants were self-pollinated to obtain T2 plants and used for further analysis.

PCR and RT-PCR analyses

The presence of the foreign cDNA sequences in generated transgenic arabidopsis was detected by PCR. Plant extracts were prepared by macerating leaves (~10 mg) with pestle and mortar in 300 μl of a buffer containing 200 mM Tris–HCl, pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS. The resulting extract was mixed with 150 μl of 3 M CH_3COONa, pH 5.2, and incubated for 10 min at −20°C. Then, samples were centrifuged, and the DNA contained in the supernatant was precipitated and resuspended in 30 μl of TE buffer. PCR was performed on 0.5 μg of DNA with a pair of primers that specifically amplify a 1389-bp fragment of the glycoprotein S gene (sense primer, 5′-GCGCGGATCCATGAAAAACTATTTGTGG-3′; antisense primer, 5′-GCGCGGTACCCGATGTGAAGCTATTG-3′).

Glycoprotein S mRNA in transgenic plants was analyzed by RT-PCR. Total RNA from the leaves of transformed plants was isolated using the Fast RNA kit (BIO 101) according to the manufacturer's instructions. RNA was treated with 10 units of DNase–RNase free (RQ1; Promega) during 15 min at 37°C. Then, 1 μg of total RNA was diluted in 4 μl of RT buffer [250 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 375 mM KCl, and 10 mM dithiothreitol (DTT)] containing 0.5 mM concentration of each dNTP (Pharmacia), 10 units of RNasin (RNase inhibitor, human placenta; Boehringer-Mannheim), 200 units of reverse transcriptase (Moloney murine leukemia virus RT; GIBCO BRL), and 100 pM concentration of the antisense primer (5′-GCGCGGTACCAAACCAAGGTTGTACAG-3′) in a final volume of 20 μl. The mixture was incubated for 1 h at 37°C. Denaturation of RNA–cDNA hybrids and inactivation of the reverse transcriptase were done by boiling the reaction for 5 min. To the RT mixture, we added 50 pM concentration of the above mentioned sense primer and 5 units of *Taq* DNA polymerase (GIBCO BRL). This reaction specifically amplifies a 197-bp fragment of the glycoprotein S gene. Treatments of purified RNA samples with RNase (Promega) were carried out with 10 units of the enzyme for 15 min at 20°C.

Detection of glycoprotein S in transgenic plants

Proteins from leaves were obtained by homogenization of leaves in a blender with liquid nitrogen, and the resulting powder was resuspended in buffer (0.3 g of fresh wt/ml) containing 10 mM MES, pH 6, 10 mM NaCl, 5 mM EDTA, 0.6% Triton X-100, 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. The extract was filtered and centrifuged 10 min at 12,000 *g*, and the resulting supernatant was used for glycoprotein S polypeptides expression analyses.

ELISA plates were coated with 100 μl (10 μg/ml of PBS) of a mixture of two monoclonal antibodies, 6AC3 and 8DH8 (kindly provided by Dr. L. Enjuanes, Centro Nacional de Biotechnología, CSIC, Spain), recognizing the antigenic sites A and D of the glycoprotein S, respectively (Correa *et al.,* 1988). Antibodies were incubated for 12 h at 4°C, and then plates were washed and blocked 1 h at 37°C with 5% fetal bovine serum in PBS containing 0.05% Tween 20. After washing the plates, leaf proteins from transgenic plants (15 μg of total soluble protein per well, diluted in 200 μl of PBS, pH 7), containing full-length or the N-terminal domain of glycoprotein S, were added to react with the previously adsorbed antibodies in the microtiter ELISA plates during 12 h at 4°C. Plates were then washed six times with 0.05% Tween 20 in PBS, and 100 μl of rabbit anti-S protein, obtained after three immunization doses with the baculovirus-expressed N-terminal fragment of glycoprotein S and diluted at 1:100 in PBS containing 0.05% Tween 20, was added per well and left to react for 1 h at 37°C. Plates were washed again six times with PBS–Tween 20 buffer, and immunocomplexes were incubated with Protein A–peroxidase (Sigma) diluted 1:1000 in PBS–Tween 20 for 1 h at 37°C. Finally, plates were washed again, and 200 μl of a freshly prepared solution of *o*-phenylenediamine dihydrochloride (Sigma) and H_2O_2 was added. Reactions were stopped with 2 N H_2SO_4, and the absorbance was measured at 492 nm.

Induction of anti-glycoprotein S antibodies

BALB/c mice (one per arabidopsis plant) were immunized intramuscularly on days 0, 15, and 30 with leaf extract in PBS (40 μg of total protein per animal per injection) in complete Freund's adjuvant for the first inoculation and in incomplete adjuvant for the others.

Mice sera were evaluated for anti-glycoprotein S-specific antibodies by ELISA using purified TGEV as antigen. Coated ELISA plates with 100 μl of PBS, pH 7.4, containing 0.2 μg of virus were blocked as described above with

5% fetal bovine serum, and after washing of the plates six times, sera diluted 1:10 in PBS–Tween 20 were added (100 μl per well) and incubated for 1 h at 37°C. Then, plates were washed again to remove unbound antibodies, and goat anti-mouse antibodies (1:500) were added to reveal immunocomplexes. After being washed and developed with *o*-phenylenediamine dihydrochloride substrate as described above, reaction was stopped with 2 N H_2SO_4, and plates were read at 492 nm.

Immunoprecipitation of glycoprotein S by sera from a mouse after different immunization doses was carried out essentially as previously described for mouse antibodies (Bullido *et al.,* 1996). Briefly, ST cells infected with TGEV (m.o.i. 5) were incubated for 14 h, pulse labeled for 2 h with 200 μCi/ml of ^{35}S-methionine (800 Ci/mmol; Amersham International, Amersham, England)/ml, and lysed with lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.4, 1 mg/ml bovine serum albumin, and 1 mM phenylmethylsulfonyl fluoride). The lysate (10^6 cpm) was incubated with a control mouse serum (15 μl) for 1 h and precleared with a 25% (v/v) suspension of Protein G–Sepharose (Pharmacia, Sweden) in lysis buffer. The precleared ^{35}S-labeled cell extract was incubated with mice sera (15 μl) for 2 h at 4°C, and immunocomplexes were incubated with 25% suspension of Protein G–Sepharose for 1 h with gentle mixing. Beads were washed three times with lysis buffer and boiled in SDS–electrophoresis buffer. The antigen–antibody complexes were analyzed in 7.5% SDS–PAGE.

A plaque reduction assay with sera from immunized mice was performed as described previously (Jiménez *et al.,* 1986). The neutralization index of each serum was expressed as the $\log_{10}$ of the ratio of the pfu/ml of virus obtained using a normal serum and that observed in the presence of a given anti-glycoprotein S mouse serum.

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