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Immunization with Potato Plants Expressing VP60 Protein Protects against Rabbit Hemorrhagic Disease Virus

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The major structural protein VP60 of rabbit hemorrhagic disease virus (RHDV) has been produced in transgenic potato plants under the control of a cauliflower mosaic virus 35S promoter or a modified 35S promoter that included two copies of a strong transcriptional enhancer. Both types of promoters allowed the production of specific mRNAs and detectable levels of recombinant VP60, which were higher for the constructs carrying the modified 35S promoter. Rabbits immunized with leaf extracts from plants carrying this modified 35S promoter showed high anti-VP60 antibody titers and were fully protected against the hemorrhagic disease.

Rabbit hemorrhagic disease (RHD), which is caused by a calicivirus (19, 20), is a rapidly lethal infection of adult animals that is characterized by acute liver damage and disseminated intravascular coagulation (1). This disease is easily identified and can be effectively controlled in commercial and domestic rabbit populations by slaughter and vaccination. Nevertheless, the production of the available commercial vaccines (4), which are made from tissues collected from experimentally infected rabbits, involves a high biological risk which should be avoided in second-generation vaccines. VP60, the major structural protein of RHD virus (RHDV), has been produced in several heterologous hosts to date (6–9, 15), and in all cases the recombinant protein obtained was able to induce protection against a lethal challenge with RHDV in rabbits.

Plant-based production of biologically active recombinant proteins has been reported, and these proteins have been successfully used for immunization of animals. Potato, tobacco, and *Arabidopsis thaliana* plants have been shown to synthesize Norwalk virus capsid protein (17), hepatitis B virus surface antigen (16, 23), foot-and-mouth disease virus VP1 (11), and bacterial enterotoxins (2, 13). All of these plant-expressed proteins were able to induce specific immune responses, even when administered by the oral route (3, 13, 17, 22). Here we report the production of recombinant VP60 protein in transgenic potato plants and its use as an immunogen in rabbits.

For the construction of plant expression vector pK2-VP60, a 1.8-kbp *Bgl*II cassette containing the VP60 coding sequence (9) was cloned into the unique *Bam*HI site of the binary plasmid pRok2 (14). In the resulting construct, the initiator ATG codon of the VP60 gene was placed 4 nucleotides (nt) from the *Bam*HI-*Bgl*II junction and included the complete VP60 coding region from the Spanish AST/89 isolate of RHDV followed by 89 nt after the VP60 termination codon (9). The binary vector pRok3 was made by inserting a 650-bp *Hin*dIII-*Bam*HI fragment from vector pBI-364 (5) into plasmid pRok2. The resulting vector, pRok3, had a cauliflower mosaic virus (CaMV) 35S promoter that included two copies of a 343-bp region containing a strong transcriptional enhancer. Plant expression vector pK3-VP60 was made by cloning the 1.8-kbp *Bgl*II cassette, containing the VP60 sequence, into the unique *Bam*HI site of the binary plasmid pRok3.

Agrobacterium tumefaciens LBA4404 containing either the pK2-VP60 or pK3-VP60 vector was used to transform potato plants (*Solanum tuberosum* cv. Desirée). Leaf explants from 4-week-old potato plants were infected with recombinant *A. tumefaciens*. The inoculated leaves were blotted dry and placed on solidified shoot-promoting Murashige and Skoog (MS) medium (18) supplemented with 30 g of glucose per liter, 6 μM zeatin riboside, and 0.02 μM naphthalenacetic acid (CM medium). After 2 days of cocultivation, the explants were placed on CM medium supplemented with 300 mg of ticarcillin and 100 mg of kanamycin per liter (CMKT medium). After 3 to 4 weeks, regenerants appeared on CMKT medium at a frequency of 55%. Each leaf explant usually gave rise to four or five shoots. Regenerated shoots were subcultured and rooted on selective MS medium supplemented with 10 g of sucrose and 50 mg of kanamycin per liter. The putative transgenic plants were transferred to a sterile peat-perlite (2:1) mixture and incubated in a growth chamber under normal humidity and light conditions.

The presence of the VP60 gene in the rooted plants was investigated by PCR. The DNA from fresh leaves, obtained as described elsewhere (12), was amplified by using a forward primer, 5′-AGAACACACCCATCATGTTCGC-3′ (nt 6674 to 6695 of the RHDV genome, isolate AST/89; EMBL accession no. Z49271, and a reverse primer, 5′-TGTAGTTGCAGATCT TGCCTGC-3′ (nt 7098 to 7119). PCR amplification of a fragment of the neomycin phosphotransferase gene was also carried out, using the forward primer NPT1 (5′-GAGGCTATTC GGCTATGACTG-3′) and the reverse primer NPT2 (5′-ATC GGGAGCGGCGATACCGTA-3′). Agarose electrophoresis analysis of the amplified products consistently showed the presence of the expected band of 445 bp in all putative VP60-transgenic clones (Fig. 1, lanes D to G). This amplification product was absent in VP60-unrelated transformed clones derived from vector pRok2 (Fig. 1, lane B). An amplification product of 700 bp was also present in all plants rooted in the presence of kanamycin (Fig. 1, lanes B and D to G). Both 445- and 700-bp products were absent in a wild-type potato (Fig. 1, lane A).

To analyze VP60 mRNA in transgenic potato leaves, a North-

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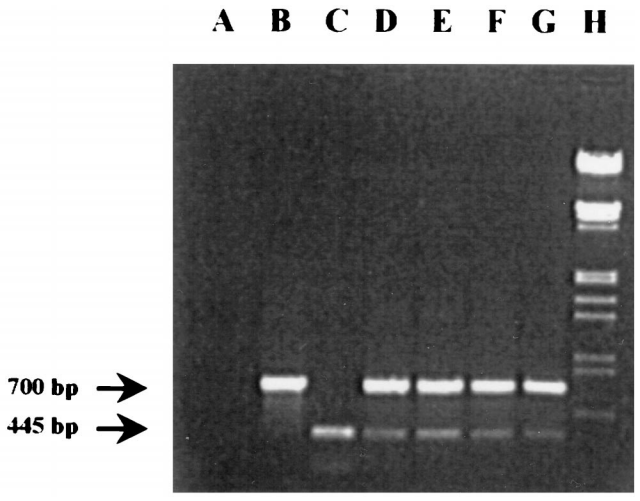

FIG. 1. Detection of the VP60 and neomycin phosphotransferase genes by PCR amplification of plant genomic DNA. Lanes: A, DNA from a wild-type potato; B, DNA from a pRok2-derived transformed plant; C, a 1.8-kbp *Bgl*II cassette containing the VP60 coding sequence (9); D and E, DNA from pK2-VP60-transformed plants; F and G, DNA from pK3-VP60-transformed plants; H, DNA markers (*Eco*RI-*Hin*dIII-digested lambda phage DNA).

ern blot assay was performed. Individual transformants showed specific bands of 1.8 kb in length, whereas control plants transformed with genes other than VP60 or those of a wild-type potato did not give detectable signals (data not shown).

The plants expressing the VP60 mRNA (nine of nine pK2-VP60 transformants and seven of nine pK3-VP60 transformants) were transferred to a sterile peat-perlite (2:1) mixture and grown in a greenhouse under high-humidity (95%) conditions. After 2 weeks, the plants were transplanted to pots and grown under normal humidity and light conditions.

For detection and quantification of recombinant VP60 in leaf extracts, a sandwich enzyme-linked immunosorbent assay (ELISA) was performed. Monoclonal antibodies 1H8 and 6G2 (10) diluted in phosphate-buffered saline (PBS) were bound to 96-well microtiter plates (Maxisorp; Nunc) for 12 to 15 h at 4°C, after which the plates were blocked with 1.5% horse serum in PBS containing 0.05% Tween 20. Protein extracts obtained by homogenization of leaves in PBS plus 0.05% Tween 20 were centrifuged at 10,000 × *g* for 3 min, added to the microtiter plates, and incubated for 1 h at 37°C. A polyclonal antibody against VP60 protein and peroxidase-labeled protein A were added in subsequent steps. The plates were developed by the addition of *o*-phenylenediamine-H_2O_2 in citrate buffer (pH 5). Each ELISA plate included positive controls containing 1 to 60 ng of recombinant VP60 (15). The amount of protein in each of the extracts was determined by the Bio-Rad protein assay. Low levels of VP60, ranging from 0.06 to 0.43 μg per mg of soluble protein, were observed in most pK2-VP60-derived transformants (Fig. 2). The pK3-VP60-derived transformants showed substantially higher levels of VP60, ranging from 0.820 to 2.97 μg per mg of soluble protein (Fig. 2). Extracts from a wild-type potato and a VP60-unrelated transformant showed no detectable levels of VP60 (Fig. 2). The maximum level of VP60 accumulation in the leaves of pK3-VP60-transformed potatoes was 0.30% of the total soluble protein. The mean level of recombinant VP60 in potato plants was 320 ng per mg of total soluble protein in pK2-VP60-derived plants and 1,600 ng per mg of total soluble protein in pK3-VP60 transformants. These data suggested that the double-enhancer CaMV 35S promoter allowed a three- to fivefold increase in VP60 gene expression compared to the levels obtained with the CaMV 35S promoter carried by vector pRok2. A number of pK2-VP60 and pK3-VP60 clones were used to produce microtubers of 1 to 5 g. The tuber VP60 expression levels were 195 and 702 ng per mg of total soluble protein in pK2-VP60- and pK3-VP60-derived plants, respectively, which were comparable to those in leaves. Recombinant VP60 was also detected (data not shown) in leaf extracts from selected pK2-VP60 and pK3-VP60 transformants by Western blotting and enhanced chemiluminescence (Boehringer Mannheim). The size of the VP60 protein expressed by the transgenic potato plants corresponded to that of native VP60, which has an apparent molecular mass of 60 kDa. The reaction of the recombinant polypeptides found in pK2-VP60 and pK3-VP60 transformants to specific antibodies also supported the hypothesis that in those constructs the translation started at the authentic initiation codon described for the VP60 found in the

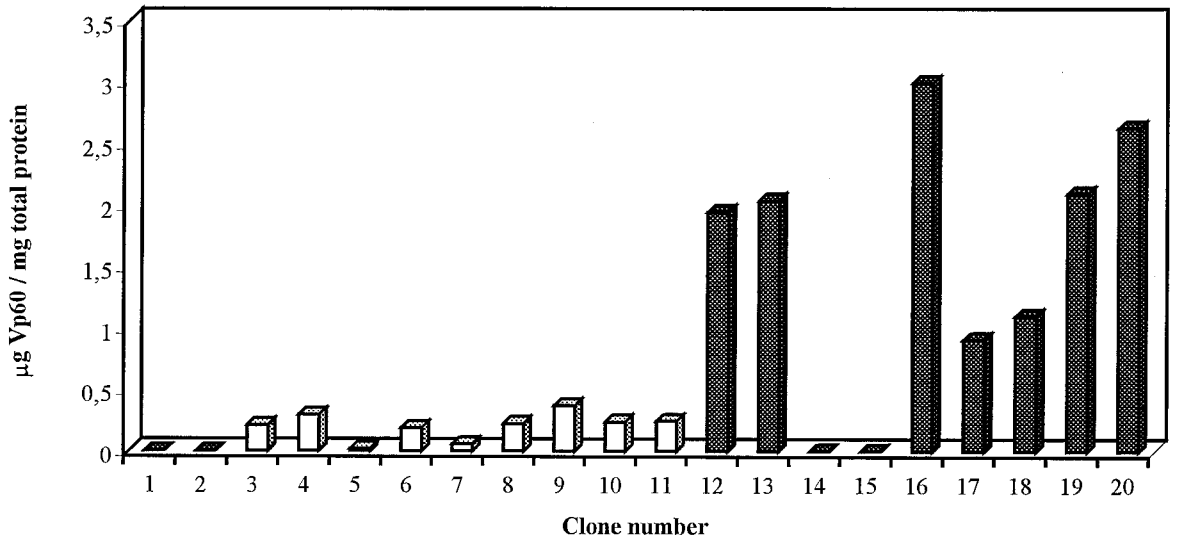

FIG. 2. Detection of VP60 in potato plant leaf extracts by sandwich ELISAs. Lanes: 1, wild-type potato extract; 2, pRok2-derived transformed-plant extract; 3 to 11, extracts of pK2-VP60-transformed clones; 12 to 20, extracts of pK3-VP60-transformed plants.

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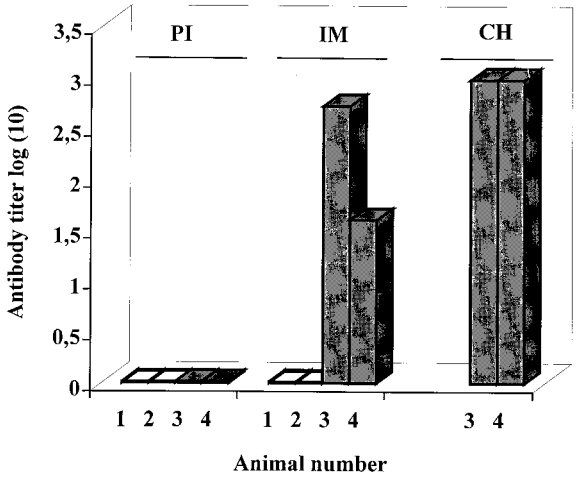

FIG. 3. ELISA titers of anti-VP60 antibodies in sera of immunized and control rabbits. Lanes: 1 and 2, titers for animals inoculated with wild-type potato leaf extracts; 3 and 4, titers for rabbits injected with pK3-VP60-transformed potato leaf extracts. PI, preimmune sera; IM, serum samples obtained 67 days after the first immunization; CH, serum samples obtained from survivors 5 days after the RHDV challenge.

virions (21). This also indicated that recombinant VP60 did not undergo major posttranslational processing in the plant system. Using leaf extracts, attempts were made to examine VP60 self-assembly, as has been described for other caliciviruses (17). Nevertheless, no RHDV VP60 virus-like particles could be observed, possibly due to the very small amount of plant material that was available for those experiments.

To investigate the induction of protective immune responses to plant-derived VP60, two adult rabbits were inoculated on day 0 with 1 ml of leaf extract, containing 12 μg of recombinant VP60 from pK3-VP60-derived transgenic potatoes, emulsified in complete Freund's adjuvant and on days 30, 60, and 90 with 1 ml of the same extract emulsified in incomplete Freund's adjuvant. The first immunization was done by the subcutaneous route at several points, whereas the boosters were done intramuscularly. As negative controls, two rabbits were injected with equivalent amounts of normal potato leaf extract. The animals were bled before the first immunization and after the second and third boosters. The endpoint titer of specific antibodies against RHDV VP60 was tested by ELISA, using recombinant VP60 as the antigen (15), of serial dilutions of sera from immunized and control rabbits. Anti-VP60 titers in the sera of the animals injected with wild-type potato leaf extract were not detectable 67 days after immunization (Fig. 3). By contrast, at this time the two rabbits immunized with pK3-VP60-derived potato leaf extract showed high antibody titers (Fig. 3) which did not increase after the last boost.

To evaluate the protective efficacy of immunization with the plant-derived VP60, all rabbits were challenged with 16,000 hemagglutination units of virulent RHDV. The animals injected with wild-type potato extract died 40 h postchallenge, showing the characteristic signs of the disease. Moreover, specific regions of the RHDV genome were amplified by PCR from liver extracts of the dead animals (data not shown). The two rabbits immunized with pK3-VP60-derived transgenic potatoes, which showed high antibody titers, were fully protected and survived the RHDV challenge infection. It should be mentioned that the small number of rabbits used, which in more conventional circumstances does not allow a valid statistical analysis, can be considered adequate in this case, taking into account that under the experimental conditions used the mortality occurring after challenge in unprotected animals approached 100% (8, 9, 15).

This article describes an alternative VP60 production method involving the use of a plant host which can be utilized for parenteral vaccination against RHDV. Unfortunately, our data also indicated that the resulting expression levels were still inadequate for the efficient use of tubers as vaccines, especially for direct animal feeding, which requires high antigen doses. Future studies must be directed to maximize VP60 expression levels in the tuber so that the oral immunogenicity of untreated potato tissue can be evaluated. The possibility of using edible plants for oral immunization is an important issue considering that RHDV also infects the wild rabbit (*Oryctolagus cuniculus*) (24), which constitutes an important staple prey for some protected carnivores (i.e., *Linx pardinus*) and is the most important small game species in Spain.

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