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Protection of Rabbits against Rabbit Hemorrhagic Disease Virus by Immunization with the VP60 Protein Expressed in Plants with a Potyvirus-Based Vector

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A new plum pox potyvirus (PPV)-based vector has been constructed for the expression of full-length individual foreign proteins. The foreign sequences are cloned between the NIb replicase and capsid protein (CP) cistrons. The heterologous protein is split from the rest of the potyviral polyprotein by cleavage at the site that originally separated the NIb and CP proteins and at an additional NIa protease recognition site engineered at its amino-terminal end. This vector (PPV–NK) has been used to clone different genes, engendering stable chimeras with practical applications. We have constructed a chimera expressing high levels of jellyfish green fluorescent protein, which can be very useful for the study of PPV molecular biology. The VP60 structural protein of rabbit hemorrhagic disease virus (RHDV) was also successfully expressed by making use of the PPV–NK vector. Inoculation of extracts from VP60-expressing plants induced a remarkable immune response against RHDV in rabbits, its natural host. Moreover, these animals were protected against a lethal challenge with RHDV.

INTRODUCTION

Plum pox virus (PPV) belongs to the *Potyvirus* genus of plant viruses. Potyviruses have a single-stranded RNA genome of positive sense. This is translated into a single polyprotein that is further processed by viral-coded proteases (Riechmann *et al.,* 1992). The potyviral genomic RNA is surrounded by approximately 2000 units of a single type of capsid protein (CP) that is encoded at the end of the genome, forming helicoidal virions (Shukla *et al.,* 1994).

The interest of using plants, and particularly plant virus vectors, for the expression of foreign proteins has led to the development of viral vectors based in different types of plant viruses (Johnson *et al.,* 1997; Scholthof *et al.,* 1996). Expression of proteins that could function as antigens for active immunization, using a plant virus, has been mostly restricted to expression of antigenic peptides fused to the capsid protein. However, a tobacco mosaic virus-based vector expressing the vp1 structural protein of the foot-and-mouth disease virus has recently been described. Mice immunized with extracts from *Nicotiana tabacum* plants infected with this chimeric virus developed an effective immune response (Wigdorovitz *et al.,* 1999).

Potyviral-based vectors for the expression of full-length independent proteins have been previously described. The first was developed based on tobacco etch virus (Dolja *et al.,* 1992). Afterward, similar vectors were developed based on other potyviruses (Choi *et al.,* 2000; German-Retana *et al.,* 2000; Guo *et al.,* 1998; Masuta *et al.,* 2000; Varrelmann and Maiss, 2000). These potyviral vectors seemed to be impaired in replication efficiency and genome stability in most of the cases in which these parameters were assessed (Choi *et al.,* 2000; Dolja *et al.,* 1993; German-Retana *et al.,* 2000; Guo *et al.,* 1998; Masuta *et al.,* 2000).

Rabbit hemorrhagic disease is a rapidly lethal infection of adult animals. Infected rabbits usually die within 48 to 72 h of necrotizing hepatitis. The causing agent of the disease, Rabbit hemorrhagic disease virus (RHDV), is a member of the family *Caliciviridae* (Ohlinger *et al.,* 1990; Parra and Prieto, 1990). Commercial vaccines against viral hemorrhagic disease of rabbits are produced from tissues, such as spleen and liver, collected from experimentally infected SPF rabbits, due to the fact that, to date, a permissible stable cell line allowing RHDV replication has not been described. In recent years, VP60—the major structural protein of RHDV—has been produced in several heterologous systems (Barcena *et al.,* 2000; Bertagnoli *et al.,* 1996a; Boga *et al.,* 1994; Fischer *et al.,* 1997; Laurent *et al.,* 1994; Marín *et al.,* 1995; Nagesha *et al.,* 1995; Plana-Duran *et al.,* 1996; Sibilia *et al.,* 1995), including transgenic potato plants (Castañón *et al.,* 1999), and in all cases the recombinant VP60 protein obtained has been shown to induce protection of rabbits against a lethal challenge with RHDV.

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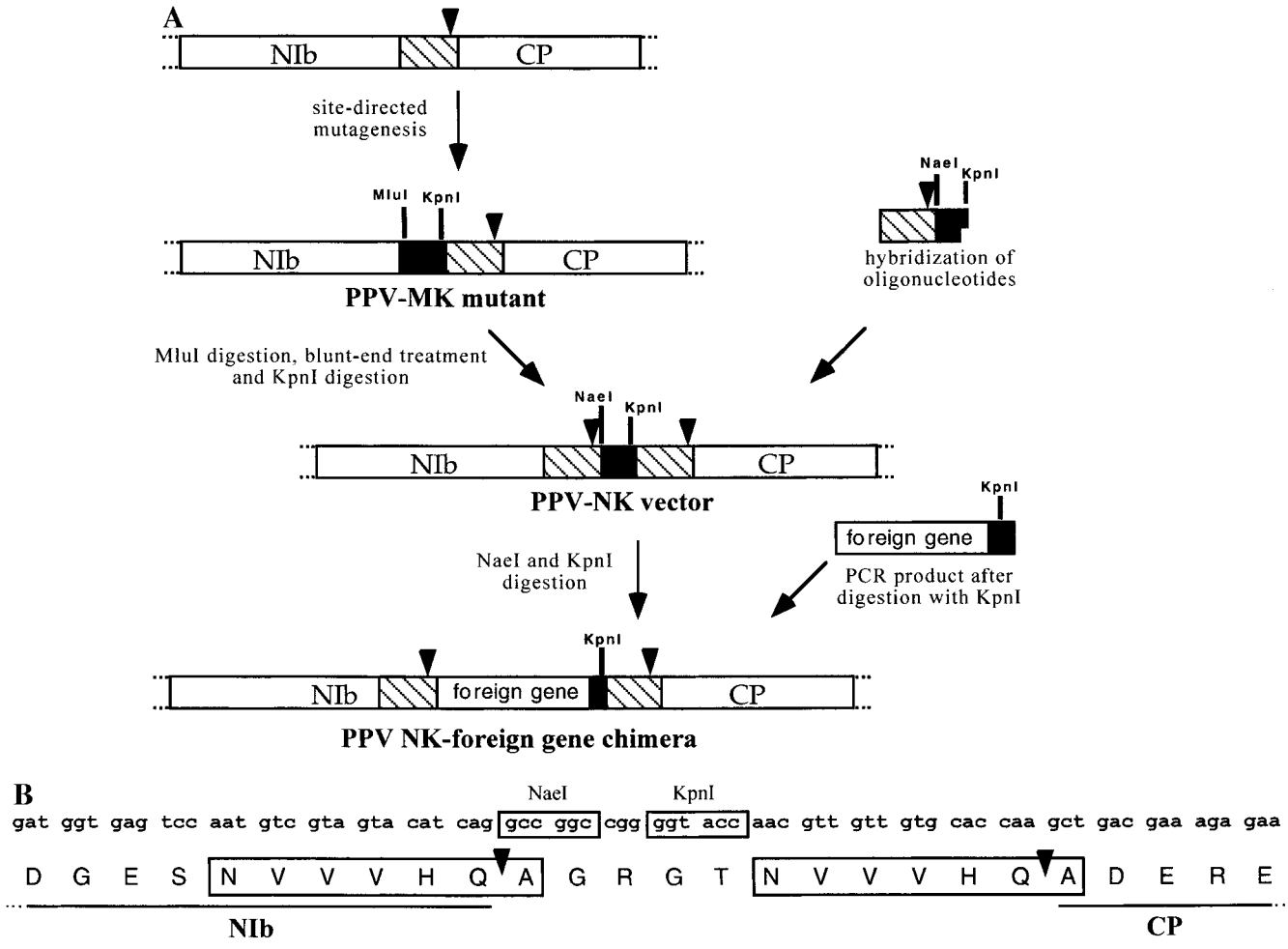

FIG. 1. (A) Strategy followed to construct the PPV–NK vector and to clone foreign genes in it. A detail of the region of the wild-type PPV genome where modifications were made is shown. Solid boxes represent engineered restriction sites and spacing sequences. Sequence coding for NIa protease recognition sites is represented by transversal grids, and the scissile bond is marked by a solid triangle. (B) Detail of the junction between NIb and CP sequences in the PPV–NK vector. The nucleotide sequence is shown in lowercase letters, and *Nae*I and *Kpn*I restriction sites are boxed. The amino acid sequence is shown in capital letters. The NIb and CP sequences are underlined. The heptapeptide recognized by the NIa protease is boxed and the scissile bond is indicated by a solid triangle.

In this paper we describe the construction of a new PPV-based vector (PPV–NK) that expresses the protein of interest between the NIb replicase and CP sequences. A chimera expressing green fluorescent protein (GFP) was constructed and it was found to be highly stable. Moreover, VP60, the structural protein of RHDV, has been expressed with the PPV–NK vector. Immunization of the natural host of RHDV (rabbits) with extracts of *Nicotiana clevelandii* plants infected with the PPV–NK–VP60 chimera induced an efficient immune response that protected animals against a lethal challenge with RHDV.

RESULTS

Construction of the PPV-based expression vector PPV–NK

A PPV-based-vector for the expression of full-length independent foreign proteins, PPV–NK, was constructed. In this vector, the foreign sequences were cloned between the NIb replicase and CP cistrons. The cloning was designed to avoid the modification of any sequence of the viral proteins, with an interest in interfering as little as possible with any viral function. The NIb/CP region of the PPV polyprotein encoded by pICPPV–NK consists of (i) an intact NIb protein, but one whose terminal six amino acids (that form part of the sequence recognized by the NIa protease) are encoded by a modified nucleotide sequence; (ii) five amino acids, including the Ala residue present at the +1 position of the natural NIb/CP cleavage site, encoded by a nucleotide sequence that contains the *Nae*I restriction site, a spacer sequence, and a *Kpn*I restriction site (*Nae*I and *Kpn*I are unique in pICPPV–NK); (iii) again the six carboxy-terminal amino acids of the NIb protein (encoded by the original sequence); and (iv) CP. The NIa protease recognition sequence engineered has the same amino acid sequence

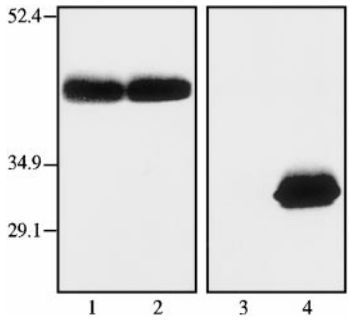

FIG. 2. Western blot analysis of extracts from *N. clevelandii* plants infected with wild-type PPV (lanes 1 and 3) and PPV–NK–GFP (lanes 2 and 4), using anti-PPV 5B MAb (lanes 1 and 2) and anti-GFP MAb mixture (Roche Molecular Biochemicals) (lanes 3 and 4). The sizes in kDa of prestained molecular weight markers (Bio-Rad) run in the same gel are indicated beside the panel.

as the original NIb/CP site, but it is encoded by a sequence in which the third nucleotide of each codon has been changed to hinder recombination events (Fig. 1B).

Full-length cDNA of PPV is cloned in pICPPV–NK-derived plasmids under the control of an enhanced cauliflower mosaic virus 35S promoter and holds a nopaline synthase termination signal, which allow the production of viral transcripts *in vivo* in plants of *N. clevelandii* directly inoculated with DNA (López-Moya and García, 2000). pICPPV–NK was able to infect plants with an efficiency similar to that of nonmutated pICPPV. The time course of infection and symptomatology were similar to those of wild-type PPV-infected plants. Western blot analysis confirmed that the level of accumulation of PPV–NK was similar to that of wild-type PPV and that, since no differences in the electrophoretic mobility of CP were observed, at least the NIa recognition heptapeptide closer to CP was fully processed (data not shown). Immunocapture RT–PCR (IC–PCR) amplification from the infected plants of a cDNA fragment of the mutated region revealed, as expected, a band with a lower mobility than that amplified from wild-type PPV. The fragment amplified from the PPV–NK-infected plants conserved the original sequence, as determined by *Nae*I and *Kpn*I digestion and nucleotide sequencing (data not shown).

Construction of a PPV chimera carrying a *gfp* reporter gene

The ease of detection of GFP activity and the expected value of a PPV–GFP chimera as a tool for the study of PPV molecular biology prompted us to choose GFP for a first attempt at expressing a heterologous protein with the PPV–NK vector. pICPPV–NK–GFP, constructed as explained under Materials and Methods, was directly inoculated in *N. clevelandii* plants, resulting in a high percentage of infected plants, similar to that of plants inoculated with pICPPV or pICPPV–NK. The time course of infection and symptomatology were similar to those observed in plants infected with wild-type PPV.

Western blot analysis showed the same electrophoretic mobility for CP accumulated in systemically infected leaves from plants inoculated with PPV–NK–GFP or with wild-type PPV (Fig. 2, lanes 2 and 1). This confirms that cleavage between GFP and CP is taking place efficiently. On the other hand, in crude extracts from PPV–NK–GFP-infected leaves, we were also able to detect GFP by Western blot (Fig. 2, lane 4). The protein showed the expected mobility. No products of lower or higher mobility were observed, ruling out both an inefficient processing between NIb and GFP and the generation of deletions in the sequence coding for the foreign protein. The autofluorescence of GFP protein was easily detected by confocal or epifluorescence microscopy in leaves infected with the chimeric virus even before macroscopic symptoms of infection were evident (data not shown).

To confirm the genetic stability of the chimera, a cDNA fragment including the foreign sequence was amplified by IC–PCR from extracts of leaves infected with PPV–NK–GFP. The amplified fragments had the expected size, larger than those amplified from wild-type PPV-infected plants (Fig. 3, lanes 3 to 8 compared to lane 9). Moreover, no smaller DNA fragments could be detected, further indicating that deleted forms arising from instability of the chimera were not being generated during virus replication in the plant.

Virus and GFP accumulations were quantified in infected *N. clevelandii* plants by ELISA using purified virions and commercial recombinant GFP (Roche Molecular Biochemicals) as standards. Virus accumulation levels in plants infected with wild-type PPV and PPV–NK–GFP (38.8 ± 3.9 and 63 ± 6.9 mg per 100 g of infected leaf tissue, respectively) seemed to be only slightly higher

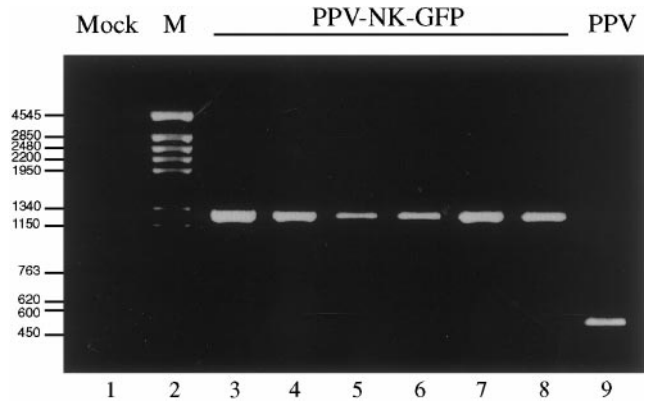

FIG. 3. IC–PCR analysis of extracts from *N. clevelandii* plants infected with PPV–NK–GFP chimeric virus after a plant passage from the progeny (lanes 3 to 8) or with wild-type PPV (lane 9), 30 dpi, and from a mock-inoculated plant (lane 1) (the amplified fragments correspond to nt 8390 to 8900 in the wild-type PPV genome). Molecular weights of *Hin*dIII restriction fragments of phage ø29 DNA used as size markers (lane 2) are indicated beside the panel.

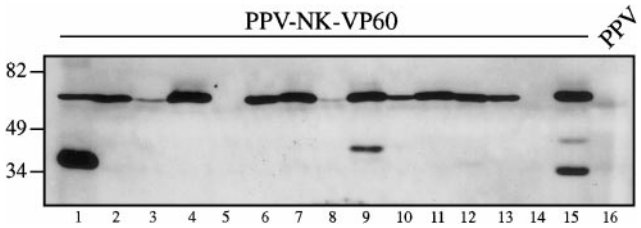

FIG. 4. Western blot analysis of extracts from *N. clevelandii* plants inoculated with PPV–NK–VP60 (lanes 1 to 15) or with wild-type PPV (lane 16) using a polyclonal serum against RHDV VP60. Plants in lanes 3, 5, 8, and 14 were symptomless (data not shown). Bands in lanes 3 and 8 are probably due to leakage from flanking wells. The sizes in kDa of prestained molecular weight markers (Bio-Rad) run in the same gel are indicated beside the panel.

than GFP accumulation in these latter plants (25 ± 2 mg per 100 g of leaf tissue).

Expression of RHDV structural protein VP60 in *N. clevelandii* plants

To test the possibility of using the PPV–NK vector to produce in plants antigenic proteins useful for active immunization, we constructed a chimera that expressed the VP60 structural protein from RHDV. pICPPV–NK–VP60 was able to infect *N. clevelandii* plants with similar efficiency to that of pICPPV. The symptomatology and time course of PPV–NK–VP60 infection were similar to those of wild-type PPV infection. The VP60 protein was easily detected by Western blot analysis at 15 days postinoculation (dpi), reaching higher accumulation levels at 21 dpi (Fig. 4). Fifteen days postinfection no other band apart from that of the full-length protein was detected in the Western blot assays reacting with anti-VP60 (data not shown); however, 21 dpi bands with electrophoretic mobilities corresponding to those of truncated proteins of approximately 38 kDa (plant 1), 44 kDa (plant 9), and 46 and 33 kDa (plant 15) were apparent in the anti-VP60 Western blot (Fig. 4). Although we cannot rule out that some of these bands correspond to degraded protein, it is probable that they were mainly the result of some instability of the chimeric genome. This assumption was supported by the detection of partial deletions of the foreign sequence in a virus cDNA fragment amplified by IC–PCR from infected tissue (data not shown). In any case, the full-length VP60 was the predominant form in most plants, demonstrating the capability of the system to efficiently express rather large proteins of interest.

Immunization of rabbits with extracts from PPV–NK–VP60-infected plants and protection against a RHDV challenge

The immunogenicity of the VP60 protein expressed in plants with the PPV–NK vector was studied in the natural host of RHDV, rabbits. Twenty-seven days after the first antigen administration, serum samples were analyzed for the presence of specific antibodies by the hemagglutination inhibition (HI) test (Table 1). Nine of 10 rabbits of group I (vaccinated with extracts from plants infected with PPV–NK–VP60 expressing the full-length VP60) exhibited VP60-specific antibodies with variable titers, ranging from 1/20 to 1/320. Similar titers were observed in all the animals from group III (vaccinated with extract from an infected plant accumulating an approximately 44-kDa deleted form in addition to the full-length VP60). Three of 4 rabbits from group II (vaccinated with extract from a plant primarily accumulating an approximately 38-kDa deleted form of VP60) gave a lower specific serological response (titers of 1/20 to 1/40). The antibody response was somehow higher and more homogeneous in the cases of animals vaccinated with the commercial vaccine CYLAP HVD (group V) (with animals showing titers of 1/320 to 1/640). As expected, animals vaccinated with wild-type PPV-infected plant extracts (group IV) and nonvaccinated control animals (group VI) did not show specific antibodies against RHDV. Thirty-three days after the initial immunization (D33), all the animals were given a booster using extracts with the same characteristics as those of the originals. On D67, the antibody titers were analyzed by HI test and a lethal challenge was performed. While control groups (IV and VI) remained negative, the serological response increased in all the other vaccination groups. Again, the highest titers were observed in groups vaccinated with CYLAP HVD and exclusively the complete VP60 (groups V and I), followed by groups vaccinated with extracts from plants accumulating VP60-deleted forms (group III being better than group II).

The challenge was adequate because the mortality rate due to RHDV in the group vaccinated with the wild-type PPV-infected plant and in the nonvaccinated control group was 100%, and RHDV was detected in their livers (Table 1). During the experimental infection period with the virulent RHDV, while all except one of the animals from control groups IV and VI died within 3 days after challenge, no clinical symptoms of the disease were observed in animals vaccinated with CYLAP HVD (group V) or with PPV–NK–VP60-infected plants (groups I, II, and III). Two weeks after challenge, surviving animals were bled and slaughtered. In general, antibody titers of surviving animals, determined by HI test, were higher 2 weeks after challenge (Table 1). No RHDV was detected in the livers of surviving animals. Therefore, it can be concluded that animals vaccinated with extracts from plants expressing the VP60 protein developed an efficient humoral immune response and were protected against a lethal challenge with RHDV.

TABLE 1

Antibody Titers against RHDV and Challenge Results

Group[a]	Animal No.	HI titers[b] D27[c]	HI titers[b] D67	Challenge results: Death	Challenge results: HI titers DY + 15	Challenge results: HA titers liver samples[d]
I						
PPV–NK–VP60 (full-length VP60, plants 2, 4, 6, 7, 11, 13)	818	1/320	1/2560	S	1/10,240	<2
	821	1/160	1/2560	S	1/5,120	<2
	822	1/80	1/640	S	1/2,560	<2
	823	1/80	1/1280	S	1/5,120	<2
	826	1/20	1/640	S	1/5,120	<2
	827	1/20	1/320	S	1/160	<2
	831	<1/20	1/1280	S	1/2,560	<2
	834	1/40	1/160	S	1/2,560	<2
	837	1/80	1/640	S	1/5,120	<2
	897	1/160	1/5120	S	1/2,560	<2
II						
PPV–NK–VP60Δ1 (full-length VP60 and ~38-kDa deleted form from plant 1)	845	1/40	1/320	S	1/5,120	<2
	846	<1/20	1/160	S	1/1,280	<2
	847	1/40	1/320	S	1/640	<2
	848	1/20	1/320	S	1/1,280	<2
III						
PPV–NK–VP60Δ9 (full-length VP60 and ~44-kDa deleted form from plant 9)	839	1/20	1/320	S	1/640	<2
	840	1/320	1/1280	S	1/5,120	<2
	842	1/20	1/640	S	1/10,240	<2
	844	1/40	1/640	S	1/2,560	<2
IV						
PPV–wt (no insertion, plant 16)	900	<1/20	<1/20	DY + 3		≥4096
	809	<1/20	<1/20	DY + 3		≥4096
	813	<1/20	<1/20	DY + 3		≥4096
	816	<1/20	<1/20	DY + 3		≥4096
V						
CYLAP HVD (Fort Dodge vaccine)	851	1/320	1/5120	S	1/10,240	<2
	853	1/320	1/2560	S	1/5,120	<2
	854	1/640	1/2560	S	1/10,240	<2
	855	1/320	1/640	S	1/10,240	<2
VI						
Control (nonvaccinated)	804	<1/20	<1/20	DY + 3		≥4096
	824	<1/20	1/20	DY + 3		≥4096
	825	<1/20	1/20	DY + 3		≥4096
	863	<1/20	<1/20	DY + 5		≥4096
	864	<1/20	<1/20	DY + 3		≥4096
	866	<1/20	<1/20	DY + 3		≥4096

[a] Plant numbering according to that in Fig. 4.

[b] HI test: rabbit sera antibody level against RHDV was determined by hemagglutination inhibition test of human red blood cells. Rabbits were seronegative to RHDV at D0 (HI titers <1/20).

[c] Rabbits were vaccinated on day 0 (D0), bled on D27, given a booster on D33, bled again (D67), and challenged (DY) on the same day. Surviving animals (S) were bled and slaughtered 2 weeks after challenge (DY + 15).

[d] HA test: presence of RHDV was determined by hemagglutination of human red blood cells. Titers <2 were considered negative.

DISCUSSION

Plants that have traditionally been used as sources of food, fibers, and fuel are now being considered as alternative factories for the production of molecules of interest, with low expenses, high biological security, and ease in manipulation (Arntzen, 1997). The use of plant virus-based vectors instead of transgenic plants for the production of novel proteins presents several advantages (Arntzen, 1997; Scholthof *et al.,* 1996), although both systems are of great value under different circumstances.

The first vectors developed for the expression of full-length independent proteins, based on potyviruses, explored the genomic region between the P1 and HC

cistrons as the site of insertion of foreign sequences (Dolja *et al.,* 1992; German-Retana *et al.,* 2000; Guo *et al.,* 1998). Although these have been of great value in the study of the molecular biology of potyviruses and other plant viruses (for instance, Carrington and Whitham, 1998; Dolja *et al.,* 1997; Kasschau *et al.,* 1997; Verchot and Carrington, 1995; Whitham *et al.,* 1999), their utility for overproduction of proteins of interest could be limited by the susceptibility of foreign sequences expressed in them to suffer deletions under certain circumstances (Choi *et al.,* 2000; Dolja *et al.,* 1993, 1997; German-Retana *et al.,* 2000; Guo *et al.,* 1998). A notable decrease in replication efficiency and/or pathogenicity has also been observed in most of these virus vectors.

A chimeric PPV containing the *gus* gene cloned between the NIb and CP cistrons has been reported; terminal sequences of these cistrons were duplicated, resulting in the production of a GUS protein fused to the 27 amino-terminal amino acids of CP and the 18 carboxy-terminal amino acids of NIb (Varrelmann and Maiss, 2000). No data are available on the biological performance or the genomic stability of this chimeric clone, although a very rapid loss of the foreign sequences cloned between duplicated viral sequences has been reported in other systems (Dawson *et al.,* 1989; Donson *et al.,* 1991). Recently, a vector based in wheat streak virus, a member of the genus *Tritimovirus,* with a genome organization typical of the family *Potyviridae,* has been developed (Choi *et al.,* 2000). The stability of chimeras based in this virus, which uses the NIb/CP junction as the insertion site, appears to depend on the gene inserted and the host used. The vector that we have constructed, PPV–NK, also uses the NIb/CP junction as the cloning site, but avoids duplication of viral sequences. The cloning strategy warrants that foreign proteins expressed with this vector only have one additional amino acid in the amino end and eight extra amino acids in the carboxyl end to ensure proteolytic processing. The sequence encoding GFP was shown to be stable when cloned in pICPPV–NK–GFP. In the case of the sequence encoding VP60, cloned in pICPPV–NK–VP60, it was also quite stable, although deleted forms appear at late times postinfection in some plants. It has been reported that genomic instability of tobacco mosaic virus-derived chimeras seems to rise with the size of the foreign insert (Donson *et al.,* 1991). Thus, the larger size of the VP60 gene (1737 nt) with respect to that of the *gfp* gene (716 nt) could account for its lower stability. However, differences in the suitability for recombination of the foreign sequences, as well as selective pressure derived from effects of the foreign products on virus fitness, might also be crucial to determine the stability of the cloned genes.

An expected advantage of viral vectors with genomic expression through single polyproteins is that all proteins, including the foreign one, are synthesized in the same quantity, and differences in accumulation levels will depend exclusively on their stability in the infected plants. GFP seems to be quite a stable protein and, according to ELISA data, accumulates in PPV–NK–GFP-infected plants at high levels, similar to those of the viral CP, in spite of the high stability that virion assembly confers on CP. It is also important to note that inclusion of the *gfp* gene in the PPV genome does not seem to negatively affect virus accumulation and that a slightly higher CP amount was observed in PPV–NK–GFP-infected plants compared to that in plants infected with wild-type PPV. The first plant virus chimera expressing GFP was derived from potato virus X and has proven to be very useful in the analysis of the mechanism of infection of this virus (Baulcombe *et al.,* 1995). Thus, in addition to its relevance as a paradigm of the suitability of PPV–NK as an expression vector, PPV–NK–GFP is of specific interest as a tool for the study of PPV molecular biology.

This paper reports the first evidence of the use of a potyviral-based vector for the expression of an antigenic foreign protein capable of conferring protection on host animals (rabbits) against a viral disease. RHD is responsible for high economic losses in rabbitries and high mortality rates in wild rabbit populations (Barcena *et al.,* 2000). For this reason the development of alternatives to classic vaccines has become a focus of interest. It is especially important to produce highly effective, low-cost and low-risk vaccines. In this regard, plants expressing foreign antigens become a very attractive system to explore. RHDV VP60 had been previously expressed in potato plants (Castañón *et al.,* 1999) using a transgenic approach. Although the results were very promising, transgenic plants have certain constraints that the use of viral vectors overcomes. Plant virus-based vectors usually allow the production of higher and more reproducible amounts of the protein of interest, manipulation techniques are easier, proteins deleterious to the plant can be produced without the requirement of complex inducible promoters, and the time necessary to get the protein of interest is shorter. Although we have not made a precise quantification of the levels of VP60 accumulation in plants infected with PPV–NK–VP60, they seem to be quite high and the protein is easily detected by Western blot in crude extracts.

The serological responses of animals vaccinated with plant extracts producing full-length VP60, although somewhat less homogeneous, were almost as high as those of animals treated with the commercial CYLAP HVD vaccine. Thus, the presence of one additional amino acid in the N-terminus and eight in the C-terminus of the recombinant VP60 apparently did not affect substantially the antigenicity of the protein. Interestingly, extracts not only from plants that accumulate mainly full-length VP60 but also from plants in which most of the VP60 is in deleted forms were able to elicit a notable antibody response and confer protection against RHDV.

If deletions in the VP60 sequence enhance the stability of the parental chimera, the demonstration that truncated forms of the protein could be effective immunogens opens a way to improve the present system that should be further analyzed.

Vaccinated animals need to exhibit HI titers in serum of at least 1/80 to ensure protection against a lethal challenge with RHDV (M.M. and J.P.-D., unpublished data). Thus, approximately half of the animals immunized with plant extracts containing full-length VP60 alone or with its 44-kDa truncated form were expected to be efficiently protected after administration of the first dose of vaccine. However, since some animals had titers below 1/80 after the first vaccination, a booster was needed to warrant protection of the entire group of rabbits.

We have demonstrated that two doses of 1 ml of extract of plants infected with PPV–NK–VP60, which represents approximately 2 g of fresh leaves, were enough to confer on animals full protection. Making a space and time quantification, merely 1 m^2 of greenhouse space and 21 days are sufficient to obtain enough material to immunize and fully protect 50 rabbits against RHDV.

Both RHDV-like particles (Plana-Duran *et al.,* 1996) and a vaccinia–RHDV recombinant virus (Bertagnoli *et al.,* 1996b) were shown to protect against RHDV when they were administered by the oral route. Taking into account the amount of VP60 produced in plants infected with PPV–NK–VP60 and the positive results obtained in the immunization experiments reported in this paper, the development of oral vaccines against RHDV in edible PPV hosts infected with PPV–NK–VP60 appears now as an appealing challenge.

MATERIALS AND METHODS

Construction of pICPPV–NK

pICPPV–NK was constructed according to the following strategy (Fig. 1A). An intermediate clone designed as pGPPV–MK was constructed by the PCR-based mutagenesis method described by Herlitze and Koenen (1990). In addition to the mutagenic oligodeoxinucleotide (oligo) mMK (5′ CAACAACGTTGGTACCCCGACGCGTGGACTCACCA 3′), two flanking oligos, which contained the direct PPV sequence from nt 8067 to 8082 and the reverse PPV sequence from nt 9114 to 9127 [numbering is according to Laín *et al.* (1988)] were used for the PCR amplifications. The mutagenic oligo created *Mlu*I and *Kpn*I unique restriction sites (underlined in the above oligo sequence). The resulting PCR product was digested with *Cla*I and *Sac*I and introduced into the full-length clone pGPPV (Riechmann *et al.,* 1990) by triple ligation using *Sal*I as the third enzyme. The accuracy of the PCR-derived sequence was verified by sequencing of the full-length clone.

The *Bam*HI–*Sac*I fragment of pGPPV–MK containing the MK mutation was subcloned in pUC18 to aid the following cloning steps. Hybridization of partially complementary oligos 5′ ATGTCGTAGTACATCAGGCCGGCCGGGGTAC 3′ and 3′ TACAGCATCATGTAGTCCGGCCGGCCC 5′ (restriction sites are underlined) created a DNA fragment that held, from 5′ to 3′, the coding sequence for a NIa protease recognition site and a NaeI restriction site. This DNA fragment, which had a blunt 5′ end and a cohesive 3′ end compatible with *Kpn*I, was inserted into pUC18–MK digested with *Mlu*I, treated with mung bean nuclease to remove the resulting protruding ends and with *Kpn*I, and yielded pUC18–NK. The accuracy of the construction was verified by sequencing. pICPPV–NK was obtained by replacing the *Cla*I–*Sac*I fragment (PPV nt 8310–9021) of pICPPV, a plasmid that contains the full-length PPV cDNA sequence placed under the control of the cauliflower mosaic virus 35S promoter (Lopez-Moya and García, 2000), with the corresponding fragment of pUC18–NK by a triple ligation that used *Sal*I as the third enzyme.

Cloning of foreign sequences in the pICPPV–NK plasmid

The reporter gene *gfp* was amplified by PCR, using as primers the oligos 5′ ATGGTGAGCAAGGGCGAG 3′ and 5′ CAGGTACCCTTGTACAGCTCGTC 3′ (a *Kpn*I restriction site is created and is underlined in the sequence of the oligo). The PCR product was digested with *Kpn*I and cloned in pUC18–NK digested with *Nae*I and *Kpn*I, giving rise to pUC18–NK–GFP. pICPPV–NK–GFP was obtained by replacing the *Cla*I–*Sac*I fragment (PPV nt 8310–9021) of pICPPV with the corresponding fragment of pUC18–NK–GFP by a triple ligation that used *Sal*I as the third enzyme. The sequence coding for the RHDV VP60 structural protein was amplified by PCR using as primers the oligos 5′ ATGGAGGGCAAAGCCCGC 3′ and 5′ CAGGTACCGACATAAGAAAAGCC 3′ (a *Kpn*I restriction site is created and is underlined in the sequence of the oligo). The VP60–PCR product was digested with *Kpn*I and cloned in pUC18–NK digested with *Nae*I and *Kpn*I, giving rise to pUC18–NK–VP60. The *Eco*RV–*Kpn*I fragment of pUC18–NK–VP60 was introduced into pICPPV–NK by a triple ligation using *Xho*I as the third enzyme, giving rise to pICPPV–NK–VP60.

Plant inoculation

For plant inoculation, full-length cDNA clones pICPPV, pICPPV–NK, pICPPV–NK–GFP, and pICPPV–NK–VP60 were diluted in 5 mM sodium phosphate, pH 7.5, to a concentration of between 100 and 500 ng/μl. Ten microliters of the diluted samples was used to inoculate three leaves per plant of *N. clevelandii.*

Western blot analysis

Samples from infected plants homogenized in 5 mM sodium phosphate, pH 7.5, were separated by SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to immunoreac-

tion as previously described (García *et al.,* 1992). The anti-PPV monoclonal antibody (MAb) mixture Ingezyme (INGENASA), anti-PPV MAb 5B (Durviz), a commercial anti-GFP MAb mixture (Roche Molecular Biochemicals), and an anti-VP60 polyclonal antibody were used as primary antibodies. The secondary antibodies were peroxidase-conjugated goat anti-mouse or anti-rabbit IgGs purchased from Jackson ImmunoResearch Laboratories. The peroxidase reaction was developed either with 4-chloro-1-naphthol (Sigma) or with the ECL kit (Amersham Pharmacia Biotech).

Immunocapture reverse transcription–PCR

Samples from infected plants homogenized in 5 mM sodium phosphate, pH 7.5, were incubated for 2 h at 37°C in tubes previously coated with anti-PPV IgGs, and then, after two washing steps with PBS–Tween buffer (1 × PBS, 0.5 g/L Tween 20), reverse transcription–PCR (RT–PCR) was performed as previously described (Candresse *et al.,* 1995).

ELISA

Three leaf discs were collected from plants infected either with wild-type PPV, or with PPV–NK–GFP or from mock-inoculated plants. The discs were homogenized in 150 μl of carbonate buffer, pH 9.6. Two microliters of each extract was diluted in 98 μl of carbonate buffer and applied to 96-well plates (Maxisorp, Nunc). Increasing amounts of recombinant GFP (Roche Molecular Biochemicals) and PPV purified virions added to 2 μl of a healthy plant extract were used to make standard curves. The plates were incubated overnight at 4°C. After washing twice with PBS buffer, the wells were saturated with 1% BSA in PBS buffer for 2 h at 37°C. The plates were then washed with PBS buffer containing 0.5% BSA and 0.05% Tween and incubated at 37°C for 3 h with anti-GFP MAb mixture (Roche Molecular Biochemicals) (1:2000 dilution) or Ingezyme anti-PPV mixture (INGENASA) (1:2000 dilution). After washing with PBS–BSA–Tween buffer, the plates were incubated at 37°C for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgGs (Jackson ImmunoResearch Laboratories). After a final washing step with PBS–BSA–Tween buffer, the plates were developed with *p*-nitrophenyl phosphate (Sigma) and the optical densities of samples were determined at 405 nm.

Formulation of vaccines

Leaves from plants, infected either with wild-type PPV or with PPV–NK–VP60, were homogenized in PBS [1:1 (w/v) or 1:2 (w/v)] and cell debris were eliminated by centrifugation. The vaccines were formulated by mixing the antigenic phase (leaf extracts) with an oily adjuvant, at a ratio of 53:47 (v/w), until a double water/oil/water emulsion was formed. The oily phase consisted of a mixture of marcol-52, simulsol-5100, and montanide-888. Four different plant-based vaccines were prepared. Also, a commercially available inactivated purified vaccine for the prevention of viral hemorrhagic disease of rabbits with oily adjuvant (CYLAP HVD, Fort Dodge Veterinaria) was used as a control for the experiment.

Hemagglutination inhibition test

RHDV agglutinates human red blood cells of type O. Hemagglutination (HA) is inhibited by specific antiserum. The HI test has been used for the detection of anti-RHDV antibodies in serum as previously described (Pu *et al.,* 1985).

Vaccination of rabbits

A total of 32 New Zealand white female rabbits, 15–16 weeks old, were used in this experiment. Animals were seronegative to RHDV by the HI test using human red blood cells and 4 hemagglutination units (HAu) of RHDV. The different groups (I to V) were vaccinated by the subcutaneous route with 1 ml of vaccine. A booster was given 33 days later. Animals from group VI remained unvaccinated as negative control. Vaccinated and nonvaccinated animals were bled by cardiac puncture on days 0, 27, and 67 postvaccination, and antibody titers against RHDV were evaluated by the HI test.

Challenge of rabbits and detection of RHDV in the liver

Sixty-seven days after the first vaccination, all the rabbits (vaccinated and nonvaccinated) were challenged by the intranasal route with 10280 HAu in 0.5 ml of PBS of an RHDV virulent strain (equivalent to 1125 LD_{50}). The animals were observed daily and deaths were monitored until 15 days postinfection. Two weeks after challenge, surviving animals were bled and slaughtered. The livers from all the challenged animals were frozen at −80°C immediately after death. Later, they were thawed, homogenized in PBS, and tested for the presence of RHDV by HA of human red blood cells type O.

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