Plant Biotechnology Journal

Plant Biotechnology Journal (2009) **7**, pp. 537–549 doi: 10.1111/j.1467-7652.2009.00422.x

Pea-derived vaccines demonstrate high immunogenicity and protection in rabbits against rabbit haemorrhagic disease virus

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Received 22 December 2008;
revised 27 March 2009;
accepted 30 March 2009.
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Keywords: adjuvant, cholera toxin B subunit (CTB), plant-derived vaccines, rabbit haemorrhagic disease virus (RHDV), VP60.

Summary

Vaccines against rabbit haemorrhagic disease virus (RHDV) are commercially produced in experimentally infected rabbits. A genetically engineered and manufactured version of the major structural protein of RHDV (VP60) is considered to be an alternative approach for vaccine production. Plants have the potential to become an excellent recombinant production system, but the low expression level and insufficient immunogenic potency of plant-derived VP60 still hamper its practical use. In this study, we analysed the expression of a novel multimeric VP60-based antigen in four different plant species, including *Nicotiana tabacum* L., *Solanum tuberosum* L., *Brassica napus* L. and *Pisum sativum* L. Significant differences were detected in the expression patterns of the novel fusion antigen cholera toxin B subunit (CTB)::VP60 (*ctbvp60*$_{SEKDEL}$) at the mRNA and protein levels. Pentameric CTB::VP60 molecules were only detected in *N. tabacum* and *P. sativum*, and displayed equal levels of CTB, at approximately 0.01% of total soluble protein (TSP), and traces of detectable VP60. However, strong enhancement of the CTB protein content via self-fertilization was only observed in *P. sativum*, where it reached up to 0.7% of TSP. In rabbits, a strong decrease in the protective vaccine dose required from 48–400 μg potato-derived VP60 [Castanon, S., Marin, M.S., Martin-Alonso, J.M., Boga, J.A., Casais, R., Humara, J.M., Ordas, R.J. and Parra, F. (1999) Immunization with potato plants expressing VP60 protein protects against rabbit hemorrhagic disease virus. *J. Virol*. **73**, 4452–4455; Castanon, S., Martin-Alonso, J.M., Marin, M.S., Boga, J.A., Alonso, P., Parra, F. and Ordas, R.J. (2002) The effect of the promoter on expression of VP60 gene from rabbit hemorrhagic disease virus in potato plants. *Plant Sci*. **162**, 87–95] to 0.56–0.28 μg antigenic VP60 (measured with VP60 enzyme-linked immunosorbent assay) of crude CTB::VP60 pea extracts was demonstrated. Rabbits immunized with pea-derived CTB::VP60 showed anti-VP60-specific antibodies, similar to RikaVacc®-immunized rabbits, and survived RHDV challenge.

Introduction

Rabbit haemorrhagic disease (RHD) is an acute and highly infectious viral disease of the European rabbit (*Oryctolagus cuniculus*). RHD causes high economic losses in rabbit meat production and has dramatically reduced the wild rabbit population. First identified in China (Liu *et al*., 1984), RHD subsequently spread to other parts of the world (Huang,

1991; Mitro and Krauss, 1993). After an incubation period of up to 3 days, morbidity achieves 100% and, in adults, a mortality of up to 90% is observed. The pathology is characterized by severe liver damage and disseminated intravascular coagulation (Alonso *et al*., 1998). The RHD virus (RHDV) belongs to the *Caliciviridae* family (Parra and Prieto, 1990). It harbours a 7.5-kb positive sense single-stranded RNA (ssRNA) (Meyers *et al*., 1991) which encodes the 60-kDa major structural protein and seven non-structural proteins. The capsid protein VP60 forms a 27–40-nm virion with a structured surface of regularly arranged cup-shaped depressions (Ohlinger *et al*., 1990). At present, all commercially available vaccines are prepared from the liver of experimentally infected rabbits. *In vitro* cell culture systems for RHDV propagation do not exist. As a result of serious reservations about the use of infectious animal material, as well as concerns for animal welfare, replacing this classical procedure with new recombinant systems is a challenge to the scientific community. In recent years, VP60 has been expressed in several heterologous systems, and successful immunization experiments in rabbits have been described. However, these recombinant vaccines have not been licensed and have not been available commercially to date (Boga *et al*., 1994, 1997; Laurent *et al*., 1994; Marin *et al*., 1995; Nagesha *et al*., 1995; Castanon *et al*., 1999; Fernandez-Fernandez *et al*., 2001).

Plants are considered to be a promising production platform for vaccines (Streatfield and Howard, 2003; Ma *et al*., 2005a; Thanavala *et al*., 2006), because many different immunogens have been expressed by higher plants (Biemelt *et al*., 2003; Warzecha *et al*., 2003; Webster *et al*., 2005; Mett *et al*., 2007). The most important advantages of plant-derived vaccines include the low production costs, capability for large-scale processing, convenient storage of the material, the absence of human or animal pathogens and reduced downstream processing (reviewed by Ma *et al*., 2005b; Streatfield, 2005a,b; Daniell, 2006). However, 25 years after the first antigen expression in plants (Mason *et al*., 1992), only one plant-derived antigen has received regulatory approval by the US Department of Agriculture (Vermij, 2006). The main problems with plant-derived vaccines seem to be low yields and vaccines of insufficient and inconsistent quality (Schillberg *et al*., 2005). In addition to the quantity of plant-derived antigens, their immunogenicity is one of the most crucial points. Immunization experiments against RHDV using transgenic potato demonstrated that between 48 and 400 μg of plant-derived VP60 was needed to induce protective immunity via the parenteral route (Castanon *et al*., 1999, 2002), and an oral application of at least 2000 μg of plant-derived VP60 was necessary to achieve protection in one of 10 rabbits (Martin-Alonso *et al*., 2003). The minimum protective dose of plant-derived VP60 seems to be considerably higher than that of baculovirus-expressed VP60, which requires a parenteral dose of 1 μg VP60 (Plana-Duran *et al*., 1996), or immunization with commercial vaccines (RikaVacc®, Riemser Arzneimittel AG, Greifswald-Insel Riems, Germany). Adjuvants, commonly used in immunization because of their stimulating effects on the immune system (Freytag and Clements, 2005; Azad and Rojanasakul, 2006; Lavelle and O'Hagan, 2006), seem to be necessary for further developments in plant-derived vaccines. One candidate is CTB, the non-toxic B subunit of cholera toxin (CTA-CTB_5), which demonstrates both mucosal and parenteral adjuvant activity in immunization procedures (Rask *et al*., 2000; Zhang *et al*., 2005). Pentameric CTB molecules bind to the oligosaccharide domain of a membrane glycolipid receptor (Lencer and Tsai, 2003) and enhance specific immune reaction of coupled antigens (Czerkinsky *et al*., 1989; George-Chandy *et al*., 2001). *In planta*, different genetically *ctb*-fused sequences form pentameric CTB::antigen complexes of between 70 and 210 kDa in size (Yu and Langridge, 2001; Kim and Langridge, 2003; Choi *et al*., 2005) with detectable adjuvant function (Yu and Langridge, 2001).

The aim of this study was to enhance the immunogenicity of the capsid protein VP60 in plant expression systems through genetic fusion to CTB. In order to produce immunogenic CTB::VP60, four different plant species, including *Nicotiana tabacum*, *Solanum tuberosum*, *Brassica napus* and *Pisum sativum*, were evaluated for expression levels. Finally, immunogenicity and protection against RHDV were tested in rabbits.

Results

Comparative expression studies of the novel multimeric protein complex CTB::VP60 in four plant species

The integration of the binary vectors pGK and pGK*ctbvp60*$_{SEKDEL}$ in *Agrobacterium tumefaciens* LBA4404, MP90 and EHA105 (Table 1) was confirmed by plasmid isolation and restriction analysis (data not shown). Transgenic plants transformed with pGK*ctbvp60*$_{SEKDEL}$ (Figure 1a) were analysed by polymerase chain reaction (PCR) using specific primers to detect *ctb* and *vp60*. Five to 19 transgenic plants, which demonstrated specific PCR products for these three coding regions, were selected per species (Table 1).

Transgenic plants were characterized by Northern blot analysis using a specific *ctb* probe. T0 plants of *N. tabacum*

Table 1 Summary of the characterization of regenerated transgenic plants. *Nicotiana tabacum*, *Solanum tuberosum*, *Brassica napus* and *Pisum sativum* transformed with pGK*ctbvp60*$_{SEKDEL}$ for the constitutive expression of CTB::VP60

		Transgenic plants			
Plant species	*Agrobacterium tumefaciens* (strain)	Regenerated plants	*ctbvp60*$_{SEKDEL}$ DNA	*ctbvp60*$_{SEKDEL}$ mRNA	CTB::VP60 protein
Nicotiana tabacum	LBA4404-GK*ctbvp60*$_{SEKDEL}$	24	19	3	1
Solanum tuberosum	LBA4404-GK*ctbvp60*$_{SEKDEL}$	12	5	3*	0
Brassica napus	MP90-GK*ctbvp60*$_{SEKDEL}$	8	5	3*	0
Pisum sativum	EHA105-GK*ctbvp60*$_{SEKDEL}$	15	14	8	6

*Truncated mRNA fragments.

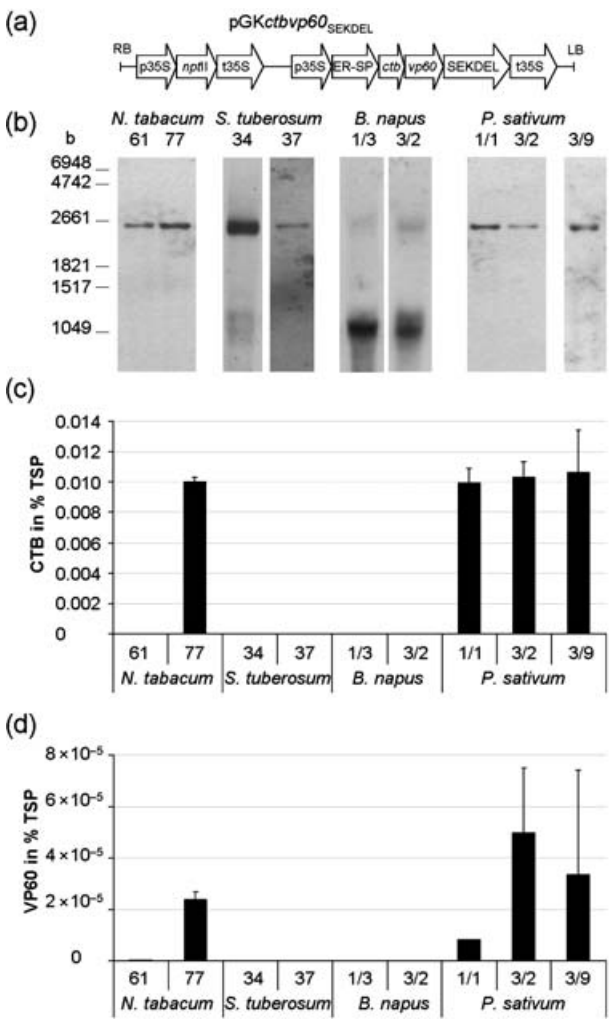

Figure 1 Expression of CTB::VP60 in four different plant species: *Nicotiana tabacum*, *Solanum tuberosum*, *Brassica napus* and *Pisum sativum* (*in vitro* cultivation, except for Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3/9). Transgenic *B. napus* and *P. sativum* plants were analysed in the T1 generation.* (a) Schematic diagram of the construct used for plant transformation to generate plants expressing CTB::VP60. The constructs contain a synthetic gene encoding cholera toxin B subunit (CTB) with signal sequence in translational fusion to the synthetic *vp60* open reading frame in a pLH9000 vector background with the cauliflower mosaic virus (CaMV) 35S promoter (p35S) and terminator (t35S): pGK*ctbvp60*$_{SEKDEL}$. The marker gene neomycin phosphotransferase II (*npt*II), regulated by p35S and t35S, was integrated into the constructs (RB, right border of the T-DNA; LB, left border of the T-DNA). (b) Northern blot analysis performed for the confirmation of *ctb*vp60 mRNA expression in transgenic plants transformed with GK*ctbvp60*$_{SEKDEL}$. The numbers at the top represent individual plants. (c) CTB enzyme-linked immunosorbent assay (ELISA) of plant leaf material. ELISA was performed with two specific CTB antibodies and CTB standard (bacterial CTB, Sigma) to calculate the amount of plant-derived CTB. (d) VP60 ELISA of plant leaf material. ELISA was performed with two specific VP60 antibodies and VP60 standard (baculovirus-derived VP60) to calculate the amount of plant-derived antigenic VP60. TSP, total soluble protein. *The transgenic plants (*B. napus* and *P. sativum*) were produced at the Plant Biotechnology Institute, National Research Council of Canada. The seeds of the transgenic plants were sent to the University of Rostock. The T1 and subsequent generation(s) were further analysed at the University of Rostock.

and *S. tuberosum* and T1 plants of *P. sativum* and *B. napus* were analysed. As demonstrated in Figure 1b, all four species showed *ctb*-specific mRNAs at the expected size of approximately 2.5 kb. However, in transgenic *B. napus* plants, truncated *ctb*-specific mRNAs of approximately 1 kb were mainly detected, in addition to that expected at 2.5 kb. A similar variation of small truncated *ctb*-specific mRNAs was detected in *S. tuberosum* plants, although in small amounts. Neither *N. tabacum* nor *P. sativum* plants showed any truncated *ctb*-specific mRNAs (Figure 1b). Plants of all four species with relatively high *ctb*-specific mRNA expression levels were selected for protein analysis (Figure 1c,d). In contrast with CTB levels of around 0.01% of total soluble protein (TSP) in several tobacco and pea plants (Figure 1c), only traces of VP60 were detectable (Figure 1d). The highest concentrations of VP60 were demonstrated in *N. tabacum* (SRI/GK*ctbvp60*$_{SEKDEL}$ #77 with 0.2 ng VP60/mg TSP) and *P. sativum* (Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3 with 0.5 ng VP60/mg TSP) (Figure 1d). There was no CTB in *S. tuberosum* and *B. napus* plants, but very small amounts of VP60 (less than 0.001 ng/mg TSP, $< 1 \times 10^{-7}$) were detected.

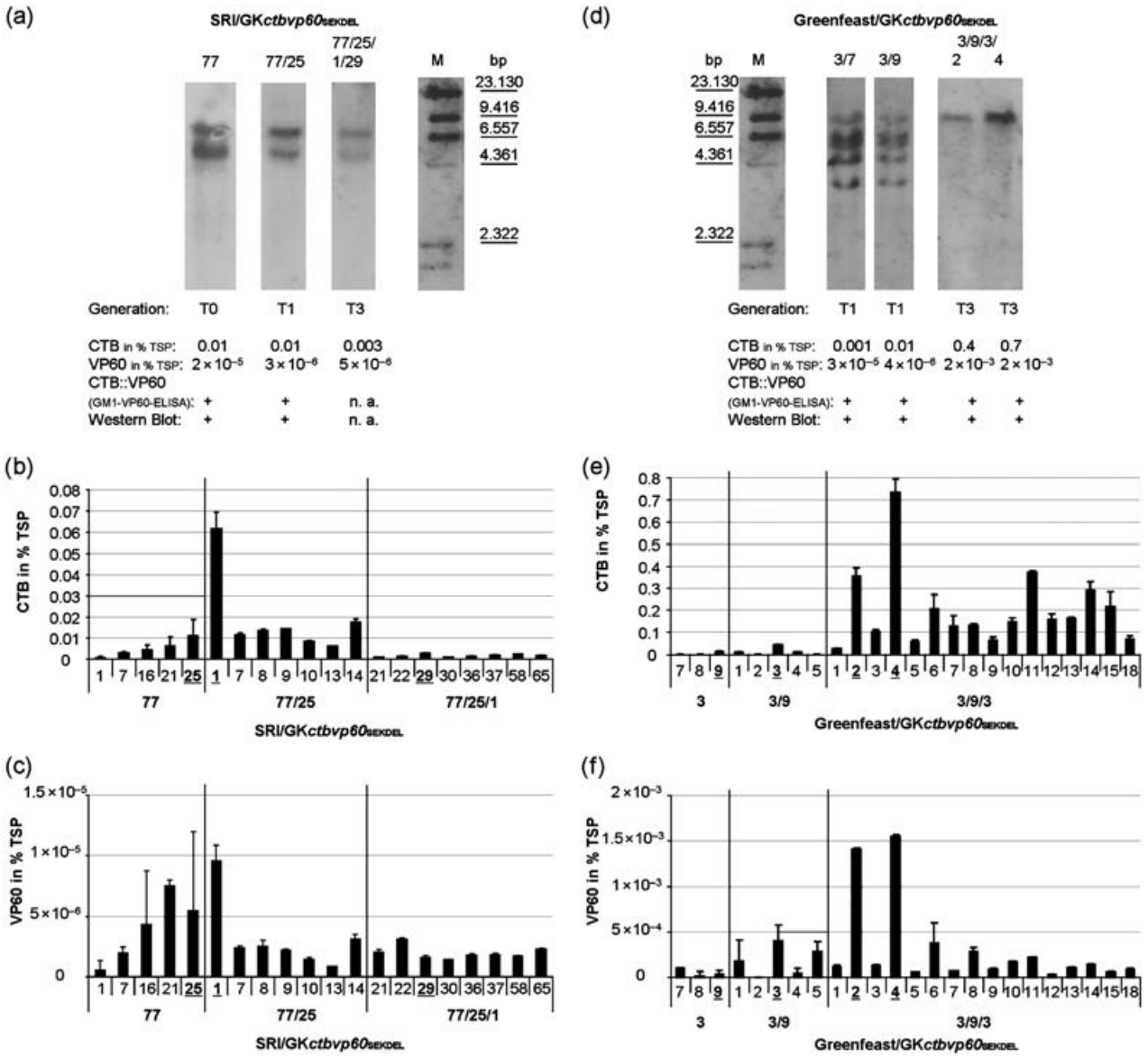

Figure 2 Analysis of *ctbvp60* transgene copy number and expression level in tobacco plant SRI/GK*ctbvp60*$_{SEKDEL}$ #77 (T0, T1 and T3) and pea plant Greenfeast/GK*ctbvp60*$_{SEKDEL}$ (T1 and T3) (*in vivo* cultivation). (a) Southern blot analysis showing the number of *ctbvp60* gene copies integrated in the genome of transgenic SRI/GK*ctbvp60*$_{SEKDEL}$ #77, 77/25 and 77/25/1/29; 50 μg of DNA digested with *Hin*dIII was probed with the *ctb* coding sequence. The fragments are indicated by the marker (M). Concentration of specific CTB and VP60 content in the transgenic *Nicotiana tabacum* plants and the results of non-quantifiable methods [combined GM1-VP60 enzyme-linked immunosorbent assay (ELISA), CTB Western blot] are listed. (b) CTB ELISA of selected SRI/GK*ctbvp60*$_{SEKDEL}$ #77 (T1, T2 and T3) plants. (c) VP60 ELISA of selected SRI/GK*ctbvp60*$_{SEKDEL}$ #77 (T1, T2 and T3) plants. (d) Southern blot analysis showing the number of *ctbvp60* gene copies integrated in the genome of transgenic Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3/7, 3/9, 3/9/3/2 and 3/9/3/4; 50 μg of DNA digested with *Hin*dIII was probed with the ctb coding sequence. The fragment sizes are indicated by the marker (M). Concentration of specific CTB and VP60 content in transgenic *P. sativum* plants and the results of non-quantifiable methods (combined GM1-VP60 ELISA, CTB Western blot) are listed. (e) CTB ELISA of selected Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3 (T1, T2 and T3) plants. (f) VP60 ELISA of selected Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3 (T1, T2 and T3) plants.

Glasshouse-cultivated Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3/9 and SRI/GK*ctbvp60*$_{SEKDEL}$ #77 were used for further multiplication of plant material via self-fertilization. The progeny of transgenic plants were analysed, and plants with a high concentration of CTB::VP60 were used to produce the next generation by self-fertilization. The progeny of tobacco plant #77 showed a small increase in CTB content in the T2 generation only (Figure 2b). In T3 plants, the CTB::VP60 concentration decreased. The number of transgene integrations (two) was stable within the progeny (Figure 2a). In contrast,

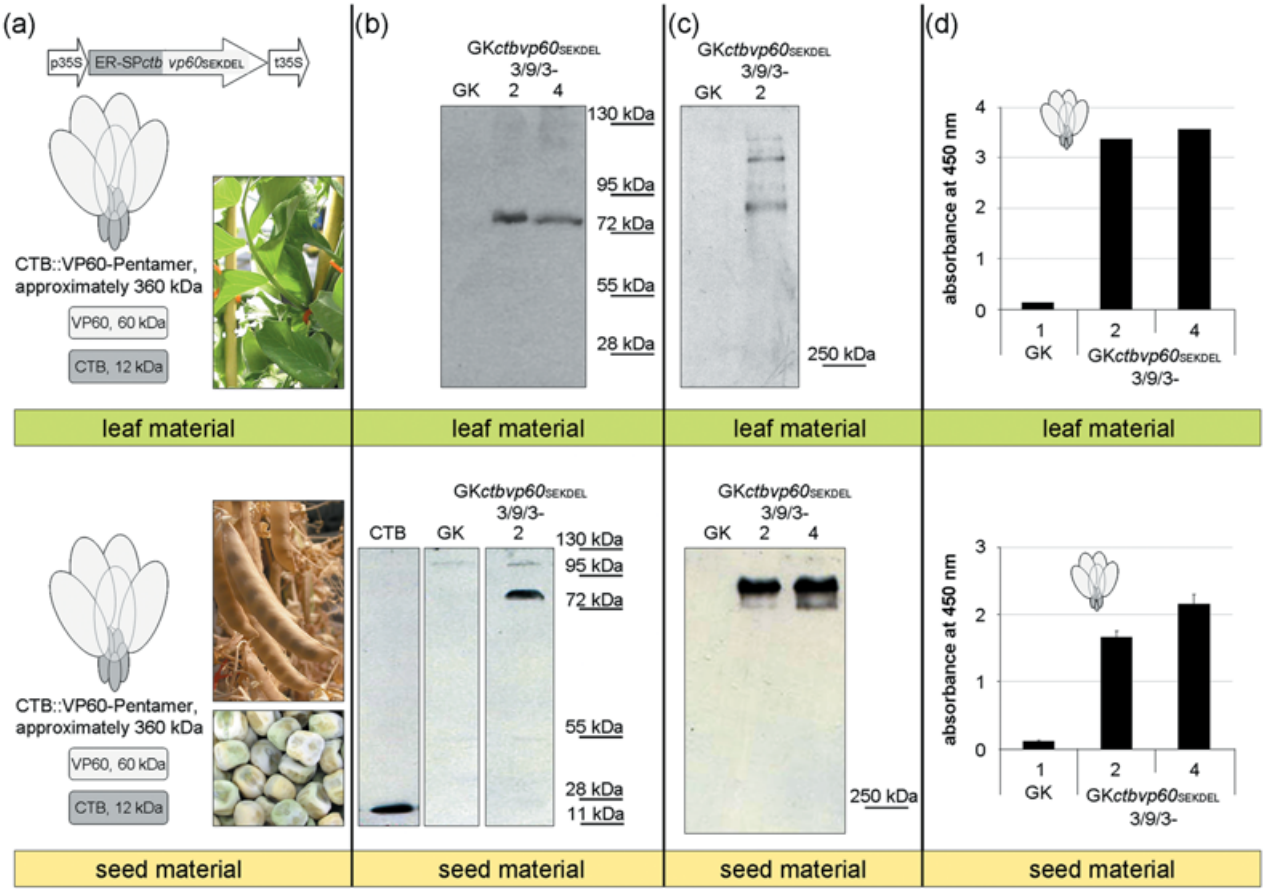

Figure 3 Analysis of multimeric CTB::VP60 molecules in the T3 generation of pea plants Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3. (a) Schematic diagram of the construct and schematic overview of the pentameric structure of CTB::VP60 molecules caused by the formation of a pentameric CTB complex in pea leaves and seeds. (b) Western blot showing CTB-specific molecules separated by 6% and 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. (c) Western blot showing CTB-specific molecules separated by 6% SDS-PAGE under non-reducing conditions. (d) Enzyme-linked immunosorbent assay (ELISA) of multimeric CTB::VP60 molecules. ELISA was performed with GM1-monoganglioside and specific VP60 antibody (sandwich ELISA).

in Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3/9/3/4, this strategy led to an increase in the concentration of immunogenic CTB::VP60 to up to 0.7% TSP (7 μg CTB/mg TSP; Figure 2d,e) in the leaf material, measured by CTB, GM1 and VP60 enzyme-linked immunosorbent assay (ELISA) in about 300 pea plants of the T3 generation (Figure 2d,f). The number of transgene integrations decreased from five in the T1 generation to one in the T3 generation high-expressing plants (Figure 2d).

Multimeric protein complexes were further analysed by a combined ELISA in order to detect pentameric CTB molecules with fused VP60 antigens. All of the tested pea plants with positive CTB- and VP60-specific signals showed an ELISA response in this mixed assay (Figure 3d). Western blot analysis with polyclonal CTB-specific antibodies confirmed the presence of high-molecular-weight fusion proteins. Primarily, two to four different bands with sizes greater than 250 kDa were detected under non-reducing sample buffer conditions (Figure 3c). By using reducing conditions, a specific protein band with a size of approximately 78 kDa has been documented (Figure 3b). When compared with pea plant Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3/9/3/2 and 4, tobacco plants SRI/GK*ctbvp60*$_{SEKDEL}$ #77/25 and #77/25/1 also showed antigenic and pentameric CTB::VP60 molecules (Mikschofsky *et al*., 2009). In Western blot analysis with monoclonal VP60-specific antibody, CTB::VP60 was not visible under reducing or non-reducing conditions (data not shown).

In pea plants, the CTB::VP60 content of the seeds was lower than that in leaves, and decreased during maturation (data not shown). However, based on the high protein content in mature seeds (around 25%), the CTB::VP60 content per dry weight was similar to that in lyophilized leaf material (data not shown). Again, seed-derived CTB::VP60 was detected by VP60, CTB and GM1-VP60 ELISA, as well as by Western blot analysis with polyclonal CTB-specific

Table 2 Seroconversion and rabbit haemorrhagic disease virus (RHDV) challenge of rabbits immunized with pea seed-derived CTB::VP60

		Optical density (490 nm) of anti-VP60 antibodies in sera of immunized rabbits					Challenge results	
Group, vaccine	Animal no.	Day 1*	Day 31	Day 54	Day 68	Day 77 (DY + 7)	Death	HAE†
I, 1.2 µg VP60 of pea CTB::VP60	1	0.02	–	0.78	0.86	0.92	S	< 2
	2	0.03	–	0.62	0.89	1.09	S	< 2
	3	0.02	–	0.88	0.89	1.38	S	< 2
	4	0.05	–	0.4	1.07	1.34	S	< 2
	5	0.05	–	0.82	0.95	1.15	S	< 2
	6	0.03	–	1.15	0.98	1.63	S	< 2
II, *npt*II pea	7	0.05	0.03	0.05	0.12	–	DY + 3	2^{12}
	8	0.03	0.04	0.04	0.05	–	DY + 2	2^{9}
	9	0.05	0.05	0.07	0.08	–	DY + 3	2^{10}
	10	0.05	0.08	0.04	0.07	–	DY + 2	2^{13}
	11	0.07	0.05	0.06	0.08	–	DY + 4	2^{11}
	12	0.02	–	0.06	0.12	–	DY + 4	2^{12}
III, RikaVacc®	13	0.04	0.37	0.65	0.78	1.21	S	< 2
	14	0.05	0.39	0.54	0.71	1.14	S	< 2
	15	0.03	0.55	0.43	0.95	1.28	S	< 2
IV, Control (non-vaccinated)	16	0.03	–	–	–	–	Dy + 4	2^{12}
	17	0.05	–	–	–	–	DY + 3	2^{13}
	18	0.05	–	–	–	–	DY + 2	2^{12}

*Rabbits were vaccinated on day 1, bled on day 31, given a booster on days 42 and 49, bled again on day 54, given a third booster on day 56, bled again on day 68 and challenged (DY) on day 70. Surviving animals (S) were bled again after 7 days [on day 77, DY + (days after challenge)]. Non-surviving animals died 2–4 days after challenge (DY + 2/3/4).

†HAE test, presence of RHDV was determined by haemagglutination of human red blood cells. Titres < 2 were considered to be negative.

–, anti-VP60 antibodies in sera of rabbits were not measured.

antibodies using reducing and non-reducing conditions (Figure 3).

In order to analyse the genotype of high-expressing transgenic pea line Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3, 18 of 55 cultivated plants (T3 generation) were analysed for transgene integration. All 18 siblings showed one identical *ctb*-specific signal, as illustrated in Figure 2c, indicating one integration with the same locus in all individuals. This is underlined by the fact that the same signal was demonstrated using a neomycin phosphotransferase II (*npt*II)-specific probe (data not shown). The mature seeds of these 55 plants (T3) were selected for immunization analysis. The mean concentration of multimeric CTB::V60 in seed material used for immunization was approximately 10 ± 3.8 ng antigenic VP60/mg TSP (0.001% TSP) and 380 ± 8.5 ng CTB/mg TSP (0.04% TSP).

Immunogenicity and protection of pea-derived CTB::VP60 in rabbits

The immunogenicity of the CTB::VP60 protein expressed in both seed and leaf material of peas was studied in the natural host of RHDV. In order to allow a direct comparison of previous immunization experiments with plant-derived VP60 using adjuvants to strengthen the immune response (Castanon *et al*., 1999, 2002; Fernandez-Fernandez *et al*., 2001), we added the adjuvant Polygen to plant-derived CTB::VP60. For the same reason, we selected a four-time immunization regime. Six rabbits immunized by the intramuscular route (one time priming, three times boosting) with extract from approximately 24 g of seed material (4 × 6 g) from CTB::VP60-expressing pea plants demonstrated a high level of anti-VP60-specific antibodies in the sera (Table 2). These concentrations were similar to or even higher than those of rabbits immunized with commercial vaccine RikaVacc®. As shown in Table 2, rabbits immunized with seed extract from control plants expressing NPTII showed no VP60 antibodies. Afterwards, all rabbits immunized with CTB::VP60 pea seed extract (approximately 1.12 µg antigenic VP60, measured by VP60 ELISA) and RikaVacc® (approximately 1 µg VP60) survived exposure to the RHDV strain 'Eisenhüttenstadt' (Schirrmeier *et al*., 1999) and demonstrated an enhanced VP60-specific antibody level.

In order to identify the minimal immunogenic dose of pea-derived antigens, the application dose of antigenic VP60

Table 3 Seroconversion and rabbit haemorrhagic disease virus (RHDV) challenge of rabbits immunized with pea leaf-derived CTB::VP60

		Optical density (490 nm) of anti-VP60 antibodies in sera of immunized rabbits								Challenge results	
Group, vaccine	Animal no.	Day 1*	Day 22	Day 44	Day 51	Day 61	Day 67	Day 70	Day 77 (DY + 7)	Death	HAE†
V, 1.12 μg VP60 of pea CTB::VP60	19	0.01	0.05	0.23	0.80	0.93	0.89	0.92	0.81	S	< 2
	20	0.02	0.04	0.36	0.92	1.43	1.54	1.46	1.26	S	< 2
VI, 0.56 μg VP60 of pea CTB::VP60	21	0.04	0.02	0.10	0.68	0.90	0.97	0.80	0.81	S	< 2
	22	0.06	0.02	0.06	0.14	0.21	0.24	0.18	0.21	S	< 2
VII, 0.28 μg VP60 of pea CTB::VP60	23	0.03	0.01	0.36	0.54	1.01	1.05	0.97	0.78	S	< 2
	24	0.03	0.01	0.03	0.09	0.28	0.29	0.17	–	DY + 2	2^{11}
VIII, *npt*II pea	25	0.02	0.03	0.03	0.06	0.06	0.01	0.05	–	DY + 2	2^{13}
	26	0.05	0.02	0.02	0.05	0.01	0.02	0.04	–	DY + 3	2^{13}
RikaVacc®	27	0.01	0.89	0.90	0.72	0.99	1.08	1.12	1.28	S	< 2
Control (non-vaccinated)	28	0.01	0.01	0.01	0.03	0.00	0.02	0.02	–	DY + 2	2^{12}

*Rabbits were vaccinated on day 1, bled on day 31, given a booster on days 42 and 49, bled again on day 54, given a third booster on day 56, bled again on day 68 and challenged (DY) on day 70. Surviving animals (S) were bled again after 7 days [on day 77, DY + (days after challenge)]. Non-surviving animals died 2–4 days after challenge (DY + 2/3/4).

†HAE test, presence of RHDV was determined by haemagglutination of human red blood cells. Titres < 2 were considered to be negative.

–, anti-VP60 antibodies in sera of rabbits were not measured.

was reduced by factors of two and four. Again, rabbits were immunized four times (one time priming, three times boosting) with 0.07, 0.14 and 0.28 μg antigenic VP60 (measured with VP60 ELISA) of the complex CTB::VP60 of lyophilized pea leaf material (0.7 g). After the first boosting procedure, anti-VP60-specific antibodies were detectable in rabbits immunized with CTB::VP60 pea leaf material (Table 3, days 44 and 51). Again, the anti-VP60-specific antibody level was similar to that of RikaVacc®-immunized rabbits (Table 3; days 51–77). One rabbit of each lower vaccine dose (rabbits #22 and #24) had lower levels of anti-VP60-specific antibodies. Only one rabbit (#24) which had been immunized with 0.28 μg VP60 did not survive RHDV challenge. This rabbit demonstrated nearly the same anti-VP60-specific antibody concentration as rabbit #22, which had been immunized with 0.56 μg VP60 and survived the challenge (Table 3).

Discussion

Until now, relatively large amounts of plant-derived VP60 (> 40 μg) have been used in immunization studies of rabbits. In previous experiments with the model plant *N. tabacum* (SRI-GK*vp60*), we demonstrated that approximately 1.1 μg of tobacco-derived VP60 was insufficient to induce VP60-specific antibodies in mice (Mikschofsky *et al*., 2009). In order to enhance the immunogenic potential, a synthetic coding region for the common adjuvant CTB was fused to the open reading frame (ORF) of VP60 (Mikschofsky *et al*., 2009).

In this study, we evaluated the expression of the novel multimeric protein complex CTB::VP60 in four different plant species. In contrast with *S. tuberosum* and *B. napus*, *N. tabacum* and *P. sativum* showed a detectable amount of multimeric CTB::VP60 complexes. The partly fragmentary transcription of the integrated construct *ctbvp60*$_{SEKDEL}$ by transformed potato and canola plants had already indicated an incorrect expression at the mRNA level. In addition, low translation frequency or protein instability of the recombinant fusion protein can cause the absence of CTB::VP60. This is in contrast with earlier comparative expression studies of the antibody scFv84.66 in tobacco, rice, wheat, peas and tomatoes (Stoger *et al*., 2002), and of phytase in tobacco, rice and *Medicago truncatula* (Abranches *et al*., 2005). These studies demonstrated broadly comparable production levels (Schillberg *et al*., 2005). An event-specific basis for the incorrect expression in *B. napus* can almost be excluded, as all PCR-positive transgenic plants showed identical expression patterns at the mRNA level. However, this observation may be caused by the transgene itself and may not hold true for other transgenes, as observed by Stoger *et al*. (2002). Therefore, it may be the case that the optimal species for producing a pharmaceutical may differ depending on the transgene.

We showed that, on the basis of careful selection of parental lines, the expression of the novel multimeric CTB::VP60 antigen could be increased from 0.01% TSP (T1 generation) to 0.7% TSP (T3 generation) in one transgenic pea line and to 0.06% TSP (T2 generation) in one transgenic tobacco line. This

corresponds to the enhancement of the *Escherichia coli* heat-labile enterotoxin subunit B (LTB) antigen content in maize kernels (Chikwamba *et al*., 2002) and cyanophycin expression in tobacco (Huhns *et al*., 2008) in the offspring after selection. The reduction of T-DNA integrations to one copy in selected *P. sativum* plants may be responsible for the increase in CTB::VP60 (Figure 2). Huhns *et al*. (2008) also demonstrated a higher cyanophycin expression in plants after a reduction in the number of T-DNA integration sites; nevertheless, an increase in further generations could also be observed without a concomitant reduction in integration sites.

The detection of multimeric CTB::VP60 at approximately 350 kDa was carried out using different non-quantifiable methods (combined GM1-VP60 ELISA, CTB Western blot); quantification was performed using single quantitative double-sandwich ELISA of each fusion partner CTB and VP60 alone. Interestingly, different amounts of both partners CTB and VP60 were demonstrated in each plant analysed using specific antibodies (double-sandwich ELSIA), with less VP60 than CTB. This may result from a reduced detection of antigenic VP60 caused by sugar residues on VP60 molecules, rather than by a reduced amount of VP60. In Western blot analysis (reducing conditions), CTB::VP60 molecules were detected at the expected size of approximately 78 kDa with CTB-specific, but not with VP60-specific, antibodies. Hence, VP60 must be present in the same amounts as CTB; nevertheless, it seems to be less detectable possibly because of a masking factor that is still present under reducing conditions. There are nine putative *N*-glycosylation sites, two within *ctb* and seven within the *vp60* sequence (Caragea *et al*., 2007). In addition, there are 13 putative *O*-glycosylation sites within the *vp60* sequence (Caragea *et al*., 2007). We assume that sugar molecules attached in the secretory pathway mask VP60 epitopes and prevent optimal binding by VP60-specific antibodies generated in guinea pig and mice using non-glycosylated VP60. Naturally, VP60 is not expressed via a secretory pathway (Meyers *et al*., 1991). However, because of the needs of the endoplasmic reticulum or chloroplast environment to create biologically active CTB molecules *in planta*, *ctbvp60* must also be produced within the chloroplast or within the secretory expression system (Mikschofsky *et al*., 2009). Until now, only natural secretory antigens or parts of antigens have been fused to the *ctb* ORF, and their concentration has always been estimated via the CTB reaction (Arakawa *et al*., 2001; Kim and Langridge, 2003; Choi *et al*., 2005; Li *et al*., 2006).

The immunogenic potential of tobacco-derived CTB::VP60 was tested first in mice with a one-fold intramuscular immunization procedure (Mikschofsky *et al*., 2009). In comparison, *Arabidopsis thaliana*-derived VP60 demonstrated a specific anti-VP60 antibody titre in mice after a three-fold intraperitoneal immunization procedure, as well as with an oral immunization strategy with five oral feedings of leaf material and one additional boosting of baculovirus-derived VP60 (Gil *et al*., 2006). In order to estimate the immunogenic and protective potential of pea-derived CTB::VP60, different organs (seed and leaf material) of constitutively expressing Greenfeast/GK*ctbvp60*$_{SEKDEL}$ #3 siblings were used for the immunization of rabbits. Most of the serologic responses of rabbits immunized with crude pea seed and leaf extracts expressing CTB::VP60 were as high as those of animals treated with the commercial RikaVACC® vaccine (Tables 2 and 3). An analysis of different plant-derived CTB::VP60 doses revealed a minimal protective dose of approximately 0.28–0.56 μg antigenic VP60, which was sufficient for three of four rabbits. The amount of antigenic VP60 has been measured by VP60 ELISA only; possibly larger amounts of VP60 without or with less capacity for antibody binding cannot be excluded. Hence, the reduction in the protective dosage might be lower than considered here. Although rabbit #24 was not protected (0.28 μg antigenic VP60), it showed an antibody level similar to that of rabbit #22 (0.56 μg antigenic VP60), which survived virus challenge. Similar results were obtained in oral immunizations with potato-derived VP60, where only one rabbit survived the RHDV challenge. However, three other rabbits demonstrated similar anti-VP60-specific antibodies in the blood (Martin-Alonso *et al*., 2003). Thus, different immunity in rabbits with very low antibody levels [optical density (OD) at 490 nm: 0.17 and 0.18, Table 3] seems to indicate the threshold of the protecting antibody level.

In order to allow a direct comparison with previous immunization experiments, we decided to use similar conditions to previous experiments (Castanon *et al*., 1999, 2002; Fernandez-Fernandez *et al*., 2001). Nevertheless, this is disproportionate compared with the conditions used for RikaVacc®, as this commercial vaccine is applied only once. Hence, in order to commercialize plant-derived RHD vaccines, the immunization procedure needs to be shortened to only one parenteral application. In addition, the protection provided by vaccination must be available within 2–3 weeks. Concomitantly, procedures for large-scale isolation, formulation and galenics of CTB::VP60 expressed in *P. sativum* need to be developed. Although both leaf and seed material demonstrates similar and high protective potential, seed-based expression systems offer advantages in accumulation in comparison with leaves in terms of both stability and the storage of proteins. Enhancement of CTB::VP60 concentration

in mature seed material using a seed-specific promoter may be an effective strategy to replace commercial rabbit-based RHD vaccines. The high immunogenity of pea-derived CTB::VP60 creates an excellent basis for the further development of parenteral plant-derived RHD vaccines and innovative oral immunization strategies against RHD.

Experimental procedures

Gene and vector construction

The amino acid sequences of CTB and VP60 were back-translated using the codon preference of tobacco, and the resulting artificial ORFs were assembled from synthetic oligonucleotides using a combined ligase chain reaction and PCR, essentially as described previously (Au *et al*., 1998; Hohle *et al*., 2005). The synthetic ORFs (GENBANK FJ807679 and FJ807680) were then cloned into the plasmid vector pSP73 (Promega, Mannheim, Germany). The ORF sequences *ctbvp60*$_{SEKDEL}$ were verified by automated nucleotide sequencing, and were subsequently cloned into the plant transfer vector pGK, a derivative of pLH9000 (Hausmann and Töpfer, 1999) that encodes an NPT. *Ctbvp60*$_{SEKDEL}$ was integrated between the cauliflower mosaic virus (CaMV) 35S promoter and terminator of pGK using *Sma*I and *Sal*I. The cloning procedures were performed using standard methods (Sambrook, 1989).

Plant transformation and tissue culture

The binary vectors pGK and pGK*ctbvp60*$_{SEKDEL}$ were transferred into three different *A. tumefaciens* strains, EHA105 (Hood *et al*., 1993), MP90 (Koncz and Schell, 1986) and LBA4404 (Hoekema *et al*., 1983) via electroporation. Afterwards, plasmids were re-isolated and control restriction was performed with *Not*I. *Pisum sativum* cv. Greenfeast was transformed using *A. tumefaciens*-mediated gene transfer, as described by Polowick *et al*. (2000). Briefly, the pea seed coat and one cotyledon were removed from each seed and the radicle was excised. The remaining embryo axis tissue was sliced longitudinally into approximately five slices and these slices were placed on a co-cultivation medium. A 5-μL droplet of *Agrobacterium* suspension culture was added to each of the pea seed slices for 4 days The slices where incubated for 4 days and, afterwards, the explants were transferred in the following order to callus, shoot and root induction media (Polowick *et al*., 2000) with 150 mg/L Timentin® and 50 mg/L kanamycin. *Brassica napus* Westar was transformed according to Moloney *et al*. (1989) using 4–5-day-old cotyledons. Each explant was dipped briefly into *Agrobacterium* suspension culture and transferred to co-cultivation medium (Moloney *et al*., 1989) for 3 days. Subsequently, explants were cultivated on callus, shoot and root induction media (Moloney *et al*., 1989) with 300 mg/L Timentin® and 20 mg/L kanamycin. *Nicotiana tabacum* cv. Petit Havana SRI and *S. tuberosum* cv. Albatros were transformed according to Wohlleben *et al*. (1988) and Horsch *et al*. (1985), respectively, with slight modifications. Potato leaves were wounded with razor blades and co-cultivated for 3 days with *Agrobacterium*. To induce callus, explants were cultivated for 10 days on Murashige and Skoog (MS) medium supplemented with 5 mg/L α-naphthaleneacetic acid (NAA), 0.1 mg/L 6-benzylaminopurine (BAP), 100 mg/L kanamycin and 250 mg/L cefotaxime. Explants were transferred to MS medium supplemented with 2 mg/L zeatinribose, 0.02 mg/L NAA, 0.002 mg/L gibberellic acid (GA3), 100 mg/L kanamycin and 250 mg/L cefotaxime. The explants were transferred to fresh medium every 2 weeks. Shoots were transferred to hormone-free MS medium containing 100 mg/L kanamycin and 250 mg/L cefotaxime for root induction.

Analysis of putative transgenic plants

Plant DNA was extracted from 100 mg of *in vitro* plant material using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The presence of the different transgenes, including *vp60*, *ctb* and *npt*II, was confirmed by PCR amplification using the following specific primer pairs: VP60syn-fw (5′-AGGCTA GAACTGCTCCACAA-3′) and VP60syn-rv (5′-CCATTAGCAGTTC CTCCAAG-3′); ctB-fw (5′-CTTCCTCCACACCACAGAAC-3′) and ctB-rv (5′-GTCCGTTTGCCATTGAGATA-3′); nptIIfw (5′-TCCGGCCGCTT GGGTGGAGAG-3′) and nptIIrv (5′-CTGGCGCGAGCCCCTGATGCT-3′).

Northern blot analysis

Total RNA was isolated from 100 mg of plant material, as described by Logemann *et al*. (1987). The RNA was denatured, separated by 1.2% formaldehyde/agarose gel electrophoresis, and transferred to Hybond-N membranes (Amersham Biosciences, Bucks, UK) by capillary blotting. The blotting, preparation of probes (*ctb*-specific) and hybridization were performed according to the methods described by Neumann *et al*. (1997).

ELISA for the quantification of VP60

The plant material was ground in liquid nitrogen, and the resulting powder was extracted three times with phosphate-buffered saline (PBS, pH 7.2) by mixing and subsequent centrifugation (5 min, 16 000 ***g***). The TSP content of each extract was determined according to Bradford (1976).

An ELISA was performed as described previously (Schirrmeier *et al*., 1999). Briefly, plates were coated with an RHDV antiserum raised in guinea pigs. After the plant extract had been added, a mixture of three murine monoclonal antibodies against VP60 was used to detect recombinant VP60. Bound antibodies were visualized using peroxidase-labelled anti-mouse immunoglobulin G (IgG) (Sigma, St. Louis, MO, USA) and 3,3′,5,5′-tetramethyl-benzidine (TMB) as the substrate (Sigma). Between each of the incubation steps, the plates were washed three times with PBST [PBS, 0.05% Tween20 (v/v)]. Finally, the absorbance (OD) at 450 nm was measured using an ELISA reader (BioTek Synergy™ HT, BioTek Instruments, Inc., Winooski, VT, USA). To calculate the relative amount of VP60 in the plant material, 100 ng of VP60, purified from insect cells infected with His-tagged VP60-expressing recombinant baculovirus, served as standard to prepare the dilution series. All of the plant samples were analysed twice, and the analysis of variance was carried out using the statistical analysis program Excel (Microsoft®).

ELISA for the quantification of CTB

The CTB protein level in transgenic pea plants was investigated by quantitative ELISA. A 96-well microtitre plate was coated with rabbit antiserum against CTB (NatuTec GmbH, Frankfurt, Germany), diluted 1 : 2000 in a carbonate buffer (50 mM Na_2CO_3, pH 9), and incubated for 2 h. It was then blocked with a 1% bovine serum albumin (BSA) solution for 1 h. Plant samples were incubated in duplicate for 2 h on coated plates, followed by incubation with goat antiserum against CTB diluted 1 : 5000 in PBS. After incubation with the anti-goat IgG–horseradish peroxidase conjugate (Dianova, Hamburg, Germany), diluted 1 : 5000 in PBS, bound antibodies were visualized with peroxidase-labelled anti-rabbit IgG (Sigma) and TMB as the substrate (Sigma). All incubation steps were performed at 37 °C, and three washes with PBST were performed between each step. Finally, the absorbance was measured at 450 nm using an ELISA reader (BioTek Synergy™ HT). CTB (Sigma) was used as positive standard. All plant samples were analysed twice, and the analysis of variance was carried out using the statistical analysis program Excel (Microsoft®).

GM1-ganglioside binding assay

The binding capacity of plant-derived CTB for the GM1-ganglioside receptor was evaluated using a GM1 ELISA for duplicate samples. The plate was coated with GM1-ganglioside receptor (Sigma) at 1 μg per well in carbonate buffer and incubated for 2 h at 37 °C. The subsequent steps were similar to those of CTB ELISA described above.

Combined GM1-ganglioside–VP60 assay

The multimeric character of the pea-expressed fusion protein CTB::VP60 was tested by a combined application of pentameric binding GM1-monoganglioside and monoclonal VP60-specific antibody mix. The plate was coated with GM1-ganglioside receptor (Sigma) at 1 μg per well in carbonate buffer, incubated for 2 h at 37 °C and then blocked with 1% BSA solution for 1 h. After the plant extract had been added, a mixture of three murine monoclonal antibodies against VP60 was used to detect recombinant VP60. Bound antibodies were visualized using peroxidase-labelled anti-mouse IgG (Sigma) and TMB as substrate (Sigma). All incubation steps were performed at 37 °C, and three washes with PBST were performed between each step. Finally, the absorbance was measured at 450 nm using an ELISA reader (BioTek Synergy™ HT). All plant samples were analysed twice, and analysis of variance was carried out using the statistical analysis program Excel (Microsoft®).

Western blot analysis

Leaf material from plants transformed by the different constructs (200 mg) was ground in liquid nitrogen, and the resulting powder was extracted three times with PBS by mixing and subsequent centrifugation (5 min, 16 000 ***g***). The TSP content of each extract was determined using the Bradford assay. Reducing and non-reducing sample buffer was added, and 100 μg of protein was separated on 6% and 10% sodium dodecylsulphate polyacrylamide gels. Proteins were transferred on to membranes (Amersham), blocked for 2 h in 5% skimmed-milk-TBST (20 mM Tris-HCl, 137 mM NaCl and 0.1% Tween 20, pH 7.0), and incubated with rabbit antiserum against CTB (NatuTec GmbH), diluted 1 : 2000 in TBS, and ECL™ anti-rabbit IgG (GE Healthcare, Munich, Germany), diluted 1 : 1500 in TBS. Bound antibody was detected using the ECL system.

Immunological assessment of pea-derived CTB::VP60 in rabbits

To investigate the immunogenicity of pea-derived VP60 in rabbits, two animal experiments (TV1 and TV2) were performed. First, 1 g of mature pea seeds of Greenfeast-NPTII and Greenfeast-CTB::VP60 were ground and extracted in 6 mL PBS and centrifuged twice for 10 min at 14 000 ***g***. After formulation of the supernatant with the adjuvant Polygen (MVP Laboratories, Inc, Omaha, NE, USA) at 15% (v/v), 4 mL was administered to 12–14-week-old rabbits by intramuscular route at two application sites. Vaccination was performed four times (one priming and three boosting at days 1, 42, 48 and 56). Group I (six rabbits) was immunized with pea seed extract of Greenfeast/GK*ctbvp60*$_{SEKDEL}$ and group II (six rabbits) with Greenfeast/GK extract. Three rabbits received commercial RHDV vaccine RikaVacc® (Riemser Arzneimittel AG) (Group III). Three rabbits remained unvaccinated (Group IV). Sera were collected prior to immunization and at days 31, 54, 68 and 77.

In a second trial, eight rabbits, aged 12–14 weeks, were separated into four groups; 0.7 g of lyophilized pea leaf material of Greenfeast/GK and Greenfeast/GK*ctbvp60*$_{SEKDEL}$ was extracted in 6 mL PBS and centrifuged three times for 15 min at 14 000 ***g***. After formulation of clear supernatant with the adjuvant Polygen at 15% (v/v), group V was immunized intramuscularly with 4 mL, group VI with 2 mL and group VII with 1 mL pea leaf extract of Greenfeast-*ctbvp60*$_{synSEKDEL}$/Polygen. Rabbits of group VIII were immunized with 4 mL of Greenfeast-NPTII/Polygen (negative control plasmid). Again, vaccination was performed four times (one priming and three boosting at days 1, 22, 48 and 56). Additionally, one rabbit served as a negative control and another rabbit was immunized with RikaVacc®. Sera were collected prior to immunization and at days 22, 44, 51, 61, 67, 70 and 77.

VP60 antibody ELISA

The sera of rabbits were tested by indirect ELISA. RHDV 'Eisenhüttenstadt', Accession number Y15440 (1 : 4000 in Tris-NaCl, pH 7.6), was coated on 96-well plates and incubated overnight at 4 °C. After the incubation of the sera of immunized rabbits (1 : 50 and 1 : 200) at 37 °C for 1 h, peroxidase labelled-anti-rabbit (1 : 20 000) (Sigma) was incubated again at 37 °C for 1 h. Bound antibodies were visualized using TMB as substrate (Sigma). Finally, the absorbance was measured at 450 nm using an ELISA reader (BioTek Synergy™ HT). All serum samples were analysed twice, and the analysis of variance was carried out using the statistical analysis program Excel (Microsoft®).

RHDV challenge

At day 70, all rabbits were inoculated intramuscularly with 2 mL of the virulent RHDV 'Eisenhüttenstadt', Accession number Y15440, containing $10^{4.5}$ lethal dose –50. The rabbits that died after the

challenge were necropsied; to confirm RHD, a liver sample was tested by haemagglutination test as described previously (Schirrmeier *et al*., 1999).

Acknowledgements

We thank Gisela Schirrmeier, Claudia Finck, Sonja Konopka, Marko Kranz, Joe Hammerlindl and David Baliski for their skilled technical assistance. This research was supported by grants from the Bundesministerium für Bildung und Forschung (BMBF, #0312744) and a DAAD German Academic Exchange Service scholarship.

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