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ScienceDirect
Virus Research 129 (2007) 80–86
Virus Research
www.elsevier.com/locate/virusres

Assessing the expression of chicken anemia virus proteins in plants

Cristiano Lacorte [a,b], Hendrikus Lohuis [a], Rob Goldbach [a,*], Marcel Prins [a,1]

[a] *Laboratory of Virology, Wageningen University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands*
[b] *EMBRAPA Recursos Genéticos e Biotecnologia, Parque Estação Biológica, Brasília, DF, Brazil*

Received 11 December 2006; received in revised form 24 June 2007; accepted 25 June 2007
Available online 14 August 2007

Abstract

Chicken anemia virus (CAV) is an important pathogen of chicken worldwide, causing severe anemia and immunodeficiency. Its small single-stranded DNA genome (2.3 kb) encodes three proteins: VP1, the only structural protein, VP2, a protein phosphatase, and VP3, also known as apoptin, which induces apoptosis. In this study, CAV proteins were expressed in plants as an alternative for recombinant protein production in animal cells. Additionally, the effect of VP3 expression was tested to evaluate possible involvement in programmed cell death in plants. The CAV genes were cloned in binary vectors with the Green fluorescent protein (GFP) as N terminal fusion, and into a Potato virus X (PVX) and Tobacco Mosaic Virus (TMV)-based vectors. *Nicotiana benthamiana* plants were inoculated with *Agrobacterium tumefaciens* containing the binary vector constructs or the PVX and TMV constructs. Upon transient expression GFP:VP1 and GFP:VP2 were observed throughout the nucleoplasm, whereas VP3 formed compact aggregates within the nucleus, indicating functional nuclear localization signals in all three proteins. An intense fluorescence was observed for VP2 and VP3 fusions, whereas GFP:VP1 fluorescence remained faint and was only detected in a limited number of cells. Co-expression of GFP:VP1 and VP2 had a marked alteration on the distribution of GFP:VP1, forming large VP1 aggregates throughout the nucleus, indicating an interaction of the two CAV proteins. No visible alteration on GFP pattern was detected upon co-expression of GFP:VP1 and VP3, or with GFP:VP2 and VP3. Plants infected with PVX or TMV-based vectors expressing VP3 displayed strong necrosis and wilting, however, a direct association with VP3 expression and programmed cell death in plants, could not be established. Overall, our results show that all CAV proteins can be expressed in plant cells, though expression level of VP1 needs to be further optimized before testing its potential as (edible) subunit vaccine.

Keywords: CAV; Plant-based expression; Oral vaccine; Apoptin

1. Introduction

Chicken anemia virus (CAV) is an important pathogen in chickens, causing severe immunodeficiency and anemia (Noteborn, 2004; Todd et al., 2001). The virus presents a small single-stranded DNA genome (2.3 kb) and has been classified in the *Circoviridae* family, being the only species assigned for the genus *Gyrovirus* (Todd et al., 2001). The negative sense genome presents three partially overlapping ORFs. VP1 (51 kDa) is the only structural protein found in purified particles (Todd et al., 1990); VP2 is a 24 kDa protein phosphatase and has been shown to interact with VP1, possibly helping VP1 assemble (Noteborn et al., 1998; Peters et al., 2002); and VP3, also known as apoptin (Noteborn, 2004; Noteborn et al., 1994). This 13 kDa protein induces apoptosis in infected chicken cells. Additionally, it was shown to cause apoptosis in human tumor cells, but not in normal human cells (Danen-Van Oorschot et al., 1997; Noteborn, 2005).

CAV has a narrow host range infecting chickens reared for the meat and egg industry worldwide. Economic losses can be very high as a consequence of outbreaks that cause mortality and morbidity due to secondary infections, and reduction in broiler weight (Adair, 2000; Todd et al., 2001). Difficulties in obtaining attenuated strains and the very slow growth rate and low titers that can be achieved from infected cell cultures have hampered the development of effective vaccines (Cunningham et al., 2001; Todd et al., 2001). The expression of VP1, VP2 and VP3 was achieved in insect cells inoculated with recombinant baculoviruses (Noteborn et al., 1998). Co-expression of VP2 and VP1 was essential for the recombinant induction of neutralizing antibodies (Koch et al., 1995; Noteborn et al., 1998) leading to the suggestion that VP2 would help VP1 – the sole viral struc-

* Corresponding author. Tel.: +31 317 483090; fax: +31 317 484820.
E-mail address: rob.goldbach@wur.nl (R. Goldbach).
[1] Present address: Keygene N.V., Wageningen, The Netherlands.

0168-1702/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.virusres.2007.06.020

tural protein – to achieve a proper stable conformation, by acting as a scaffold protein (Noteborn et al., 1998). The induction of neutralizing antibodies that could confer protection shows the viability of using recombinant subunit vaccines against CAV (Koch et al., 1995; Noteborn et al., 1998). However, for an effective vaccination schedule, and considering the large number of animals to be vaccinated, large amounts of the recombinant antigen would have to be produced, at low cost. An oral recombinant vaccine, for example, delivered through the drinking water or feed would be very adequate (Todd et al., 2001).

The expression of heterologous proteins in plants has become an attractive alternative for the more current expression systems based on animal, yeast or bacteria cell culture (Floss et al., 2007; Ma et al., 2003). A number of proteins of pharmaceutical and industrial applications have been produced in plants, where the low cost of production, easy scale-up, and low risk of contamination with animal pathogens are the main advantages (Floss et al., 2007; Ma et al., 2003; Rigano and Walmsley, 2005). Plants expressing a recombinant antigen can be used directly as an oral vaccine, which presents low cost and high stability. Therefore, in this study, the potential use of plants for the production of CAV proteins, to be used as a recombinant vaccine, was explored. For this purpose, *Nicotiana benthamiana* leaves were infiltrated with *Agrobacterium tumefaciens* strains containing binary vectors or, alternatively, inoculated with PVX and TMV-based viral vectors carrying the CAV genes. In addition, the expression of VP3 and its potential role in inducing programmed cell death (PCD) in plants was included in the experiments.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli strain XL1Blue was used for DNA cloning. Cells were grown in LB medium, in a shaker at 37 °C. *A. tumefaciens* were cultured in LB3 medium (10 g/l peptone, 5 g/l yeast extract, 4 g/l NaCl, 1 g/l KCl, 3 g/l $MgSO_4$, pH 7.0), at 28 °C. Competent cells were transformed by electroporation using a Bio-Rad apparatus, following the manufacturer's instructions.

2.2. DNA amplification and cloning

CAV genes were amplified by PCR from cloned DNA of the Cux-1 strain, obtained from Dr. Harry Flore (Lohmann Animal Health, Cuxhaven, Germany). Restrictions sites were included as extension on the primers for VP2 and VP3. PCR fragments were cloned on pGem-T easy vector (Promega) and then digested with BamHI and SstI, for cloning into a pCambia 2300 binary vector, containing the CaMV35S promoter and nos terminator. The *gfp* gene (Chiu et al., 1996) was PCR amplified excluding the termination codon, and cloned into the NcoI site of the pCambiaCaMV35S-VP2 and pCambiaCaMV35S-VP3 to obtain *in frame* GFP C terminal fusions. VP2 and VP3 fragments were also cloned in the pEntr11 vector (Invitrogen). VP1 was amplified with primers containing the Gateway attB1/attB2 (Invitrogen) extensions. PCR fragment was recombined with pDonr207 using the PB clonase mix (Invitrogen), as recommended by the manufacturer. Entry vectors (pDonor207 and Entr11), containing the VP1, VP2 or VP3 genes were recombined with destination vectors using the LR clonase mix (Invitrogen). Destination vectors used were the binary vectors pK2GW7 and pK7WGF2 (Karimi et al., 2002), both containing the CaMV35S promoter, and a Gateway compatible PVX-based vector (PVX-GW and PVX-GFP-GW) (Chapman et al., 1992). Recombination reactions were transformed into *A. tumefaciens* strain GV3101. The PVX-GW vectors were transformed in a GV3101 strain containing the pSoup plasmid (Hellens et al., 2000). DNA restriction and cloning was done essentially as described by Sambrook et al. (1989).

2.3. Plant material and inoculation conditions

N. benthamiana plants (4–5-week old) were used for *A. tumefaciens* transient assay (ATTA). Young, fully expanded leaves were infiltrated with *Agrobacterium* suspensions using a 5 ml syringe without needle at the abaxial surface. Bacterial suspensions were obtained from an overnight culture (600 μl) centrifuged and resuspended in 3 ml induction medium [10.5 g/l K_2HPO_4, 4.5 g/l KH_2PO_4, 1.0 g/l $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 0.2% (w/v) glucose, 0.5% (v/v) glycerol, 50 μM acetosyringone, and 10 mM morpholineethanesulfonic acid (MES), pH 5.6]. After overnight incubation at 28 °C, cultures were centrifuged and resuspended in 5 ml MS medium (Murashige and Skoog, 1962) containing 10 mM MES and 150 μM acetosyringone and the OD_{600} adjusted to 0.5. An *Agrobacterium* strain (LBA4404) carrying the suppressor of gene silencing, HcPro, from Cowpea aphid-born mosaic virus (CAbMV) was used for co-inoculation by mixing one volume of strain LBA4404 pBin-HcPro (Bucher et al., 2003) with two volumes of strains carrying the binary vectors with the CAV genes. Strains carrying the *gfp* gene, either in a binary vector or in a viral vector were included as controls. For each construct, two to three plants were inoculated and each experiment was repeated at least three times. After infiltration, plants were maintained in a growth chamber at 25 °C and 12 h photoperiod.

2.4. Sample preparation and microscope imaging

Samples from infiltrated tissues were mounted with water on a glass slide. Images were obtained with a digital camera (CoolSNAP^{TM}) mounted on an epifluorescence microscope (Leica), with UV light and a blue filter set (465 nm). LSM images were obtained in a Zeiss LSM 510 confocal microscope and the software Zeiss LSM Data Server. GFP fluorescence was observed through excitation by a blue light laser light (488 nm) and emission through a 505–530 bandpass filter. For DAPI staining, samples were incubated in 0.1 M phosphate buffer with 10 mg/ml DAPI for 15–20 min at 60 °C. Samples were observed in an epifluorescence microscope, using a 380 nm filter set.

2.5. Western blot analysis

Four leaf discs (9 mm) were collected with a cork borer and macerated with a pestle in a tube with 200 μl of phosphate-saline

buffer (PBS), pH 7.2. Samples (20 μl per well) were loaded on a 12% SDS-acrylamide gel on an electrophoresis apparatus (Bio-Rad) and run at 150 V. The gels were blotted to an Immobilon membrane (Millipore), using semi-dry transfer (Bio-Rad). Membranes were incubated for 1 h in 5% (w/v) non-fat milk, in PBS, and subsequently incubated with an anti-GFP polyclonal antibody (1:3000). An alkaline-phosphatase conjugate anti-rabbit antibody (Sigma) was used as a secondary antiserum (1:4000). The blot was developed by adding BCIP/NBT. Hexahistidine tagged GFP was purified from *E. coli* culture using the TALON® CellThru Resin (Clontech), according to the manufacturer's instructions. A standard curve with purified GFP was used to estimate the amount of GFP and GFP fusions present in the purified samples by using a Fujifilm FLA 3000 scanner and the Multigauge software (Fujifilm, Japan). Concentration of bacteria-purified GFP and total soluble protein in plant extracts was determined using the Bio-Rad kit, based on the Bradford assay (Bradford, 1976).

3. Results

3.1. Expression and subcellular localization of CAV-GFP fusion proteins

N. benthamiana plants were infiltrated with *Agrobacterium* cultures containing binary vectors with GFP C-terminal fusions to the VP1, VP2 and VP3 genes of CAV, hereafter referred to as GFP:VP(1, 2 or 3). To enhance transgene expression levels, each strain was co-infiltrated with an *Agrobacterium* strain carrying the HcPro gene, a suppressor of gene silencing. Three days after infiltration the leaves were visualized under UV light using an epifluorescence microscope. GFP:VP1 fusion was observed as a weak fluorescence signal primarily localized in the nucleoplasm and in a subnuclear location (Fig. 1A) and detectable from epidermal cells (Fig. 1G). Leaves inoculated with GFP:VP2 and GFP:VP3 examined under the epifluorescence microscope revealed that both protein fusion were also localized to the nucleus. Nuclear localization was confirmed by staining the tissues with DAPI, as shown for GFP:VP3 (Fig. 1E and F). Detailed cytology using the LSM showed small compact aggregates of GFP:VP3 clustered in the nucleus, while GFP:VP2 had a nucleoplasmic distribution (Fig. 1B and C). Western blot analysis of samples from infiltrated leaves confirmed the expression of the GFP:VP2 and GFP:VP3, at the expected sizes of 40 and 51 kDa, respectively. The signal of GFP:VP1 was too low to be detected using Western blot analysis (Fig. 2). Expression level of the fusion proteins was estimated to be 1.2% of total soluble protein for GFP:VP2 and 2.6% TSP for GFP:VP3. Non-fused GFP was expressed in concentrations up to 5.4% TSP.

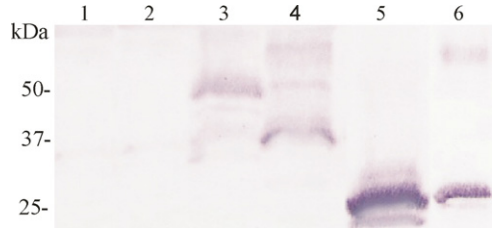

Fig. 2. Western blot from *N. benthamiana* leaves infiltrated with *A. tumefaciens* strains carrying various CAV:GFP fusion constructs. The blot was incubated with antiserum against GFP. Lane 1: non-infiltrated plant; lane 2: GFP:VP1; lane 3: GFP:VP2 (51 kDa); lane 4: GFP:VP3 (40 kDa); lane 5: free GFP (27 kDa); lane 6: GFP purified from bacteria. Precision Plus Protein™ standard (Bio-Rad) was used a size marker.

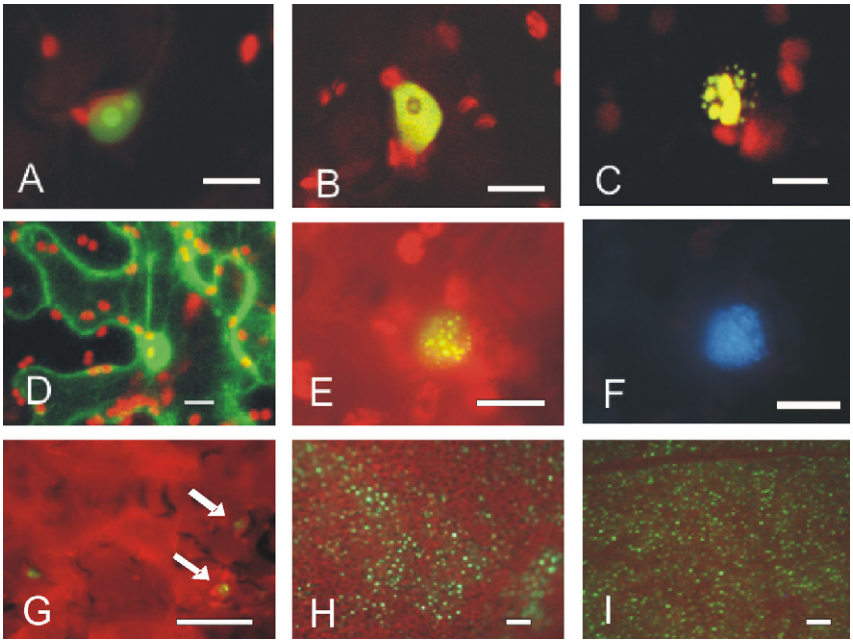

Fig. 1. Fluorescence microscopy images of GFP in *N. benthamiana* leaves expressing GFP-CAV fusion proteins. LSM images showing GFP fluorescence in the nucleus of cell expressing GFP:VP1 (A), GFP:VP2 (B) and GFP:VP3 (C). Leaf cells expressing non-fused GFP (D). Epifluorescence images showing the nucleus of a cell in a leaf expressing GFP:VP3 (E and F). The same cell is shown with a GFP filter (E) and a DAPI filter (F). Bar = 10 μm. Leaf cells expressing GFP:VP1 (G) (arrows), GFP:VP2 (H) and GFP:VP3 (I). Bar = 100 μm. The red background is due to autofluorescence of chlorophyll.

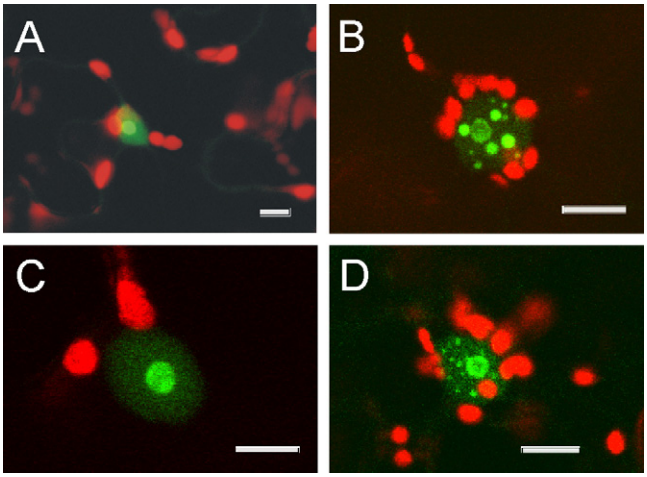

Fig. 3. LSM images of *N. benthamiana* leaf cells showing GFP:VP1 fluorescence in the nucleus. Leaves were infiltrated with *Agrobacterium* lineages carrying GFP:VP1 (A) and co-infiltrated with GFP:VP1 and VP2 (B), GFP:VP1 and VP3 (C) and GFP:VP1 and VP2 and VP3 (D). Bar = 10 μm.

3.2. Co-infiltration of GFP:VP1 and VP2/ VP-3

To test possible effects of co-expressing CAV proteins on their expression and localization, GFP:VP1 was co-infiltrated with binary vectors carrying VP2 or VP3 (devoid of the GFP fusion). A suspension of the *Agrobacterium* strain carrying the HcPro gene was also included (a ratio of 2:1:1 of VP1:VP2/VP3:HcPro). In leaves co-infiltrated with GFP:VP1 and VP2, the fluorescence pattern of GFP:VP1 clearly altered as compared to expression of GFP:VP1 alone (Fig. 1A and B). Co-expression of GFP:VP1 and VP2 lead to the formation of previously unobserved aggregates inside the nucleus (Fig. 3B), adding to an earlier suggestion that VP2 may effect the stability of VP1 and thereby its expression (Koch et al., 1995). No alteration in the GFP:VP1 fluorescence pattern was seen when co-infiltrated with VP3 (Fig. 3C). Triple co-infiltration of GFP:VP1 with VP2 and VP3 followed the pattern of aggregate formation as observed for co-expression of GFP:VP1 and VP2 (Fig. 3D). The inverse combinations, VP1 and GFP:VP2 or GFP:VP3, were also tested but did not cause an alteration in the fluorescence pattern of VP2 or VP3 when compared to the pattern observed for these proteins when expressed alone (results not shown). Also co-infiltration of GFP:VP2 and VP3 or GFP:VP3 and VP2 also did not reveal any change of GFP fluorescence pattern.

3.3. Expression of CAV proteins from a PVX-based viral vector

CAV genes VP1, VP2 and VP3 were cloned into a PVX-based vector. This PVX replicon contains a GFP gene and the *attR* sites for recombination using the Gateway LR clonase. *Agrobacterium* containing these PVX-based GFP fusions, hereafter referred to as PVX-GFP:VP(1, 2 or 3), were agroinoculated in *N. benthamiana* and GFP fluorescence was monitored. PVX-GFP:VP2 showed identical fluorescence patterns to those observed in leaves infiltrated with the binary vector constructs. For PVX-GFP:VP1, GFP fluorescence was observed in a few cells (approximately 10 cells per infiltrated leaf), showing both nuclear and cytoplasmic localization (data not shown). Systemically infected leaves showed typical PVX symptoms but no GFP fluorescence was detected. Co-inoculation of PVX-GFP:VP1 and VP2 (with an *Agrobacterium* strain containing a binary vector) did not alter this VP1 localization. The differences in expression pattern and efficiency could be due to the large insert size of GFP:VP1 (~2 kb) leading to instability of the foreign sequence and the generation of deletion mutants (Chapman et al., 1992). Expression of VP1 using this PVX-based expression vector was not further explored.

3.4. Plant cell death induced by VP3 expression

Plants inoculated with PVX-GFP:VP3 expressed the fusion proteins after 3 days. However, infiltrated areas severely wilted after 4 days and died after 7–10 days. Systemically infected leaves showed small necrotic spots. Similar results were obtained with PVX-VP3 (lacking the GFP fusion part-

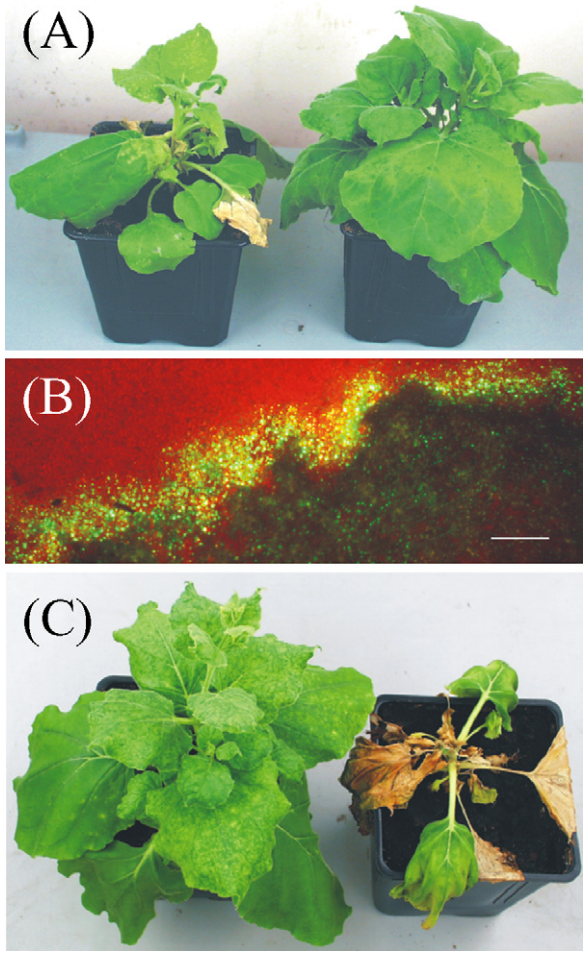

Fig. 4. *N. benthamiana* plants systemically infected (10 dpi) with PVX-GFP:VP3 (left) and PVX (right) (A). PVX-GFP:VP3 infected leaf tissue showing GFP fluorescing cells (arrow) and dead cells (B). *N. benthamiana* plants systemically infected (15 dpi) with TMV:VP3 (right) and TMV (left) (C).

ner). Observation of PVX-GFP:VP3-infected tissues under the epifluorescence microscope showed an area of dead tissue surrounded by a ring of cells expressing GFP:VP3, suggesting cell death following VP3 expression as the virus moved from cell-to-cell (Fig. 4). DNA extracted from infiltrated area after 3, 6 and 10 dpi. was analyzed in agarose gel electrophoreses, but no low molecular weight DNA laddering pattern typical for PCD was observed (data not shown). Expression of VP3 from a TMV-based vector was also tested and also in this case plants showed severe wilting and died after 10 days (Fig. 4).

Since no evident sign of cell death was observed in leaves agroinfiltrated with GFP:VP3, or VP3, expressed from binary vectors, the relation between PVX or TMV infection and VP3 expression was further studied. For this purpose, leaves were co-inoculated with a strain containing the PVX vector without a foreign gene insert (pGR107) and a strain carrying the VP3 gene or the GFP:VP3 (binary vectors). In addition, leaves infiltrated with GFP:VP3 or VP3, were mechanically inoculated after 3 days with extracts from PVX or TMV infected plants. In all these inoculations no evident signs of cell death were observed. No difference in the pattern of GFP fluorescence from agroinoculated GFP:VP3 was noticed after co-infiltration with either PVX or TMV.

4. Discussion

The expression of CAV proteins in *N. benthamiana* leaves was evaluated. VP2 and VP3 GFP-fusions were readily observed at higher levels relative to the GFP:VP1 fusion which was only detected by GFP fluorescence at low levels and in only a few cells (10–20 cells per inoculated leaf). VP1, VP2 and VP3 fused to GFP all showed nuclear localization, which indicates that nuclear localization signals of the three CAV proteins are functional in plants. CAV proteins all possess putative nuclear localization signals and are known to be directed to the nucleus in infected chicken cells where virus assembly takes place (Adair, 2000). However, this nuclear localization is not always observed, since VP3, e.g. does not localize in the nucleus of normal human cells (Noteborn, 2004).

VP1 is the only structural protein found in CAV purified particles and is the main choice as candidate antigen for a recombinant vaccine (Cunningham et al., 2001; Koch et al., 1995). Expression of VP1 from *E. coli* was attempted but recombinant proteins were unstable leading to truncated products (Pallister et al., 1994). Successful expression of VP1 was obtained in insect cells inoculated with a recombinant baculovirus. It was also shown that co-expression of VP1 and VP2 was necessary to induce neutralizing antibodies (Koch et al., 1995). The association of VP1 and VP2 was also demonstrated by co-immunoprecipitation assays, leading to the suggestion that VP2 could act as a chaperone, helping VP1 to achieve the correct conformation necessary for stability and effective epitope presentation that would lead to neutralizing antibodies (Noteborn et al., 1998). The need for a correct VP1 conformation or proper particle assembly is also suggested by the fact that monoclonal antibodies poorly recognized denatured VP1 in Western blot experiments. VP1 expressed alone in insect cells was also poorly recognized in contrast to VP1 co-expressed with VP2 (Koch et al., 1995; Noteborn et al., 1998).

Besides its interaction with VP1, and its possible role as a chaperone or scaffold protein, VP2 was shown to be a dual protein phosphatase (Peters et al., 2002, 2005). However, as these studies were carried out *in vitro*, the role of VP2 in CAV infection is still not established (Noteborn, 2005). As shown in this study, in *N. benthamiana* cells, co-expression with VP2 clearly alters the GFP:VP1 distribution within the nucleus, indicating a possible interaction between these proteins. On the other hand, the reverse combination did not cause VP2 to assemble in a pattern similar to VP1, which might suggest that the association is only transient and – only – leads to a different location of VP1 after refolding by VP2. No alteration was observed by co-expressing GFP:VP1 and VP3 or VP2 and VP3, when either one was expressed as GFP fusion. Further research will be needed to test whether co-expression of VP2 would also be required for proper VP1 conformation in plants, suitable to induce neutralizing antibodies.

Besides testing the expression of VP1 as a potential candidate for a recombinant vaccine against CAV, the effect of VP3 expression was tested for a possible involvement in programmed cell death (PCD) in plants. Programmed cell death is a complex process involved in many developmental and physiological processes. In animal cells, PCD or apoptosis, has been extensively studied in recent years, and is essentially distinct from plant PCD (Hengartner, 2000; van Doorn and Woltering, 2005). Nevertheless, some morphological features related to apoptotic animal cells including chromatin condensation, cell shrinkage and degradation of DNA into nucleosomal fragment sizes resulting in a laddering pattern have also been observed in plants and are generally described as “apoptotic-like” (Huckelhoven, 2004; Ryerson and Heath, 1996; van Doorn and Woltering, 2005). Based on sequence similarity, most genes known to be involved in apoptosis in animals are not present in plants (Higashi et al., 2005; Huckelhoven, 2004), but some studies have shown that the expression of apoptosis related genes in plants, like the Bcl-2 family, causes PCD related effects (Huckelhoven, 2004; Lacomme and Santa Cruz, 1999).

The VP3 gene of CAV causes apoptosis in infected chicken cells and in transfected human tumor cells, but not in normal human cells (Danen-Van Oorschot et al., 1997). This fact has called the attention for the potential use of VP3 as a drug in cancer therapy (Noteborn, 2005). Induction of apoptosis by VP3, also called apoptin, is p53 and Bcl-2 independent and involves an activation of caspases (Rohn and Noteborn, 2004). In the nucleus, VP3 forms aggregates, interacting with the heterochromatin (Leliveld et al., 2004; Leliveld et al., 2003).

In *N. benthamiana* cells, VP3 is targeted to the nucleus and locally forms large aggregates. When expressed from a binary vector no evidence of cell death was observed. However, when expressed from a viral vector, either TMV or PVX, areas expressing VP3 showed extensive necrosis, eventually leading to plant death. No DNA laddering was observed from these

infected tissues, which does not exclude PCD induction as a result of VP3 expression, since DNA laddering is often not observed in plant PCD related processes (Hengartner, 2000; Lacomme and Santa Cruz, 1999; van Doorn and Woltering, 2005).

Cell death observed in viral vector infected tissue expressing VP3 could result from a necrotic reaction not related to PCD. Necrotic reactions are frequently observed upon viral vector-based expression of different genes unrelated to PCD (Gleba et al., 2004; Pogue et al., 2002). Furthermore, PCD in plants may not be easy to demonstrate or to distinguish from a necrotic reaction and further biochemical analysis would be necessary (Greenberg and Yao, 2004; van Doorn and Woltering, 2005). Therefore, we consider it premature to assign any involvement of VP3 in PCD in plants as yet. Nevertheless, the relatively high level (~2.6% TSP) of expression of VP3 in plant cells offers an attractive alternative source for apoptin production. Considering its importance as a potential in anti-cancer therapy, purification of VP3 from plant extracts and biological activity tests are promising enough to warrant further studies.

Overall, our results show that all CAV proteins can be expressed in plant cells. Current expression levels of VP1 may be too low to be exploited as an oral vaccine, but optimizing expression level in plant cells to obtain recombinant VP1 protein capable of inducing neutralizing antibodies, most likely by co-expression with VP2, represents an attractive low cost strategy towards novel, affordable vaccines against CAV.

Acknowledgments

We would like to thank Dr. Harry Flore for providing the CAV clones. This work was partially financially supported by Lohmann Animal Health, Cuxhaven, Germany, and by CAPES Foundation (Brazil) through a fellowship to C. Lacorte. We also thank Daniel Todd for kind collaboration.

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