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Development and assessment of a *Potato virus X*-based expression system with improved biosafety

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Over the last decade, plant virus-based vectors have been developed and successfully exploited for high-yield production of heterologous proteins in plants. However, widespread application of recombinant viruses raises concerns about possible risks to the environment. One of the primary safety issues that must be considered is the uncontrolled spread of the genetically engineered virus from experimental plants to susceptible weeds or crops. Using a movement-deficient *Potato virus X* (PVX)-based transient gene expression vector which harbors the β-glucuronidase (*gus*) gene, we established a plant viral expression system that provides containment of the recombinant virus and allows for safe and efficient protein production. By deletion of the viral *25k* movement protein gene, systemic spread of the modified virus in non-transgenic *Nicotiana benthamiana* plants was successfully inhibited. In transgenic *N. benthamiana* plants expressing the 25K viral movement protein, this deficiency was complemented, thus resulting in systemic infection with the movement-deficient virus. While no differences in virus spread and accumulation were observed compared to infection caused by wild-type PVX in non-transgenic plants, the movement protein transgenic plants exhibited none of the normal symptoms of viral infection. Several biosafety aspects were investigated including the potential for recombination between the defective virus and the movement protein transgene, as well as complementation effects in non-transgenic plants doubly infected with the defective and the wild-type virus. Furthermore, the applicability of the safety system for the production of heterologous proteins was evaluated with *gus* as a model gene. With respect to the stability of the *gus* insert and the expression level of the GUS protein, there were no differences between the novel system developed and the conventional PVX-based expression system.

Keywords: biosafety / complementation / GUS / movement deficiency / plant virus / *Potato virus X* / recombinant protein expression / transgenic plants / plant viral expression system

INTRODUCTION

In recent years, plant virus-based expression vectors gained in importance as powerful tools for the expression of recombinant proteins in plants. They have already been successfully applied for the production of commercially valuable pharmaceutical compounds such as vaccines, antigens and therapeutics (Awram et al., 2002; Breiteneder et al., 2001; Pérez Filgueira et al., 2003; Verch et al, 2004; Wagner et al., 2004). Moreover, plant virus-based systems have been used as molecular tools to address fundamental questions related to virus-host interactions (Santa Cruz et al., 1998; Selth et al., 2004; Wright et al., 2000), functional genomics (Hanley et al., 2003; Hong et al., 2003; Ratcliff et al., 2001; Valentine et al., 2004), inherent biosafety of transgenic plants (Kopertekh et al., 2004a, 2004b), or plant biology in general (Hiriart et al., 2002; Kumagai et al., 1995, 1998; Page et al., 2004; Sablowski et al., 1995).

Several inherent features of plant viruses make them ideal candidates for high-yield and large-scale production of recombinant proteins. Since many plant viruses are easily transmissible, large numbers of plants can be infected efficiently by mechanical inoculation. Also, many plant viruses have small genomes that can be easily manipulated and tolerate genetic modification to a relatively great extent. These features allow very fast and flexible application, because once a suitable viral expression vector has been constructed, various foreign genes can be expressed in a variety of different plants

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depending on the host range of the virus. Moreover, the speed with which a virus infection becomes established throughout the plant, and the high yield of virus-expressed proteins accumulating in the plant, provide efficient protein production within a very short time. Finally, a general advantage of plant-based expression systems is that plants provide one of the most economic sources of biomass for the large-scale production of commercially valuable proteins.

However, when plant viral vectors are exploited for commercial-scale production of heterologous proteins, issues of biological safety must be considered. Some of the major concerns have already been addressed in several reviews (Campbell, 1999; Joshi and Joshi, 1991; Pogue et al., 2002; Scholthof et al., 2002). According to these studies, the primary containment and safety considerations relate to the risks to the environment. Recombinant viruses could spread from experimental plants to susceptible weeds or crops resulting in possibly negative effects for the environment. Safety systems that prevent uncontrolled virus spread can help to assure proper containment of the genetically engineered viruses. Until now, there are only few approaches developing plant viral vectors with improved biosafety (Mor et al., 2003; Mori et al., 2001; Turpen, 1998; Zhao et al., 2000). All of them are based on a newly emerging technique called the "deconstructed virus" vector strategy (Gleba et al., 2004). By eliminating virus-specific functions that are either limiting or undesired, and by delegating them to a genetically engineered host plant, this strategy attempts to increase the efficiency and biosafety of foreign gene expression. Since the viruses are dependent on a specific host plant, any escape of the recombinant virus outside the experimental system is prevented.

Based on this concept, we have employed a *Potato virus X* (PVX)-derived vector for our studies. It is one of the most commonly used viral expression systems and has already been used for the production of a variety of different proteins in plants (Escobar et al., 2003; Franconi et al., 2002; Li et al., 2004; Saitoh et al., 2001; Ziegler et al., 2000). Since transcription of the full-length clone used in this study is driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter, the PVX cDNA can be applied directly onto the experimental plant via mechanical inoculation without the costly and time-consuming production of *in vitro* transcripts. Expression of the foreign gene is directed by an additional copy of the PVX coat protein subgenomic RNA promoter, which allows for high expression levels of the recombinant protein. Compared to many other plant virus vector systems, the PVX genome can accommodate large heterologous sequence insertions as demonstrated by Chapman et al. (1992) for the 1.8 kb β-glucuronidase (*gus*) gene or by Avesani et al. (2003) for the human islet autoantigen glutamic acid decarboxylase (*hGAD65*) gene that is also 1.8 kb in size.

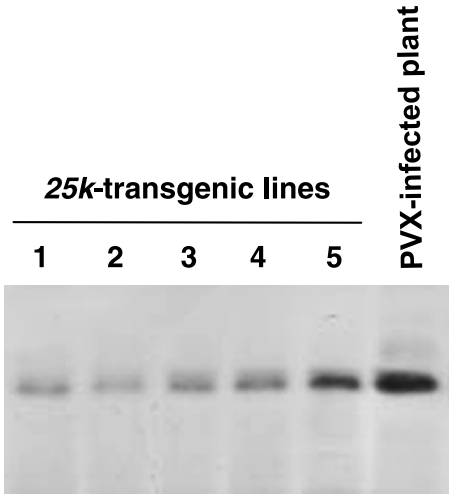

Figure 1. Western blot analysis of PVX *25k*-transgenic *N. benthamiana* lines. Equal amounts of total soluble protein extracted from five independent transgenic lines were analysed using PVX 25K-specific antiserum. Extract of total soluble protein from a non-transgenic *N. benthamiana* plant infected with unmodified PVX.GUS served as positive control.

This paper reports the development of a PVX-based expression system with improved biosafety. By combining a movement-deficient PVX.GUS full-length clone with movement protein gene-transgenic plants, containment of the chimeric virus within the transgenic system was provided. Different biosafety issues as well as the applicability for protein production are analyzed and discussed. The results obtained demonstrate that the developed system represents a basis for safe and efficient production of recombinant proteins in plants.

RESULTS

Generation of *25k*-transgenic tobacco plants

Transgenic *Nicotiana benthamiana* plants carrying a PVX *25k* movement protein gene controlled by the 35S promoter were produced by *Agrobacterium tumefaciens*-mediated leaf disk transformation. A total of 25 phosphinothricin-resistant plant lines were regenerated. PCR with transgene-specific primers was used to confirm the presence of the transgene in transformed lines (data not shown). Western blot analysis of T_1 progeny of five positively tested lines revealed consistently low levels of 25K accumulation (Fig. 1). T_1 progeny of line 5, which showed

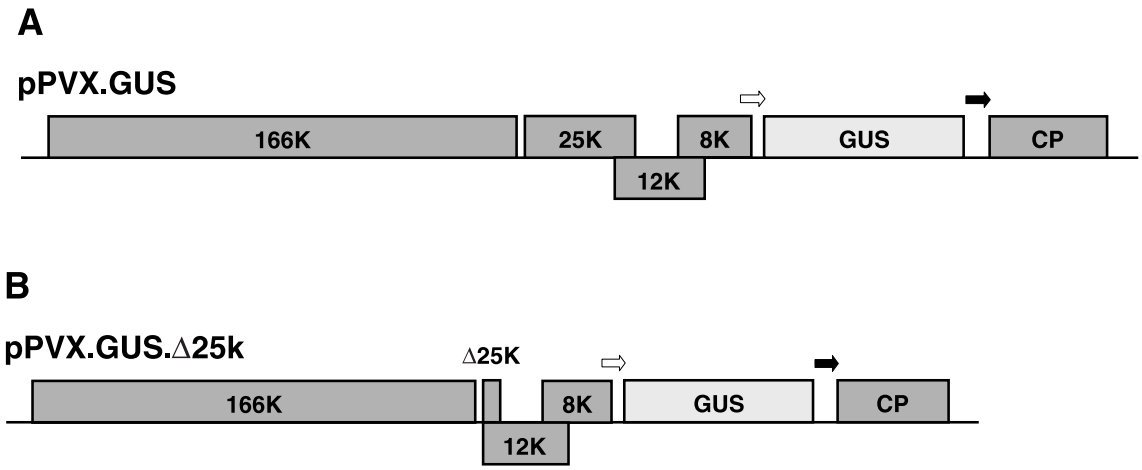

Figure 2. Schematic representation of the full-length PVX clones used in this study. (A) 35S promoter-controlled PVX cDNA clone based on pGC3 (Chapman et al., 1992). (B) Movement-deficient derivative of pPVX.GUS with a 639-bp deletion (213 amino acids) within the *25k* gene. Boxes indicate open reading frames coding for the following proteins: 166K, RNA-dependent RNA polymerase; 25K, 12K, 8K, triple gene block movement proteins; Δ25K, remaining sequence of the 25K after deletion; GUS, β-glucuronidase; CP, coat protein. The open arrow depicts the additional copy of the CP subgenomic promoter. The closed arrow denotes the CP subgenomic promoter.

the highest level of 25K expression were used for further experiments.

Trans-complementation of the movement-deficient PVX in *25k*-transgenic tobacco plants

The movement-deficient pPVX.GUS.Δ25k mutant was generated from a cDNA clone of PVX.GUS placed under the control of the CaMV 35S promoter (Fig. 2). The presence of the β-glucuronidase (GUS) reporter protein, expressed by means of an additional copy of the PVX coat protein subgenomic RNA promoter, was used to permit visualization of PVX spread. The capacity of pPVX.GUS.Δ25k to infect *25k*-transgenic and non-transgenic *N. benthamiana* plants systemically was tested by mechanical inoculation of 20 plants, respectively. Systemic virus spread was confirmed by ELISA and histochemical detection of the virus-expressed GUS activity using young apical leaves at 14 days post-inoculation (dpi). Non-transgenic plants (n = 20) inoculated with pPVX.GUS served as a positive control. Inoculation of *25k*-transgenic plants with pPVX.GUS.Δ25k resulted in systemic PVX infection of 70% of the inoculated plants, which was comparable to the infection rate obtained with the unmodified PVX.GUS in non-transgenic plants (Tab. 1). In contrast, none of the non-transgenic plants inoculated with the movement-deficient mutant was infected systemically (Tab. 1). As shown by histochemical analysis, the distribution of GUS activity was similar in PVX.GUS- and PVX.GUS.Δ25k-infected plants and revealed the same bundle sheath-restricted spreading as already reported for GFP-tagged PVX (Santa Cruz et al., 1996). According to these results, systemic spread of the movement-deficient virus was supported effectively in *25k*-transgenic plants. In contrast, in non-transgenic plants inoculated with pPVX.GUS.Δ25k, GUS activity was confined to single spots on inoculated leaves indicating that the 25K-deficient PVX mutant was still able to replicate in initially infected cells but failed to move into the neighboring cells.

Table 1. Efficiency of systemic infection following inoculation of full-length PVX clones onto non-transgenic and *25k*-transgenic plants 14 days post-inoculation. Systemic PVX infection of young apical leaves was confirmed by DAS-ELISA using polyclonal antibodies specific for PVX coat protein.

Full-length viral clone	Number of systemically infected plants	
	Non-transgenic[a]	*25k*-transgenic[a]
pPVX.GUS.Δ25k	0 (0%)	14 (70%)
pPVX.GUS	15 (75%)	n.d.

[a] For each experiment, 20 plants were inoculated.

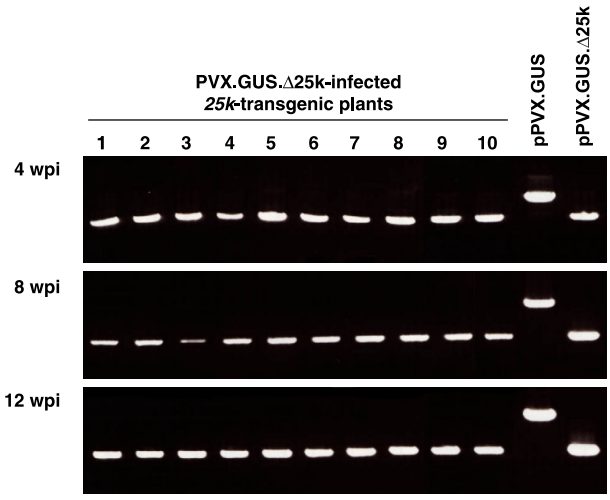

Figure 3. Analysis of the potential occurrence of virus recombinants with restored movement function in *25k*-transgenic plants infected with PVX.GUS.Δ25k. Young apical leaves of 10 individual plants were subjected to IC-RT-PCR with primers up- and downstream of the deleted PVX *25k* gene in the viral genome at 4, 8 and 12 weeks post-inoculation. Plasmid-DNA of pPVX.GUS and pPVX.GUS.Δ25k served as controls.

Although virus accumulation and virus spread of the movement-deficient virus in *25k*-transgenic plants were comparable to a normal PVX infection, striking differences were observed concerning the development of symptoms. Despite systemic infection with PVX.GUS.Δ25k, transgenic plants did not show any symptoms and were phenotypically indistinguishable from non-infected control plants over the whole observation period of 8 weeks post inoculation (wpi) (data not shown). In contrast, infection with PVX.GUS induced symptoms typical for PVX.

Analysis of safety aspects

Do virus recombinants with restored movement function arise in transgenic plants expressing the 25k movement protein gene?

Although most of the *25k* gene sequence has been deleted from the PVX cDNA, there are still some nucleotides left in pPVX.GUS.Δ25k that are homologous to the *25k* transgene. This could give rise to recombination between the viral RNA and the transgene-derived mRNA, which could result in virus recombinants with restored movement function. To test whether movement-competent virus recombinants may arise, 10 PVX.GUS.Δ25k-infected movement protein gene-transgenic plants were subjected to IC-RT-PCR with primers up- and downstream of the deleted *25k* gene in the viral genome at 4, 8 and 12 wpi. In addition, systemically infected plant material was harvested at each sampling time and used for virus particle infection assays on non-transgenic *N. benthamiana* plants. A total of 5 plants for each initially infected *25k*-transgenic plant were inoculated, and plants were tested up to 8 wpi for systemic PVX infection by ELISA. As shown in Figure 3, even at 12 wpi, only virus progeny with deleted *25k* gene could be detected by IC-RT-PCR in *25k*-transgenic plants infected with PVX.GUS.Δ25k. Also, inoculation of virus particles derived from these plants never resulted in systemic infection of non-transgenic plants (data not shown). Thus, virus recombinants with restored movement function could not be detected in *25k*-transgenic plants infected with movement-deficient PVX up to 12 wpi.

Does complementation occur in non-transgenic plants simultaneously infected with movement-deficient and wild-type PVX?

Application of the movement-deficient PVX onto non-transgenic plants that are already infected with wild-type (wt) PVX could allow for complementation by the wild-type virus, thus leading to spread and multiplication of the movement-deficient virus in mixed infected plants. To test whether complementation effects could occur, non-transgenic *N. benthamiana* plants were inoculated both with wtPVX particles and movement-deficient particles derived from *25k*-transgenic plants infected with PVX.GUS.Δ25k. Three different scenarios were investigated: I. The movement-deficient virus was applied to a non-transgenic plant that was already infected with wtPVX; II. The wtPVX was applied to a non-transgenic plant that was locally infected with the movement-deficient virus; and III. The movement-deficient and the wtPVX were applied simultaneously onto the non-transgenic plant. For scenario I., plants were pre-inoculated with wtPVX particles. 5 days later, movement-deficient virus particles were applied to one of the upper leaves. For scenario II., the movement-deficient virus was inoculated on plants followed by inoculation of wtPVX on one of the lower leaves 5 days later. For scenario III., both particles were mixed in equal quantities and inoculated simultaneously. For every scenario, 5 plants were used, and virus particles were inoculated onto one leaf per plant. Samples were taken at 2, 4, 8 and 12 weeks after the first inoculation from young apical leaves and subjected to IC-RT-PCR with primers up- and downstream of the *25k* gene in the viral genome. As depicted in Figure 4, plants inoculated

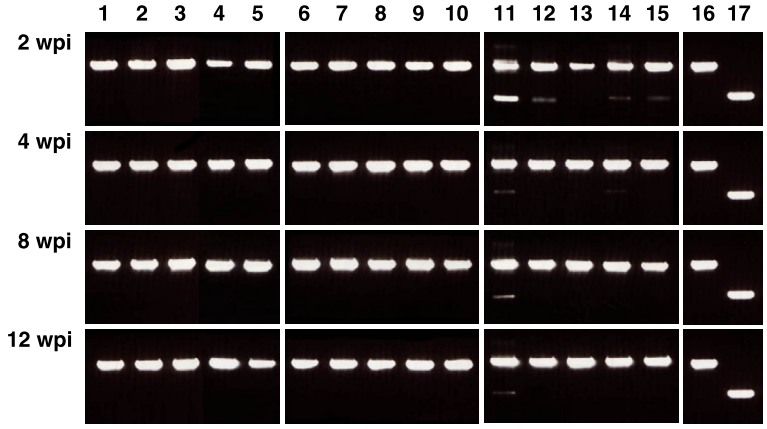

Figure 4. Analysis of potential complementation effects in non-transgenic plants inoculated with movement-deficient and wild-type PVX in different variations. Young apical leaves of five individual plants inoculated with I. wild-type PVX prior to the defective PVX (lane 1–5), II. defective PVX prior to wild-type PVX (lane 6–10), or III. defective and wild-type PVX simultaneously (lane 11–15) were subjected to IC-RT-PCR with primers up- and downstream of the PVX *25k* gene in the viral genome at 4, 8 and 12 weeks post-inoculation. Plasmid-DNA of pPVX.GUS (lane 16) and pPVX.GUS.Δ25k (lane 17) served as controls.

separately with the two viruses only showed systemic infection with wtPVX. In contrast, in plants inoculated simultaneously with both viruses, the movement-deficient PVX was also detected in systemically infected leaves. IC-RT-PCR of these plants resulted in two PCR products different in size. The smaller PVX.GUS.Δ25k-specific product was much weaker than the wtPVX-specific one, even though the smaller PCR product should be favored by PCR. This indicated that accumulation of movement-deficient virus was much lower in doubly infected plants compared to wtPVX accumulation. Moreover, the number of plants showing systemic infection with both viruses decreased in the course of time. At two wpi, movement-deficient virus was found in all plants at least in low levels. From 8 wpi on, PVX.GUS.Δ25k was only detected in one plant, which showed the highest accumulation of movement-deficient PVX from the start.

Analysis of the applicability of the safety system for protein production

Besides the safety aspects, an important requirement for a powerful viral expression system is efficient and high-yield production of the heterologous protein. With *gus* as a model gene, the suitability of the viral safety system for heterologous protein production was examined. The stability of the *gus* insert and the expression level of the GUS protein in the course of virus infection were determined and compared to the results obtained by the conventional PVX-based expression system. The experiments were carried out by using five *25k*-transgenic and five non-transgenic *N. benthamiana* plants, systemically infected with the movement-deficient or the unmodified PVX.GUS, respectively. Samples from young apical leaves were taken at 10, 14, 21 and 28 dpi and subjected to IC-RT-PCR and Western blot analysis.

Stability of the gus gene sequence in PVX full-length clones

In the full-length PVX clones used in this study, the expression of the *gus* gene was driven by an additional copy of the PVX coat protein subgenomic RNA promoter. It is already known that in viral cDNA clones harboring two homologous subgenomic promoter sequences the inserted foreign gene tends to be deleted during virus multiplication by recombination (Chapman et al., 1992; Dawson et al., 1989). On the other hand, stability of the inserted gene to a certain extent is imperative for efficient protein production by means of full-length viral clones. To proof that adequate insert stability is given, samples of PVX.GUS.Δ25k- and PVX.GUS-infected plants were examined by IC-RT-PCR with primers GUS1, which binds at the 5'-terminus of the *gus* gene, and PVXb, which is located in the PVX cDNA sequence downstream of the GUS gene (Tab. 2). Gel electrophoresis of the PCR products (Fig. 5 I.A + II.A) showed that at 10 and 14 dpi *gus*-tagged PVX was present in all plants in high quantities. As infection proceeded, the abundance of *gus*-tagged PVX decreased rapidly although virus accumulation determined by ELISA was similar at all sampling times (data not shown). These results demonstrate that the *gus* insert was lost in the course of infection and that stability of the *gus* gene was provided only during the first two weeks after inoculation. There was no difference in insert stability between the movement-deficient and the unmodified PVX.GUS.

Table 2. Oligonucleotide sequences used for IC-RT-PCR.

Primer name	Orientation	Sequence [5'→3']	Position[a]
px166kRT	forward	TAGTTTCCACAGGCTTGAGG	4107–4126
px12kRT	reverse	TACACTTTTTCAGAATTGACCG	5199–5178
GUS1	forward	ATGTTACGTCCTGTAGAAACCC	5692–5713
PVXb	reverse	TTTGTGGTAGTTGAGGTAGTTGA	7702–7680

[a] Positions of the primers in complete pPVX.GUS are shown.

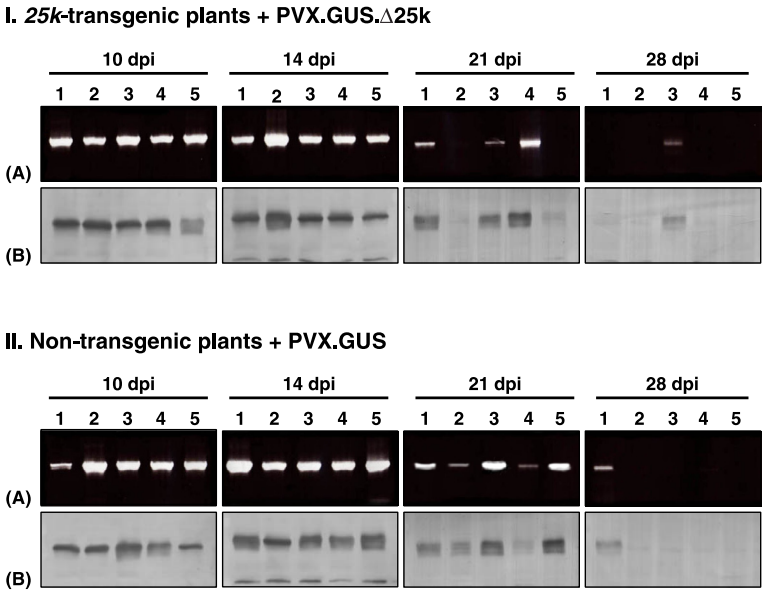

Figure 5. Analysis of the stability of the *gus* insert (A) and analysis of the GUS expression (B) in I. PVX.GUS.Δ25k-infected *25k*-transgenic plants and II. PVX.GUS-infected non-transgenic plants. At 10, 14, 21 and 28 days post-inoculation, young apical leaves of five individual plants were subjected to IC-RT-PCR with primers GUS1 and PVXb to investigate the insert stability or to Western blot analysis with GUS-specific antibodies to determine the GUS expression level.

Expression of the GUS protein from full-length PVX clones

To evaluate the expression level of the GUS protein, total soluble protein extracted from virus-infected plants was subjected to SDS-PAGE and Western blot analysis with GUS-specific antibodies (Fig. 5 I.B + II.B). At 10 and 14 dpi, high levels of GUS protein were detected in all plants with no difference in accumulation between the movement-deficient and the unmodified PVX.GUS. At 21 dpi, the amount of GUS protein decreased distinctly, and finally at 28 dpi, GUS expression was confirmed only in two plants, one of them infected either with movement-deficient or with unmodified PVX.GUS.

The stability of the *gus* gene within the viral sequence and the accumulation of the GUS protein in PVX-infected plants were correlated (Fig. 5). The expression level of the GUS protein decreased as the loss of the *gus* gene increased. In plants that did not show GUS expression, GUS-tagged PVX could not be detected as well.

With respect to the amount of GUS protein produced by the PVX-based system, comparison with defined amounts of purified GUS protein (Fig. 6) revealed an estimated yield of 0.5 µg GUS/µl of protein extract. 1 µl of the protein extract corresponds to ~0.25 mg of fresh leaf material so that the estimated yield was approximately 2 mg of virus-expressed GUS protein from 1 g of virus-infected *N. benthamiana* leaves for both PVX.GUS.Δ25k and PVX.GUS.

DISCUSSION

In recent years, plant virus-based expression systems have been developed that provide the opportunity for efficient and high-yield production of recombinant proteins in plants. Several reports have demonstrated the successful application of plant viral vectors for the expression of commercially important proteins as hormones, vaccines, antigens and therapeutics (Dirnberger et al., 2001; McCormick et al., 2003; Yusibov et al., 2002; Zhang et al.,

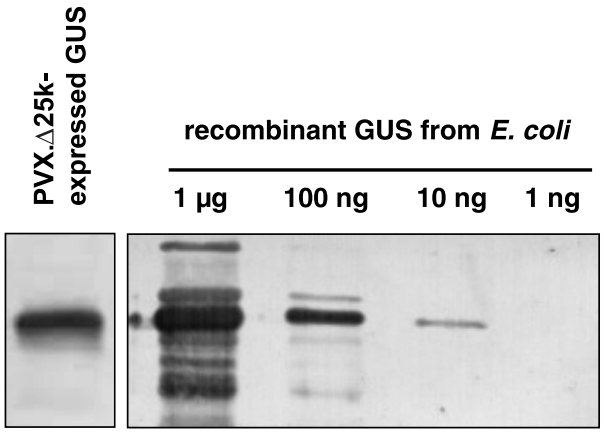

Figure 6. Comparison of the amount of GUS protein expressed from PVX.GUS.Δ25k with defined amounts of recombinant GUS purified from *E. coli*.

2000). However, large-scale production of high-value proteins requires widespread use of the recombinant virus vectors, thus resulting in large amounts of genetically modified organisms distributed during the inoculation process and in the residual plant material. This raises concerns about the biological safety of plant virus-based expression systems, especially in respect to potential risks for the environment. Spread of the recombinant virus from experimental plants to susceptible weeds or crops could result in negative effects for the environment. To meet this problem, we have established a plant viral expression system that provides containment of the engineered virus and allows for safe and efficient production of recombinant proteins in plants.

In this study, we designed a full-length PVX clone with almost complete deletion of the *25k* movement protein gene. When inoculated on non-transgenic *N. benthamiana* plants, this mutant was not able to spread systemically. The viral infection was restricted to single cells, indicating that the mutant virus could still replicate in the initially infected cells, but was not able to move cell-to-cell. Microinjection studies revealed the same single cell-restricted virus accumulation when virions of a PVX.GUS full-length clone with a 354 bp deletion within the *25k* gene were applied to *Nicotiana clevelandii* (Angell et al., 1996). The first evidence that the movement deficiency can be complemented by PVX 25K *in trans* was supplied by co-bombardment of a *25k* frameshift mutant of PVX.GUS with a 35S promoter-controlled *25k* gene in *N. benthamiana* (Agranovsky et al., 1998; Morozov et al., 1997). Transient expression of both constructs in one cell resulted in restored cell-to-cell movement. Complementation by stable expression was shown in *25k*-transgenic *Nicotiana tabacum* plants with a PVX mutant carrying a 357 bp deletion within the *25k* gene (Verchot et al., 1998). In agreement with these findings, we demonstrated the successful complementation of the movement-deficient PVX.GUS.Δ25k, lacking 640 of 681 bp of the *25k* gene, in *25k*-transgenic *N. benthamiana* plants. Inoculation resulted in systemic infection with no differences in virus spread and accumulation compared to the unmodified PVX.GUS in non-transgenic plants. In contrast, striking differences were observed concerning the development of viral symptoms. Whereas typical PVX symptoms were induced by the unmodified PVX.GUS on non-transgenic as well as *25k*-transgenic *N. benthamiana* plants no symptoms could be observed in *25k*-transgenic plants systemically infected with the movement-deficient PVX. Up to now, the reasons for this phenomenon are not clear. Ares et al. (1998) proposed that in addition to its role in virus movement, the 25K protein could be involved in symptom development. On the basis of a South American isolate (PVX strain CP; Orman et al., 1990), they reported that *25k*-transgenic *N. tabacum* plants exhibited a distinctive phenotype consisting of stunted plants with short internodes, decreased leaf number and slightly chlorotic appearance. When transgenic plants were systemically infected with a movement-deficient PVX mutant, they displayed disease symptoms similar to those induced by wild-type PVX on non-transgenic plants. Infection of transgenic plants with wild-type PVX caused even more severe symptoms. Detailed investigation of the *25k*-transgenic plants showed that developmental and physiological alterations are correlated with 25K accumulation and supported the assumption that 25K could be an important contributor to the symptoms caused by this PVX isolate (Kobayashi et al., 2004). In *25k*-transgenic *N. benthamiana* plants used in our study the 25K expression level was rather low. Possibly, the low 25K accumulation was sufficient to restore the movement-deficiency of the PVX mutant but insufficient to contribute to the development of viral symptoms. Taking into account that the PVX strain CP was rather different from the isolate used in our study (less than 80% sequence identity), an additional role of the PVX 25K movement protein in symptom development may be a possible explanation for the symptomless infection caused by PVX.GUS.Δ25k in *25k*-transgenic *N. benthamiana* plants.

After having demonstrated that systemic infection of the modified virus only occurred in transgenic plants, we analyzed several biosafety aspects of our expression system. The most important aspect was related to recombination between transgenic and viral RNA. In connection

with virus-resistant transgenic crops, issues of environmental risk associated with the release of plants harboring a virus derived-transgene have already been discussed (Aaziz and Tepfer, 1999; Tepfer, 2002), and several studies gave attention to the occurrence of recombination between challenging viruses and transgenic viral mRNA (Adair and Kearney, 2000; Borja et al., 1999; Greene and Allison, 1994, 1996; Schubert et al., 2004; Vigne et al., 2004). Our investigations were focused on the question if viral recombinants with restored movement function might arise under conditions given in our biosafety system. Even at 12 wpi, only movement-deficient virus was detected by IC-RT-PCR in *25k*-transgenic plants systemically infected with PVX.GUS.Δ25k. Inoculation of virus particles extracted from up to 12 wpi old plants on non-transgenic plants never resulted in systemic virus infection. The results indicated that movement-competent virus progeny did not accumulate to detectable levels within the viral expression system.

As mentioned above, the movement-deficient virus was not able to systemically infect non-transgenic plants. Even if it escaped into the environment, it would not be able to spread by itself outside of the transgenic system. But what happens if the mutant virus is applied to a non-transgenic plant that is simultaneously infected with the wild-type virus? In mixed infected plants, there might be the possibility of complementation of the movement-deficient virus by means of the wild-type virus, thus leading to spread and propagation of the mutant virus. To investigate this aspect, non-transgenic *N. benthamiana* plants were inoculated with particles of the mutant and the wild-type virus in different variations and analyzed by IC-RT-PCR with primers that allow for discrimination of both viruses. When particles were applied separately in time on different leaves, only wild-type virus was detected up to 12 weeks after inoculation. The likelihood that movement of the mutant virus was maintained in plants with separate infection foci has turned out to be very low. This result is supported by distribution studies performed with identical but differently labeled full-length PVX clones carried out by Dietrich and Maiss (2003). They demonstrated that in doubly infected plants the viral populations replicated predominantly in discrete areas and co-existence was restricted only to a few cells at the border of these clusters. Referred to these findings, the movement-deficient virus presumably was surrounded by the wild-type virus, thus preventing any spread even if single cells were infected with both viruses. In contrast, after simultaneous inoculation of PVX particles onto one leaf, both viruses were detected in systemically infected leaves. But, compared to the wild-type PVX, accumulation of the movement-deficient PVX was much lower, and over the time the number of doubly infected plants decreased. Obviously, complementation of the movement-deficient virus only occurred when both viruses were present in one cell right from the outset of infection. Under natural conditions, the likelihood that two viruses, which were not propagated together before, invade a host plant simultaneously at the same infection site must be considered rather low. Moreover, in mixed infections, the mutant virus was out-competed in the long run, showing clearly that it was not competitive compared to the wild-type virus.

Our biosafety considerations are based on contained use conditions. Even if the movement-deficient virus escaped into the environment it would not be able to spread by itself. For field releases potential risks related to horizontal transfer of the newly introduced gene of interest would not necessarily be mitigated by our approach. Though the probability of horizontal gene transfer is rather low, a massive introduction of a recombined gene, *e.g.* for pharmaceutical production into the open field, would need further consideration.

The results described above confirmed that our viral expression system provides a high level of biological safety. Another important characteristic of an efficient expression system is high-yield production of the recombinant protein. The effectiveness of our safety system in comparison to the conventional PVX-based system was investigated on the basis of the reporter protein β-glucuronidase. Firstly, the stability of the *gus* gene within the viral sequence was examined. It turned out that the insert was deleted in the course of infection, but adequate insert stability was given up to 14 dpi. No differences were observed between the safety and the conventional system. Loss of the inserted sequence is a common problem of viral vectors that express a foreign gene by means of a duplicated promoter (Chapman et al., 1992; Dawson et al., 1989), and there are already several approaches that meet this problem, *e.g.* by using heterologous promoter sequences from related viruses (Donson et al., 1991; MacFarlane et al., 2000; Shivprasad et al., 1999). Actually, in terms of biosafety, the inherent insert instability is advantageous because the foreign sequence by which the virus is a recombinant organism is eliminated in the long run. Assessment studies performed by Rabindran and Dawson (2001) provided insight into the fate of the foreign gene after inoculation of an engineered virus onto a host plant. Using a green fluorescent protein (*gfp*)-tagged *Tobacco mosaic virus*-based expression vector, they demonstrated that in general the foreign gene as well as repeated sequences were

deleted. The resulting recombinants retained only those sequences required for optimal replication and movement and proved to be less fit than their wild-type counterparts.

Nevertheless, stability of the foreign gene to a certain extent must be assured for efficient protein production. The GUS expression rates obtained in 25k-transgenic plants infected with PVX.GUS.Δ25k proved that the insert stability in our safety system is sufficient. Western blot analysis revealed high-level expression of the GUS protein up to 14 days post-inoculation. The approximate amount of virus-expressed GUS was estimated to be 2 mg of recombinant protein per g of fresh weight biomass. It must be considered that the insert stability as well as the expression level is strongly associated with the particular recombinant protein used for virus-based expression. Thus, it is essential to re-assess a viral expression system for each specific protein. As we did not detect any difference in their effectiveness, we assume that our biosafety system is as efficient as the conventional PVX system, which is one of the most common plant virus-based expression systems for the production of recombinant proteins in plants.

In this report, we describe the development and evaluation of a viral expression system that meets major concerns associated with the commercial application of viral full-length clones for the production of proteins in plants. Spread of the recombinant virus outside of the experimental system was prevented effectively by using a "deconstructed" virus with deletion of the *25k* movement protein gene. Systemic virus spread and multiplication was confined to transgenic plants providing the movement function *in trans*. The data presented in this paper demonstrate that the delegation of functions that are indispensable for virus propagation to a genetically engineered host plant is a promising tool for improving the biological safety of plant virus-based expression systems.

MATERIALS AND METHODS

Full-length viral clones

For construction of the movement-deficient mutant pPVX.GUS.Δ25k, the full-length cDNA clone pPVX.GUS was used. pPVX.GUS is a CaMV 35S promoter-driven derivative of the plasmid pGC3 (Chapman et al., 1992), which carries the *gus* gene under the control of an additional copy of the PVX coat protein subgenomic RNA promoter. To obtain pPVX.GUS.Δ25k, nucleotides 3940 to 4503 were amplified using pPVX.GUS as a template and primers 5'-CCTAGGCACGTTATCAATTAT-3' and 5'-CATATGACTGATGAGAATATCCCTCT-TATTC-3'. The resulting PCR fragment contained an additional 3'-terminal *Nde*I restriction site and a point mutation T→G at position 4487 of the pPVX.GUS sequence to prevent aberrant translation initiation of the 12K. The PCR fragment was subcloned in pGEM-T (Promega), excised with *Avr*II/*Nde*I and ligated into similarly digested pPVX.GUS. The resulting plasmid pPVX.GUS.Δ25k lacks almost the complete coding sequence of the 25K movement protein apart from the first 18 5'-terminal nucleotides and the last 23 3'-terminal nucleotides, which overlap with the *12k* gene.

For the analysis of complementation effects in non-transgenic plants simultaneously infected with movement-deficient and wild-type PVX, virus particles derived from pPVX.201-infected plants were used and regarded as wild-type. pPVX.201 is similar to pPVX.GUS, but lacks the *gus* gene (Baulcombe et al., 1995).

Transgenic plants

The entire PVX *25k* coding sequence was amplified using pPVX.GUS as a template and primers 5'-CCATGGA-TATTCTCATCAGTAGTTTG-3' and 5'-GGATC-CCTATGGCCCTGCGCG-3'. The PCR product was subcloned into pGEM-T, excised with *Nco*I/*Bam*HI and ligated into similarly digested pRT103 (Töpfer et al., 1993). From the resulting plasmid, the expression cassette consisting of CaMV 35S promoter, *25k* gene and CaMV polyadenylation signal was released with *Hind*III, subcloned into *Hind*III-digested pLH7000 (Hausmann and Töpfer, 1999) and introduced into *N. benthamiana* by *A. tumefaciens*-mediated leaf disk transformation (Horsch et al., 1985). Phosphinothricin-resistant T_0 plants were analyzed for the presence of the *25k* transgene by PCR using transgene-specific primers. Selected transformed lines were allowed to self-pollinate. T_1 progeny were analyzed for 25K expression by Western blot analysis with a 25K-specific antiserum kindly provided by A.G. Solovyev. To ensure transgenicity, seeds were always grown on MS medium containing 4 µg/ml phosphinothricin before being transplanted into soil.

Inoculation of plants

25k-transgenic as well as non-transgenic *N. benthamiana* plants were used for infection assays with full-length viral clones. One leaf per plant was dusted lightly with carborundum powder and inoculated by gentle rubbing with 5 µg of either pPVX.GUS or pPVX.GUS.Δ25k purified plasmid DNA, diluted in 50 µl phosphate buffer [500 mM Na_2HPO_4; 500 mM KH_2PO_4, pH 7.2]. For

infection assays using virus particles, virus-infected leaf tissue from primarily infected plants was ground in phosphate buffer, pH 7.2. Plant sap was inoculated onto non-transgenic *N. benthamiana* leaves lightly dusted with carborundum powder.

Plants were maintained in a containment greenhouse at 20 °C with a 16 h light period and an 8 h dark period.

ELISA

Systemic virus infection of inoculated plants was confirmed by DAS-ELISA. Leaf discs of young apical leaves were harvested and tested for the presence of PVX particles using a PVX coat protein-specific polyclonal antibody. DAS-ELISA was performed as described by Clark and Adams (1977).

Analysis of GUS activity

Histochemical assay for GUS activity with X-Gluc was performed as described by Jefferson et al. (1987). Incubations were carried out overnight at 37 °C before clearing the tissue in ethanol.

IC-RT-PCR

Immunocapture-RT-PCR assay was carried out essentially as described by Nolasco et al. (1993). Young apical leaves from PVX.GUS.Δ25k-infected *25k*-transgenic plants were used. Briefly, PVX particles were immunocaptured on 0.5 ml reaction tubes with PVX coat protein-specific polyclonal antibodies and denatured for 3 min at 80 °C. Viral RNA was used as template for reverse transcriptase reaction with 50 U Superscript II RNase H^- Reverse Transcriptase (Invitrogen) and 20 pmol reverse primer (Tab. 2) in a final volume of 25 µl. PCR was performed in a 20 µl volume containing 0.5 U Taq DNA polymerase (TaKaRa Bio Inc.), 2 µl 10 × PCR buffer, 0.2 mM of each dNTP, 1 µl RT solution, 20 pmol of each sense and reverse primer (Tab. 2). The samples were incubated first at 94 °C for 2 min, followed by 30 cycles of 94 °C (30 sec), 52 °C (30 sec) and 72 °C (1 min/1000 bp) with a final 10 min extension at 72 °C.

Detection and quantification of GUS protein in PVX-infected plants

Systemically infected apical leaves were harvested and total soluble protein was extracted by grinding 100 mg leaf material in 2 vol. (w/v) of 0.25 M Tris-HCl, pH 6.8. After adding 2 vol. (w/v) of extraction buffer [125 mM Tris-HCl, pH 6.8; 9 M urea; 10% SDS; 25% glycerol; 12.5% β-mercaptoethanol], protein samples were centrifuged for 5 min at 10 000 × *g* to remove all insoluble material. Aliquots of the supernatant (1 µl) were diluted 1:5 with sample buffer [625 mM Tris-HCl, pH 6.8; 10% SDS; 0.005% bromphenol blue], incubated for 2 min at 100 °C and loaded on a 10% polyacrylamide/SDS gel. After electrophoresis, the proteins were transferred to a PVDF membrane (Amersham Bioscience) by semi-dry electroblotting. For immunodetection of the GUS protein, the membranes were probed with 1:2000 diluted anti-β-glucuronidase rabbit IgG fraction (Molecular Probes), followed by a 1:2000 diluted goat anti-rabbit alkaline phosphatase conjugate (Sigma). Colorimetric detection was performed with BCIP/NBT Color Development Substrate (Promega). The relative molecular weight of the GUS protein was estimated using prestained molecular mass standards (MBI Fermentas).

For approximate quantification of the GUS protein, defined quantities (1 µg; 100 ng; 10 ng; 1 ng) of β-glucuronidase purified from Escherichia coli (Sigma) were compared with defined amounts (1 µl of protein extract) of virus-expressed GUS protein following immunodetection.

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