An efficient *Foxtail mosaic virus* vector system with reduced environmental risk

Zun Liu\(^1,2\), Christopher M Kearney\(^1*\)

### Abstract

**Background:** Plant viral vectors offer high-yield expression of pharmaceutical and commercially important proteins with a minimum of cost and preparation time. The use of *Agrobacterium tumefaciens* has been introduced to deliver the viral vector as a transgene to each plant cell via a simple, non-sterile infiltration technique called "agroinoculation". With agroinoculation, a full length, systemically moving virus is no longer necessary for excellent protein yield, since the viral transgene is transcribed and replicates in every infiltrated cell. Viral genes may therefore be deleted to decrease the potential for accidental spread and persistence of the viral vector in the environment.

**Results:** In this study, both the coat protein (CP) and triple gene block (TGB) genetic segments were eliminated from *Foxtail mosaic virus* to create the "FECT" vector series, comprising a deletion of 29% of the genome. This viral vector is highly crippled and expresses little or no marker gene within the inoculated leaf. However, when co-agroinoculated with a silencing suppressor (p19 or HcPro), FECT expressed GFP at 40% total soluble protein in the tobacco host, *Nicotiana benthamiana*. The modified FoMV vector retained the full-length replicase ORF, the TGB1 subgenomic RNA leader sequence and either 0, 22 or 40 bases of TGB1 ORF (in vectors FECT0, FECT22 and FECT40, respectively). As well as *N. benthamiana*, infection of legumes was demonstrated. Despite many attempts, expression of GFP via syringe agroinoculation of various grass species was very low, reflecting the low *Agrobacterium*-mediated transformation rate of monocots.

**Conclusions:** The FECT/40 vector expresses foreign genes at a very high level, and yet has a greatly reduced biohazard potential. It can form no virions and can effectively replicate only in a plant with suppressed silencing.

### Background

Plant expression systems have been developed as production platforms for many therapeutic proteins over the past two decades. Although many foreign proteins have been expressed in stably transgenic plants, plant viral vectors have emerged as the most efficient approach to achieving high-level expression of recombinant proteins in plants [1,2]. These self-replicating vectors produce maximum levels of foreign gene expression and require minimal set-up time. It is often possible to generate purified recombinant protein within three weeks of receiving a gene sequence [3,4].

However, the potential widespread use of recombinant viruses raises concerns about possible risks to the environment. Bio-safety issues must be considered to prevent the spread of the genetically engineered virus from experimental plants to susceptible wild plants [5-7]. Intact viral vectors have the potential to spread and infect non-target plants, but replication-defective or movement-defective viruses avoid these problems. These deleted viral vectors also address cross-contamination issues in the growth room and greenhouse. In the field, it might be possible to achieve high expression in transgenic plants carrying an inducible virus as a transgene [8,9]. In all of these cases, deleted virus vectors would be greatly preferred over full virus vectors for reduced transmission and persistence.

An obvious disadvantage to the deleted virus approach is that the vector cannot spread past the originally inoculated cells. However, this weakness can be successfully overcome by the agroinoculation technique, which uses *Agrobacterium tumefaciens* to deliver the virus...
sequence, carried in a binary vector, to the genome of the vast majority of plant cells in the infiltration zone of the leaf using whole, nonsterile plants [10]. For small scale use, a syringe is used to infiltrate leaves with *Agrobacterium*, while for large scale applications, vacuum infiltration is used to inoculate an entire greenhouse at once [10]. For both agroinoculation and transgenic use, systemic spread becomes an unnecessary property.

Agroinoculation involves the local transformation of the infiltrated leaf with the viral cDNA as a part of the T-DNA of the Ti plasmid. A plant promoter (most commonly CaMV 35S) placed upstream of the viral cDNA induces the transcription of viral genome in the plant nucleus and viral RNA is transported to cytoplasm for viral replication.

Over the past few years, several deleted viral vectors delivered by agroinoculation have been created and some are used commercially. *Tobacco mosaic virus* (TMV) lacking the coat protein (CP) gene has been used to express a large number of foreign proteins commercially [4,11,12]. Removal of the CP gene from TMV can lead to unexpectedly large increases in foreign gene expression [13]. In the *Potato virus X* (PVX) replacement virus vector, both the triple gene block (TGB) and CP viral genes were removed, leaving only the replicase gene and terminal untranslated regions, and these deleted genes were replaced with GFP [14]. The expression level of GFP from this vector was 2.5-fold higher than that of full-length PVX vector with the GFP encoding sequence between the triple gene block and the CP genes. A defective RNA TMV vector has also been shown to express at high levels [15].

*Agrobacterium* infiltration-mediated transient expression can be greatly enhanced by suppression of gene silencing. An RNA silencing suppressor, such as p19 [16] from tomato bushy stunt virus or HcPro [17] from potato virus Y, is co-inoculated in a separate strain of *Agrobacterium* along with the *Agrobacterium* carrying the viral cDNA. Using this approach, highly efficient production of GFP from a TMV-based vector was achieved with up to a 100-fold increase of the overexpression level [18]. As well, potexvirus expression was greatly increased with suppressor co-inoculation [14].

The FECT vectors are derived from foxtail mosaic virus (FoMV) which is a member of the genus *Potexvirus*. *Potexvirus* is a large group of flexuous and filamentous plant viruses with a single-stranded, positive-sense genomic RNA which has a cap structure at the 5’ terminus and a poly-(A) tail at the 3’ terminus [19,20]. The FoMV genome structure resembles that of PVX, the type species of the genus *Potexvirus*, and the gene functions are presumed to be similar as well [21,22]. The genome of FoMV contains five open reading frames (ORFs), and two subgenomic promoters directing transcription of subgenomic RNAs (sgRNAs) 1 and 2 [21]. The genomic RNA allows the expression of ORF1 encoding the RNA-dependent RNA polymerase (RdRP) with methyltransferase, helicase, and polymerase motifs in PVX [23]. The first sgRNA contains ORF2, 3 and 4 coding for the triple gene block (TGB) proteins TGB1, TGB2 and TGB3, which are required for virus cell-to-cell movement [24]. The PVX ORF2 codes for a multifunctional protein that has RNA helicase activity, promotes translation of viral RNAs, increases plasmodesmatal size exclusion limits, and acts as a suppressor of RNA-mediated post-transcriptional gene silencing (PTGS) [24]. The PVX ORF5 encodes the coat protein, which is required for viral capsidation, cell to cell movement, and long distance movement [25,26].

FoMV has a broad host range, infecting 56 species of the Poaceae and at least 35 dicot species [27]. The sequence of FoMV genomic RNA was first published in 1991 [21]. Infectious full-length clones were constructed based on the same FoMV isolate and some corrections to the published sequence were noted [28]. The significant difference between the gene organizations of FoMV and PVX is the presence of ORF 5A upstream of the CP gene in FoMV. ORF 5A initiates 143 nts upstream of the CP and extends the reading frame of CP gene. The 5A protein was produced in vivo, but it was not required for either replication or productive infection of plants [28]. Recently, the revised full-length sequence of foxtail mosaic virus clone was published in 2008, and reveals a triple gene block structure similar to *potato virus X* [22].

The potexvirus replicase is the only protein translated directly from the full-length genomic RNA, but other viral proteins are translated from 3’ coterminal sgRNAs [29,30]. Two sgRNAs of approximately 2.1 and 0.9 kb in length have their 5’ termini upstream of the TGB and CP genes, respectively [19], while a 1.4 kb bicistronic (readthrough) sgRNA provides for the translation of TGB2 and TGB3 ORFs [31]. The integrity of the subgenomic promoter in a vector is very important for the accumulation of sgRNA and target protein. However, the boundaries of sgRNA promoters have not been delineated for FoMV.

Vectors using FoMV have not been reported until this present study, but foundational work on potexvirus vectors has been completed with PVX. PVX was engineered to express reporter genes cloned just upstream of the CP gene and expressed from a duplicated copy of the CP subgenomic promoter [32]. The reporter gene was translated from a sgRNA separate from the other viral ORFs. Because PVX has a linear helical capsid, rather than an icosahedral capsid, a recombinant viral genome longer than wild type can still be encapsidated into infectious virus particles. Expression of longer ORFs
with this construct led to earlier deletion of the ORF from the vector [33]. A PVX vector with a bicistronic sgRNA, carrying the reporter gene and the CP gene, has also been constructed. An IRES site allowed for translation of the distal gene on the sgRNA [34]. As mentioned previously, a deleted PVX vector has also been constructed [14]. A vector using another potexvirus, Alternanthera mosaic virus, has been recently developed [35]. In this vector system, the potexvirus was split into two components, the replicase portion and the TGB/CP portion, each in its own binary vector and Agrobacterium culture. Following co-agroinoculation, recombination in vivo regenerates the full length virus. In vivo expressed T7 RNA polymerase is used to transcribe the transgenic virus components after agroinoculation.

In this study, a vector with the properties of high protein expression and greatly lowered environmental risk was constructed. The TGB and CP genes of FoMV were removed and replaced with heterologous sequences while the subgenomic promoter of the first TGB gene (TGB1) was reserved to direct the transcription of the heterologous coding sequence. The FoMV expression vectors driven by the 35S promoter were delivered as a T-DNA to plant cells by agroinoculation. These severely crippled viral vectors would have difficulty surviving in the wild, since they form no virions, use agroinoculation for transmission, and are unable even to productively express marker genes unless a silencing suppressor is supplied. However, expression levels are among the highest of those reported from plant expression systems when silencing suppressor co-expression is provided.

Results

Construction and agroinoculation of full length JL22/pFoMV

FoMV full length wild type viral cDNA from an in vitro transcription construct [26] was inserted into the binary vector, pJL22 [18] (Figure 1). To accomplish this, the PCR-amplified 5’ end of the FoMV sequence was first transferred, followed by the insertion of a restriction fragment containing the rest of the FoMV sequence (Figure 1). The resulting construct (JL22/FoMV) comprised the 35S promoter, the full FoMV sequence, a poly(A) tract present in the original sequence [28], and the 35S terminator.

A. tumefaciens GV3101 was transformed with pJL22/FoMV and agroinoculated to N. benthamiana. At 1 and 2 weeks, there were no visible symptoms of viral infection on any plants. To detect the presence of FoMV infection, total RNA was extracted from leaves and screened by RT-PCR at 7 days post-inoculation (dpi). Systemic movement of FoMV was demonstrated in inoculated plants by the presence of RT-PCR product, but no bands were seen in uninoculated controls (data not shown). Thus, agroinfection with the JL22/FoMV construct resulted in replication and movement of this FoMV sequence but the infection proved very mild.

Construction and agroinoculation of deletion vectors (FECT)

Primers were designed to delete the TGB and CP genes while retaining the subgenomic promoter of sgRNA1 and 3’-end of the CP gene to enable expression of foreign genes (Table 1; Figure 2). The extent of the sgRNA1 promoter region was unknown, so the entire sgRNA1 putative leader sequence and portions of the TGB1 ORF were included. Three upstream primers were used to include the first 0, 22 and 40 bases of ORF of TGB1 to create pFECT0, pFECT22 and pFECT40, respectively. The native TGB1 AUG was mutated to AUC and PacI and AvrII sites were included as cloning sites. The 3’ terminal part of CP FoMV gene between AvrII and 3’-UTR was reserved because deletion of this region drastically reduced vector accumulation in a deleted PVX vector [14]. The final form of these constructs is diagramed in Figure 3.

To test viral replication and foreign gene expression, GFP was inserted into each of the FECT vectors (Figure 3). N. benthamiana was agroinoculated with GV3101 Agrobacterium cultures carrying FECT. At 2-4 days after agroinoculation, GFP-expressing cells could be seen faintly using a hand-held UV lamp and fluorescence microscopy. At this time, there were many faint green spots showing on leaves inoculated with FECT0/GFP and FECT22/GFP (but fainter in the latter), but no green fluorescence could be detected on leaves inoculated with FECT0/GFP (Figure 4a). Furthermore, the fluorescence was transient and, by eight days post-inoculation, the GFP spots on all
plants had disappeared (Figure 4b). Apparently, the transcription of agroinfiltrated T-DNA induced posttranscriptional gene silencing (PTGS), which led to the inhibition of viral vector infection and the reduction of viral productivity [36].

**Rescue with silencing suppressors**

It has recently been demonstrated that co-inoculation of RNA silencing suppressor proteins enhances the expression of heterologous proteins from viral vectors [14,18]. To test this effect, *N. benthamiana* plants were...
The unusually high expression level led us to quantify the level of fluorescence was surprising (Figure 4a and 4b). Ds-Red was also expressed with FECT40/GFP plants, though quite fluorescent, with SDS-PAGE electrophoresis and Coomassie blue protein staining (Figure 4c). GFP expression was detected in extracts of total soluble protein were measured 2 to 7 dpi were homogenized and the relative amounts of GFP in extracts of total soluble protein were measured by densitometry analysis (Figure 6), FECT40 yielded GFP at 40% of total soluble protein (as for Figure 4c). The mg of GFP produced per gram fresh weight (gfw) of tissue was determined for FECT40/GFP (top row) matched that of JL24 (bottom row) when both were co-agroinoculated with p19 (Figure 5). Thus, even with the disadvantage of no systemic movement, FECT vector expression was equivalent to that of an excellent systemic TMV vector. FECT40/GFP expression was also equal to the TRBO-G vector [13], which is among the most strongly expressing of the deleted TMV vectors. In SDS-PAGE/Coomassie blue densitometry analysis (Figure 6), FECT40 yielded GFP at 40% of total soluble protein (as for Figure 4c). The mg of GFP produced per gram fresh weight (gfw) of tissue was determined for FECT40/GFP by comparison to a GFP standard and was 1.6-1.7 mg/gfw, similar to that of TRBO-G with 1.3-1.7 mg/gfw (Figure 6). In comparison, the single-enhancer 35S promoter driving GFP expression in the standard binary vector pGDG [37], induced by p19 expression, yielded GFP at only 0.03 mg/gfw, or more than 50-fold less than FECT/GFP expression (Figure 6).

In support of these values were calculations made by western blot analysis. The GFP band of Figure 4d represented a 10 μl load from a 100× dilution of 300 μl of extract from 150 mg of fresh leaf material. The protein content of the virus-expressed GFP band was estimated as

**Quantification and comparison to other vectors**

The unusually high expression level led us to quantify the percent of total soluble plant protein that the GFP represented in the inoculated zone. Over the course of two weeks, fluorescence had appeared to increase through the first week and then stabilize so a time course assay covering the first week was initiated. *A. tumefaciens* FECT40/GFP + *A. tumefaciens* 35S/p19 co-infiltrated leaves from 2 to 7 dpi were homogenized and the relative amounts of GFP in extracts of total soluble protein were measured with SDS-PAGE electrophoresis and Coomassie blue protein staining (Figure 4c). GFP expression was detected from the second day after inoculation (Figure 4c). The expression level of fluorescent protein increased gradually, and stabilized at 5-7 dpi (Figure 4c). GFP accumulated to 30% to 40% of the total soluble protein extracted, as measured by densitometry.

This expression level was further quantified and compared to TMV vectors, which are the most commonly used plant viral expression vectors. In a dilution comparison with the full length TMV vector, JL24 [18], the expression of FECT40/GFP (top row) matched that of JL24 (bottom row) when both were co-agroinoculated with p19 (Figure 5). Thus, even with the disadvantage of no systemic movement, FECT vector expression was equivalent to that of an excellent systemic TMV vector. FECT40/GFP expression was also equal to the TRBO-G vector [13], which is among the most strongly expressing of the deleted TMV vectors. In SDS-PAGE/Coomassie blue densitometry analysis (Figure 6), FECT40 yielded GFP at 40% of total soluble protein (as for Figure 4c). The mg of GFP produced per gram fresh weight (gfw) of tissue was determined for FECT40/GFP by comparison to a GFP standard and was 1.6-1.7 mg/gfw, similar to that of TRBO-G with 1.3-1.7 mg/gfw (Figure 6). In comparison, the single-enhancer 35S promoter driving GFP expression in the standard binary vector pGDG [37], induced by p19 expression, yielded GFP at only 0.03 mg/gfw, or more than 50-fold less than FECT/GFP expression (Figure 6).
0.2 μg by western blot, because it has the same density as the 0.2 μg GFP standard generated by a bacterial expression system. From these data we again determined a yield of 1.6 mg/gfw of GFP for FECT40/GFP. To further support this, GFP from FECT40/GFP infected tissue from three replicates from another experiment was quantified by spectrophotometry in comparison with bacterially-produced GFP standard. By this method, 1.58 ± 0.13 mg/gfw was determined for the GFP yield (data not shown).

Expression in monocots and legumes via agroinoculation
Since the natural host range of FoMV includes many grass and legume species, it was appropriate to test GFP expression of FECT40/GFP plus 35S/p19 via agroinoculation.

0.2 μg by western blot, because it has the same density as the 0.2 μg GFP standard generated by a bacterial expression system. From these data we again determined a yield of 1.6 mg/gfw of GFP for FECT40/GFP. To further support this, GFP from FECT40/GFP infected tissue from three replicates from another experiment was quantified by spectrophotometry in comparison with bacterially-produced GFP standard. By this method, 1.58 ± 0.13 mg/gfw was determined for the GFP yield (data not shown).

Expression in monocots and legumes via agroinoculation
Since the natural host range of FoMV includes many grass and legume species, it was appropriate to test GFP expression of FECT40/GFP plus 35S/p19 via agroinoculation.
Figure 4 GFP expression with differing subgenomic promoters and rescue by gene silencing suppressor coexpression. (a) 4 dpi and (b) 8 dpi. In both panels, the top row show fluorescence from *N. benthamiana* agroinoculated with FECT/GFP but without p19 silencing suppressor and the bottom row shows leaves co-agroinoculation of FECT/GFP and 35S/p19. Only a small number of very faint fluorescent spots were found in FECT22 and FECT40 leaves at 4 dpi (a), and this fluorescence disappeared by 8 dpi (b). However, a vigorous fluorescence occurred with the addition of 35S/p19. FECT0 plants did not fluoresce even in the presence of 35S/p19. (c) FECT40/GFP was co-agroinoculated with 35S/p19 onto *N. benthamiana* and samples were taken each day of the time course and protein extracted by grinding and centrifugation. Lanes: M, protein marker; 0, protein extract from uninoculated leaf; 2 to 7, extracts from FECT agroinfiltrated leaves, 2 to 7 dpi, respectively. (d) Western blot of GFP expression at 7 dpi. Lanes: 1, 0.2 μg GFP standard (E. coli generated); 2, FECT/GFP + 35S/p19 in *N. benthamiana*; 3, 35S/p19 alone in *N. benthamiana*. 
agroinoculation against a panel of grass and legume species via agroinoculation. Switchgrass, foxtail millet, barley, wheat, oat and maize were co-agroinoculated with the mixture of two *Agrobacterium* cultures containing FECT40/GFP and p19, respectively. Patches of widely spaced fluorescing cells were observed by UV microscopy in all grass species (Figure 7a) beyond the few number of autofluorescent cells in uninoculated controls. However, these isolated fluorescent cells were still quite uncommon in inoculated grass leaves. A C58 strain of *A. tumefaciens* carrying FECT40/GFP was also tried, but did not provide any improvement in expression rates in grass species. In the legume, *M. trunculata*, however, relatively larger numbers of isolated fluorescing cells were commonly encountered in inoculated tissue (Figure 7b), while few or no autofluorescent cells

---

**Figure 5** Dilution trial of agroinoculant with FECT and p19 on *N. benthamiana* plants. Top row: FECT40/GFP vector. Bottom row: JL24/GFP vector. *A.t.* cell suspensions were diluted, as noted in the figure, from an initial OD600 of 1.0. Pictures were taken under UV illumination at 4 dpi with photos taken at same exposure.

**Figure 6** Comparison of FECT40 GFP expression with TMV and 35S vectors in *N. benthamiana*, 7 dpi. Lane 1, 1 μg GFP standard, Lanes 2 & 3, FECT40/GFP + 35S/p19, Lanes 3 & 4, TRBO/GFP + 35S/p19, Lane 5, NEB prestained broad-range protein markers, Lane 6 35S/p19 alone, Lane 7, 35S/GFP (pGDG vector) + 35S/p19. Each sample lane represents approximately 5.5 mg fw of plant tissue.

**Figure 7** Fluorescence microscopy of monocots and legumes agroinoculated with FECT40/GFP/p19. (a) Maize leaf infection. For all grasses tested, occasional areas of scattered fluorescent cells were seen more commonly in FECT40/GFP/p19 agroinoculated leaves than in p19 agroinoculated or noninoculated leaves, but such cells were rare nonetheless. (b) *Medicago trunculata* leaf infection. Scattered fluorescent cells were common and FECT40/GFP/p19-infected tissue was easily distinguishable from p19-inoculated control leaves under fluorescent microscopy for both *M. trunculata* and lentils.
were seen in uninoculated controls. Somewhat fewer fluorescent cells were found in lentils, while no strong evidence for infected cells was found in bean or cowpea by fluorescence microscopy.

We have demonstrated in *N. benthamiana* that expression of FECT/GFP requires p19 co-expression and we expect that most cells are doubly infected with agrobacteria containing FECT/GFP or 35S/p19. However, in the agroinoculation of grasses, only a small fraction of cells, at best, are expected to be even singly infected by *Agrobacterium* [38]. Even fewer cells would be expected to be doubly infected with two different agrobacteria containing FECT/GFP and p19, respectively, and this might explain the difficulty in visualizing fluorescent cells in grasses. For this reason, we added to the binary vector carrying 35S/FECT/GFP another component carrying the nos promoter (Pnos) driving the expression of p19 and terminated by the nos terminator (Tnos). This cis-acting construct tested very strongly in *N. benthamiana* at 2 dpi (Figure 8), demonstrating an independence from p19 co-agroinoculation for strong fluorescence in the cis construct. By 6 dpi, the fluorescence of the trans configuration (separate binary vectors carrying FECT/GFP and 35S/p19) had increased so as to be somewhat superior to the cis configuration. In spite of these *N. benthamiana* results, when the cis-configured FECT/GFP/p19 was co-agroinoculated onto various grass species, no increase over the trans configuration in fluorescent cell numbers or brightness was seen either by eye or by fluorescence microscopy.

**Discussion**

We have demonstrated very high protein expression rates in *N. benthamiana* for the deleted viral vector, FECT, in co-agroinoculation with 35S/p19. At 40% TSP and 1.6 mg/gfw for the expression of GFP, FECT expresses well beyond expression rates published for traditional plant virus vector systems [32,39]. The GFP expression of the best nonviral 35S system is 270-340 μg GFP/gfw aided by p19 and 5-12 μg/gfw without p19 [36]. The most recently developed TMV vectors, TRBO [13] and the magnifection system [10] both report yields of up to 5 mg/gfw. In side-by-side comparison, with p19 co-inoculation for both, however, FECT expressed GFP at levels equal to those of TRBO. Thus, we have developed a simple and highly efficient protein synthesis vector system for plant-based expression.

The FECT viral vector system has many features that enhance its environmental safety when used via agroinoculation or potentially as a transgene. First, its genome is reduced by almost one-third, making it severely crippled. Second, it does not replicate efficiently unless the plant immune system is suppressed. Thus, if it were to somehow infect another plant (e.g., via its *Agrobacterium* carrier), it would replicate poorly. Third, it lacks a coat protein and cannot form a virion, making it improbable for the virus to survive outside of a host. Finally, it is derived from a virus that in most hosts causes mild infections [27]. In fact, we observed no symptoms in *N. benthamiana* with the full length virus.

We observed a novel control mechanism operational with the FECT construct, which could be very useful in further applications of FECT. In the absence of a silencing suppressor, almost no expression of GFP occurred, and what expression did occur was transient, disappearing by the seventh day after inoculation. This contrasts with the extreme expression in the presence of the silencing suppressor. This was not the case with the PVX vector of Komorova et al. [14]. Though PVX and FoMV are both potexviruses, the Komorova deletion vector performed quite well, better than the full length standard PVX vector, even in the absence of suppressor. Furthermore, this paper states that no extra bases of ORF were used to extend the subgenomic promoter, but...
we found no expression when we emulated this with FoMV. Only when extra bases of the TGB1 ORF were included was expression seen, and for significant expression, suppressor co-expression was needed. Thus, the vectors differed in design as well as performance.

Tyulkina et al. [40] examined the effect of removing the coat protein of TMV (tobamovirus family) and portions of the movement protein. They found that complete removal of the movement protein produced only small infection points, which could be rescued by the co-agroinoculation of p19 or of various sized portions of the TMV movement protein. Thus, a similar effect was found in the tobamoviruses as we found with the FoMV potexvirus. In contrast, the work of Komorova et al. [14] appeared to clearly demonstrate that, for potexviruses, the removal of coat protein and triple gene block genes produced a vector which was quite functional in the absence of suppressor. Our results with the FoMV potexvirus were thus unexpected.

There may be functional differences in the replication mechanisms between FoMV and PVX which are unexplored at present. For example, major differences in silencing suppressor activity of TGB1 have been found between different potexviruses [41] and even between variant sequences of a single potexvirus, Alternanthera mosaic virus [42]. Though TGB1 was deleted in both the FoMV and PVX vectors, it may be that other features in these viruses, affecting their ability to replicate when the TGB1 silencing suppression is eliminated, may also exhibit taxonomic diversity. It may be further speculated that host proteins that aid potexvirus replication, such as NbPC1P1 [43], might be more critical for some potexviruses than for others. The omission of, for example, the CP gene, which NbPC1P1 binds to, might be more detrimental for FoMV than for PVX.

The very low rate of infectivity of FECT in grasses is not unexpected. It is for this reason that full virus vectors, capable of obviating low agroinfection rates via cell-to-cell and long-distance movement, are used for agroinoculation studies [44,45]. There is only one paper to our knowledge that demonstrates successful visualization of single cell agroinfections of grasses [38]. In this work, only scattered cells transformed with a 35S/GUS construct were visualized. We were unable to utilize the GUS marker gene in this study because of GUS production in the agrobacterium culture itself from the FECT/GUS construct (data not shown). However, the creation of FECT/GFP/Pnosp19 opens the possibility of the use of FECT as a viral transgene in grasses, since both p19 and FECT would be expressed in each cell.

Conclusions

There are several potential applications for the use of FECT vectors. This system has the capacity for high level expression of a variety of proteins, including GFP (0.7 kb), the larger GUS (1.8 kb) and the multimeric Ds-Red (0.7 kb) proteins. The inability of FECT to replicate significantly in the absence of silencing suppression or to survive or infect in unencapsidated form greatly reduces environmental risk. This system, then, would be expected to be amenable to the production of pharmaceutical or industrial proteins via agroinoculation in the greenhouse. The tight on/off control of this system also makes it suitable for an inducible transgenic system for field use and for the expression of proteins toxic to the plant host. Finally, the use of FECT as an agroinoculation vector for screening genes and gene variants for plant expression before the construction of stably transgenic plants is facile due to high expression and lack of cross contamination of greenhouses and growth chambers.

Methods

Plants

All plants were grown in a dedicated plant growth room with temperatures ranging from 22-24°C and with automated watering. All seeds were sprouted under plant spectrum fluorescent bulbs. *N. benthamiana* seedlings were then transplanted and grown for two weeks under 400 W metal halide lamps to 10-15 cm before agroinoculation. *Panicum virgatum* cv. Blackwell (switchgrass), *Setaria viridis* (foxtail grass), *Hordeum vulgare* (barley), *Triticum aestivum* (wheat), *Avena sativa* (oat) and *Zea mays* (corn), *Medicago trunculata* (barrel medic), *Lens culinaris* (lentil), and *Cicer arietinum* (chickpea) plants were germinated from seed and grown with 24h/day illumination with plant spectrum fluorescent bulbs. Grasses and legumes 2-3 weeks from seed, with fully expanded leaves, were used for agroinoculation.

Vector construction

All FoMV viral cDNA constructs used in this study are derivatives of a wild-type FoMV cDNA clone that was a gift from Nancy Robertson of the USDA [28] and were constructed with standard recombinant DNA techniques. The binary vector, pJL22, provided by John Lindbo [18], has the mini binary plasmid, pCB301 [46], as a backbone. JL22 contains multiple cloning sites flanked by a 35S promoter and 35S polyA signal/transcription terminator (Figure 1). The source FoMV sequence had an additional 70 adenosine residues inserted after the viral 3’ terminus, followed by an XbaI site. To create JL22/FoMV, the 5’ end of FoMV was amplified by PCR with primers FoMV5’termUp and FoMV756NotDown and cut with NotI. The 5’ end of FoMV was cloned into JL22 digested with StuI and NotI to create JL22/FoMV5’. The 3’ end fragment of FoMV was prepared by restriction digest with PmlI and XbaI and then inserted into JL22/FoMV5’ also digested with PmlI and XbaI.
Agroinoculation

Agroinfiltration was performed as described [47] with modifications. Agrobacterium tumefaciens stain GV3101 was used for the agroinoculation of N. benthamiana and cereals. A. tumefaciens was transformed with plasmid constructs using the same conditions as for E. coli above. Agrobacterium transformants were selected at room temperature on Luria-Bertani plates containing 10 µg/ml rifampicin, 25 µg/ml gentamycin and 50 µg/ml kanamycin. A colony of A. tumefaciens was inoculated to 5 ml of LB-MESA medium (LB media supplemented with 10 mM MES, 20 µM acetosyringone (Phytotechnology Labs, Shawnee Mission, KS)) and the same antibiotics, and grown overnight at room temperature. The cells of the overnight culture were harvested by centrifugation and resuspended in induction media (10 mM MES, 10 mM MgCl2, 100 µM acetosyringone) to a final OD600 of 1.0 and incubated for 2 h to overnight at room temperature. The cultures of A. tumefaciens were infiltrated into the underside of attached leaves with a 3 ml syringe without needle. For co-agroinoculation of two or more bacterium cultures, cultures of A. tumefaciens were mixed in equal amounts and infiltrated together. The gene expression or virus activity was tested at 6-8 days post-infiltration and one of three plant replicates were analyzed per experiment.

RT-PCR

To detect FoMV (without GFP or DsRed) in the plant, total RNA was extracted after 7 dpi using Tri-Reagent (Sigma, St. Louis, MO) according to the manufacturer’s protocol. RT-PCR reactions were performed using the RT-PCR kit (NEB, Beverly, MA) as described by the supplier. To detect the presence of virus sequence, FoMV specific primers were used to amplify the partial viral genome.

GFP and DsRed photography

Plants were examined under long-wave UV light (UVL-56, UVProducts, Upland, CA). For macrophotography, a Canon Digital EOS Rebel XT camera (Canon Inc., Japan) equipped with a Hoya yellow (K2) filter (Hoya Corp., Japan) was used. For microscopic analysis, samples from infiltrated tissues were mounted with water on a glass slide. Images were obtained with a Nikon TE2000-U inverted microscope, captured using a Cool-Snap cf camera (Roper Scientific, Tucson, AZ) and analyzed with Metavue imaging software (version 5, Molecular Devices Co, Downingtown, PA).

GFP Quantification Assay

GFP fluorescence was analyzed and GFP protein was quantified using a standard curve determined from a purchased GFP standard (Vector Laboratories, Inc., CA).
Burlingame, CA), since the amount of GFP protein is directly proportional to the fluorescence intensity [18,48]. Total soluble protein extracts were serially diluted in 50 mM carbonate/bicarbonate buffer, pH 9.6 and loaded on the 96-well Costar black plate with clear bottom (Costar, Cambridge, MA). Fluorescent activities were assayed with a Fluoroscan Ascent FL (Thermo Fisher Scientific Inc., Waltham, MA) using a 485 nm excitation and 538 nm emission filter set.

Protein extraction, SDS-PAGE
Proteins were extracted by grinding agroinoculated leaves to a fine powder in liquid nitrogen and mixing 1:2 (w/v) with reducing protein extraction buffer (50 mM tris, pH7.5, 150 mM NaCl, 0.1% Tween 20, and 0.1% β-mercaptoethanol) or nonreducing protein extraction buffer without β-mercaptoethanol. The insoluble material was removed by centrifugation for 10 min at 16,000 × g in a benchtop centrifuge. The supernatant was collected and stored at 4°C. Clarified extract of protein samples were mixed with denaturing 3 × SDS-PAGE sample buffer (NEB, Beverly, MA) and analyzed by PAGE consisting of a 5% stacking gel and a 7.5% or 15% separation gel. Proteins in the gels were identified with Coomassie brilliant blue R-250 (Sigma, St Louis, MO).

Western Blot
Recombinant GFP detection was tested by western blot using total soluble protein (1 µg protein from FECT: GFP + p19 infections or 1 µg protein from p19 alone infections) and 2 µg bacterially expressed recombinant GFP (Victor Laboratories, CA) run on a precast linear gradient polyacrylamide gel (4-15%) (Bio-Rad, Hercules, CA). The gel was electrophorized onto a PVDF membrane (Amersham Biosciences, Piscataway, NJ) by a semi-dry transfer cell (Bio-Rad, Hercules, CA). The blot was blocked with TBST buffer (100 mM tris-HCl, 0.9% NaCl, 0.1% Tween 20, pH 7.5) containing 5% fat free milk at room temperature for 1 h. The membrane was incubated with an anti-GFP antibody (Invitrogen, Carlsbad, CA) (1 µg/ml) at room temperature for one hour. Bound rabbit IgG was detected by horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, CA). Visualization was performed via ECL detection reagents (Pierce). The gel image was taken by FluorChem SP imaging system (Alpha Innotech, San Leandro, CA) and the relative protein concentrations were analyzed by AlphaEaseFC software version 4.1.0 (Alpha Innotech).

Abbreviations
dpi: days post-inoculation; ELISA: enzyme linked immunosorbent assay; FECT: vector: FoMV eliminated CP and TGB; FoMV: Foxtail mosaic virus; CP: coat protein; GFP: green fluorescent protein, gfw: gram fresh weight; GU5: glucuronidase; MCS: multiple cloning site; ORF: open reading frame; Pnos: promoter of nopaline synthetase gene; PXM: Potato virus X; RT-PCR: reverse transcription polymerase chain reaction, SDS-PAGE: sodium dodecyl sulfae polyacrylamide electrophoresis, sgRNA: subgenomic RNA; T-DNA: transfer DNA; TGB: triple gene block; Tl: tumor-inducing; TMV: Tobacco mosaic virus; Tnos: terminator of nopaline synthetase gene; UTR: untranslated region.

References
1. Gleba Y, Marillonnet S, Klimyuk V: Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. Curr Open Plant Biol 2004, 7(2):182-188.
2. Musiychuk K, Stephenson N, Bi H, Farrance CE, Orozovic G, Brodelius M, Brodelius P, Horsey A, Ugulava N, Shamlou AM, Mett V, Rabindran S, Streafield SJ, Yusibov V: A launch vector for the production of vaccine antigens in plants. Influenza Other Respir Viruses 2007, 1(1):19-25.
3. Mett V, Lyons J, Musiychuk K, Chichester JA, Brasil T, Couch R, Sherwood R, Palmer GA, Streafield SJ, Yusibov V: A plant-produced plague vaccine candidate confers protection to monkeys. Vaccine 2007, 25(16):3014-3017.
4. Gintch A, Marillonnet S, Engler C, van Eldik G, Botteman J, Klimyuk V, Gleba Y: Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. Proc Natl Acad Sci USA 2006, 103(40):14701-14706.
5. Manske U, Schiemann J: Development and assessment of a potato virus X-based expression system with improved biosafety. Environ Biosafety Res 2005, 4(1):45-57.
6. Ploys M, Laimer M, Noris E, Schubert J, Wassenegger M, Tepfer M: Strategies for antiviral resistance in transgenic plants. Mol Plant Pathol 2008, 9(1):73-83.
7. Teycheney PY, Tepper M: Possible roles of endogenous plant viral sequences and transgenes containing viral sequences in both virus resistance and virus emergence. Environ Biosafety Res 2007, 6(4):219-221.
8. Zuo J, Niu QW, Chua NH: Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J 2000, 24(2):265-273.
9. Tremblay A, Beauchemin C, Seguin A, Laliberte JF: Reactivation of an estrogen receptor-based expression system. Plant Biotechnol J 2008, 6(4):319-325.
10. Marillonnet S, Thoeninger S, Kandzia R, Klimyuk V, Gleba Y: A novel two-component Agrobacterium tumefaciens-mediated transfection of viral replicons for efficient transient expression in plants. Nat Biotechnol 2005, 23(6):718-723.
11. Gils M, Kandzia R, Marillonnet S, Klimyuk V, Gleba Y: High-yield production of authentic human growth hormone using a plant virus-based expression system. Plant J 2006, 39(6):613-620.
12. Santi L, Gintch A, Roy CJ, Marillonnet S, Klimyuk V, Gleba Y, Webb R, Arztzen CJ, Mason HS: Protection conferred by recombinant Yersinia pestis antigens produced by a rapid and highly scalable plant expression system. Proc Natl Acad Sci USA 2006, 103(4):861-866.
13. Lindbo JA: TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. Plant Physiol 2007, 145(4):1232-1240.
14. Komarova TV, Skulachev MV, Zvereva AS, Schwartz AD, Dorokhov YL, Atabekov KS: New viral vector for efficient production of target proteins in plants. Biochemistry (Mosc) 2007, 72(18):846-850.
15. Roy G, Weissburg S, Rabindran S, Yusibov V: A novel two-component Tobacco mosaic virus-based vector system for high-level expression of
multiple therapeutic proteins including a human monoclonal antibody in plants. Virology 2010, 405(1):93-9.

16. Scholthof HB: The Tombusvirus-encoded P19: from irrelevance to elegance. Nat Rev Microbiol 2006, 4(5):405-411.

17. Andanlakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AG, Smith TH, Vance VB: A viral suppressor of gene silencing in plants. Proc Natl Acad Sci USA 1998, 95(22):13079-13084.

18. Lindbo JA: High-efficiency protein expression in plants from agroinfection-compatible Tobacco mosaic virus expression vectors. BMC Biotechnol 2007, 7:52.

19. Dolja V, Graha DP, Morozov SY, Abatkovc JC: Potato virus X-related single- and double-stranded RNAs. FEBS Lett 1987, 214:308-312.

20. Huisman MJ, Sonenberg N, Shaklin AJ, Riccardi RP, Rubin M, Goodman RM: Analysis of terminal structures of RNA from potato virus X. Nucleic Acids Res 1978, 5:2501-2512.

21. Bankcroft J, Boureau M, Johnston R, Pink J, Mackie GA: The entire nucleotide sequence of foxtail mosaic virus RNA. J Gen Virol 1991, 72(9):2173-2181.

22. Bruun-Rasmussen M, Madsen CT, Johansen E, Albrechtsen M: Revised sequence of foxtail mosaic virus reveals a triple gene block structure similar to potato virus X. Arch Virol 2000, 153(1):223-26.

23. Davenport GP, Baulcombe DC: Mutation of the GKS motif of the RNA-dependent RNA polymerase from potato virus X disables or eliminates virus replication. J Gen Virol 1997, 78(6):1247-1251.

24. Verchoit-Lubcz J: A new cell-to-cell transport model for Potexviruses. Mol Plant Microbe Interact 2005, 18(4):283-290.

25. Cruz SS, Roberts AG, Prior DA, Chapman S, Opara N: Cell-to-cell and phloem-mediated transport of potato virus X. The role of virions. Plant Cell 1998, 10(4):495-510.

26. Verchoit-Lubcz J, Ye CM, Banunusinge M: Molecular biology of potexviruses: recent advances. J Gen Virol 2007, 88(6):1643-55.

27. Short NN, Davies AW: Host ranges, symptoms and amino-acid compositions of potexviruses. Ann Appl Biol 1987, 110:213-219.

28. Robertson NL, French R, Mains T: The open reading frame 5A of foxtail mosaic virus is expressed in vivo and is dispensable for systemic infection. Arch Virol 2000, 145B:1685-1698.

29. Gama DP, Maier II: The construction of a model of the envelope proteins of potex-group viruses. Mikrobiol Zh 1990, 52(5):59-66.

30. Morozov SY, Miroshnichenko NA, Zelenina DA, Fedorin ON, Solovjov AG, Lukasheva UI, Abatkovc JC: Expression of RNA transcripts of potato virus X full-length and subgenomic cDNAs. Biochimie 1990, 72(9):657-664.

31. Verchoit J, Angell SM, Baulcombe DC: In vivo translation of the entire gene triple block of potato virus X requires two subgenomic mRNAs. J Gen Virol 1998, 72(10):8316-20.

32. Chapman S, Kavanagh T, Baulcombe D: Potato virus X as a vector for gene expression in plants. Plant J 1992, 2(4):549-557.

33. Avesani L, Marconi G, Morandini F, Albertini E, Bruschetta M, Bortesi L, Pezzotti M, Porceddu A: Stability of Potato virus X expression vectors is related to insert size: implications for replication models and risk assessment. Transgenic Res 2007, 16(5):587-597.

34. Toth RL, Chapman S, Carr F, Santa Cruz S: A novel strategy for the expression of foreign genes from plant virus vectors. FEBS Lett 2001, 489(2-3):215-219.

35. Lim HS, Vaira AM, Donnelf JL, Lee SC, Kim HG, Hammond J: Efficiency of VIGS and gene expression in a novel bipartite potexvirus vector delivery system as a function of strength of TGB1 silencing suppression. Virology 2010, 402(1):149-163.

36. Voinnet O, Baulcombe DC: A novel strategy for the expression of foreign genes from plant virus vectors. FEBS Lett 2001, 489(2-3):215-219.

37. Goodin MM, Dietzgen RG, Schichnes D, Ruzin S, Jackson AO: Improved expression of recombinant GFP using a replicating vector based on Beet curly top virus in leaf-disks and infiltrated Nicotiana benthamiana leaves. Plant Mol Biol 2007, 64(1-2):103-112.

38. Ding XS, Schneider WL, Chaluvadi SR, Mian MA, Nelson RS: Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol Plant Microbe Interact 2005, 19(11):1229-1239.

39. Xiang C, Han P, Lututzer I, Wang K, Oliver DJ: A mini binary vector series for plant transformation. Mol Biol 1999, 40(4):711-717.

40. Park MR, Park SH, Cho SY, Kim KH: Nicotiana benthamiana protein, NbP1CPt1, interacting with Potato virus X coat protein plays a role as susceptible factor for viral infection. Virology 2009, 386(2):257-269.

41. Kim K, Sunger B, Bisaro DM, Chung IS: Improved expression of recombinant GFP using a replicating vector based on Beet curly top virus in leaf-disks and infiltrated Nicotiana benthamiana leaves. Plant Mol Biol 2007, 64(1-2):103-112.

42. Anandalakshmi R, Pruss GJ, Ge X, Marathe R, Mallory AG, Smith TH, Vance VB: A viral suppressor of gene silencing in plants. Proc Natl Acad Sci USA 1998, 95(22):13079-13084.