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Transient Virus Expression Systems for Recombinant Protein Expression in Dicot- and Monocotyledonous Plants

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1. Introduction

Plants have long been a source for traditional medicinal products. Indeed, greater than four billion people utilize plants to meet their primary health care [1-2]. There are >120 distinct drugs derived from plant sources representing >70% of the approved drugs in the past 20 years [3, 4]. The manner to exploit the scale and cost advantages of agriculture while diversifying the product offerings made available by plants has been under intense investigation since the early 1980s. Traditional transgenic approaches were initially pursued, but the challenges associated with the transformation and regeneration of viable recombinant crops delayed the appearance of initial products of medicinal promise until 1989 with the production of antibodies [5] and 1990 with the production of human serum albumin [6]. During this time, the concept of using plant virus genomes as expression vectors emerged. In early investigations, researchers recognized the natural capability of virus systems to change the translational priorities within infected cells such that virally encoded proteins were produced preferentially. This ability suggested that expression vectors could be constructed from viral nucleic acids to produce recombinant proteins throughout infected plants [7]. However, for this hypothesis to be tested, the genomes of viruses, starting with positive (+) strand RNA viruses, had to be cloned and characterized [8-10]. Soon after the first full-length "infectious" clones of a (+) strand RNA plant virus were constructed, and preceding traditional transgenic systems, the virus genome was converted into an expression vector [11]. Although limited with regards *to in planta* expression, this first vector revealed the promise of virus genomes to be efficient expression systems for plants. The advantages revealed in these early studies, continue to be present: cDNA "infectious clones" offer facile subcloning vehicles allowing rapid prototyping of genetic expression constructs, and recombinant protein expression levels that exceed that offered by transgenic systems.

The rapid replication cycle of the virus systems provided amplification of messenger RNA and the resulting proteins providing for a "burst" of recombinant expression that can provide impressive yields (reviewed in [12-15]). While these early vectors were useful in plant cell systems to produce recombinant protein products with potential market value [16], these early systems could not support large scale manufacturing nor did they exploit the advantages of agriculture to provide cost-effective products. This review will provide an overview of plant virus-based expression vectors, and provide select examples how virus expression systems have evolved to offer valuable tools for the production of medically important products [17] and support the study of plant structure and metabolic function (reviewed in [18]) in dicot- and monocotyledonous plants. The growing biomedical and agricultural markets have encouraged great creativity in the construction and testing of plant virus expression systems.

2. Biomedical market for recombinant proteins

As an example of the market drivers for plant-based expression vectors, the biopharmaceutical industry market will be briefly reviewed. Recombinant proteins, including monoclonal antibodies (mAbs), enzymes, hormones, cytokines and growth factors, and vaccine antigens, are the source for new medical therapies and the pharmaceutical market. The global pharmaceutical market continues to prove to be robust, >$850B in 2010, in spite of generic pressure and biosimilars appearance [19]. Recombinant protein drugs, known as biologics, expanded their market to $149B, including $48B in sales of the top selling monoclonal antibody (mAb) products treating cancer and other disorders [19, 20]. The global cancer therapeutic market is projected to continue growth at 12.6% compound annual growth rate through 2014 [21]. Monoclonal antibody immunotherapy has revolutionized the treatment of many diseases – most notably cancer where the nondestructive nature of mAb treatment synergizes with many existing therapies to result in improved efficacy. These molecules make up the most promising part of product portfolios for biopharmaceutical companies and this market is predicted to grow by 11-14% compounded annual growth rate (CAGR) in the next five years compared with the rather tepid growth of ~3% CAGR of small molecule drugs [22, 23]. Indeed, in the midst of an economic downturn (2008-2010), the growth in mAbs continues to occur with sales of therapeutic mAbs being $48B in 2010 compared with $40B in 2009 and $37B in 2008 [24]. Adding the $10B of sales for mAbs used for diagnosis and research reagents raises the total mAb market to $58B in 2010. The clinical development of immunotherapy has been revived after several breakthroughs that have led to the approval of drugs and treatment for cancer. Indeed, four of the top 10 mAbs in terms of sales, are used for cancer treatment [24]. New drug targets and associated drug interventions are under investigation that will provide therapeutic options for traditionally underserved populations.

Recent successes and the growing market demand for more innovative biologic products to treat chronic patients has continued to fuel interest and investment to identify tools and strategies to accelerate discovery and product validation in immunotherapy fields. Further, clinical success is ultimately determined by established clinical endpoints indicative of

survival. However, these results often require monitoring for months to years after the therapy has been given to the patient. These timelines are not conducive to the iterative and experimental process that is cancer therapy. Therefore, surrogate markers are sought: more rapidly appearing measurements that correlate with longer-term clinical endpoints. These functions require access to relevant clinical samples from diseased and healthy patients as well as adapting laboratory assays to more clinical formats. Surrogate markers require specific assays used to demonstrate efficacy of immunologic therapies and, as noted above, will fuel extraordinary market growth in the coming decade. These assays are highly empirical and require well trained staff, highly controlled conditions and consistency in procedure to ensure trends in data can be validated as statistically significant. Diagnostic assays incorporating recombinant proteins or exploiting mAb for detecting and assessing medical conditions was $776M in 2010 and is expected to grow at a CAGR of 47% through 2015 [25]. This growth in the use of biologics for immunotherapy and diagnostic products continues, in part, because of recent FDA approvals and physician implementation of several new immunology tools and immunotherapeutic products to diagnose and treat patients.

3. Plant production of bio-pharmaceutical proteins

Plants have steadily gained acceptance as alternative production systems for biologics. The recent United States Food and Drug Administration approval of Protalix Biotherapeutic's Elelyso (taliglucerase alfa) [20] represents the successful realization of the goal for plant-produced human biologics, initiated soon after the formation of Agrigenetics in 1981. This product, produced in engineered carrot cells, benefits from the simpler culture conditions required by plant cells compared with Chinese hamster ovary (CHO), insect, or yeast systems. However, it still requires the capital intensive production methods, requiring multiplicative costs for increased scale [26]. Nevertheless, the approval of Elelyso, demonstrates that plants can be used to source biologics that meet the stringent demands for high quality in human products, at competitive scale and costs. Indeed, Protalix indicates that the lower production costs associated with carrot cell systems will allow pricing of Elelyso to be 75% of Cerezyme, the leading product in this market sector [20]. This approval strengthens the regulatory case for plant-based production systems that was established by the 2006 approval of a DowAgrosciences, LLC vaccine by the US Department of Agriculture for the prevention of Newcastle disease in chickens [27]. As with the Protalix product, this recombinant vaccine was derived from a transgenic plant cell culture system.

Agriculture offers several advantages as a biologic production system. Plants allow capital-efficient design of upstream manufacturing capacity at various scales providing cost savings that cannot be easily matched by fermentation technologies. The market opportunities provided by follow-on biologics and the rising capital costs associated with production using traditional systems make plants particularly attractive. Considerable capital and time is required to construct the upstream facilities for cell culture production. The upstream facility must be linked with downstream capabilities supporting product purification and characterization. Although outdated, the published costs associated with

these facilities are \$300–\$500 million and require from four to five years to complete construction, validation, and to gain regulatory approval [28]. Agriculture-based production requires less specialized upstream facilities, typically controlled growth chambers, linked with similar downstream production capabilities. The use of plants therefore reduces capital expenditures and also provides for more flexible use of space and capital.

The handling of plant biomass and its initial extraction requires unique biomanufacturing solutions [14]. Virus vector expression systems offer significant advantages at considerably reduced costs to current cell-based manufacturing systems, such as employed by Protalix and Dow AgroSciences, while avoiding concerns associated with stable plant transformation [29, 30]. Virus based expression systems have been extensively tested and shown safe and environmentally-friendly in both indoor and outdoor tests since 1991 [12] and multiple products completing early stage human clinical investigations [31, 32]. Additional advantages also exist compared with traditional cell-based fermentation approaches include: 1) speed and low cost of genetic manipulation; 2) rapid manufacturing cycles; 3) no mammalian pathogen contamination; 4) minimal endotoxin concentrations and 5) economical production [12-15, 33, 34].

4. Types of plant virus-based expression vectors

Many different types of plant viruses have been converted into vectors for the production of recombinant proteins or peptides (for complete review, see [12-15, 33, 34]). As different viruses have distinct biological limitations and gene expression potential, each vector system has its own unique opportunities. This review focuses on virus vectors that have been particularly useful to produce recombinant proteins for biomedical, therapeutic and
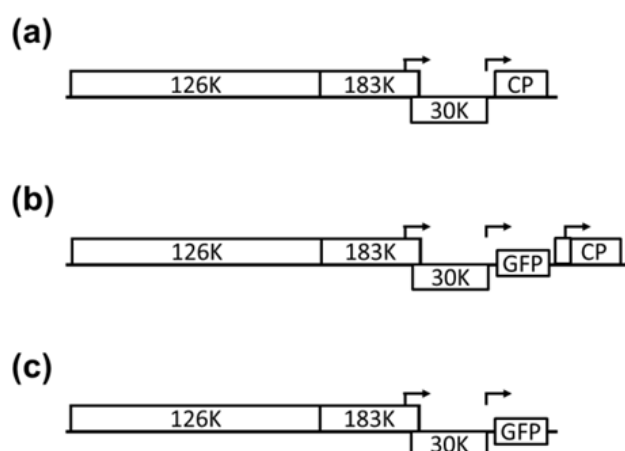
Figure 1. The genomic structure of wild type Tobacco Mosaic *tobamovirus* RNA (a) and derivative independent (b) and minimal (c) expression vectors. Boxes represent the open reading frames on each RNA and are labeled with the the viral protein they encode. The 126K and 183K proteins are required for virus replication. The 30K gene is the virus movement protein required for cell-to-cell transport. The CP is the virus coat protein that is required for encapsidation and systemic movement. The green fluorescent protein (GFP) is used as a placeholder for a gene for protein overexpression *in planta*. Arrows indicate the position of subgenomic promoters used to express the downstream proteins.

research, use. Many groups have sought to categorize virus based expression systems. For this review, we will organize our thinking by using two categories: "independent-virus" or "minimal-virus". Independent-virus vectors are replication competent vectors that can be principally inoculated to plants as virus particles or viral RNA, multiply in initially infected cells and exploit virus encoded cell to cell and systemic movement activities to infect the majority of the phloem sink tissue of a host. In contrast, minimal virus systems are replication competent systems that have be modified in order to possess greater expression capabilities. The modifications are typically replacement of a virus-encoded open reading frame, not essential for genome replication, with the gene of interest such that the minimal-virus systems lack the ability to systemically infect a host. Examples of each system will be provided in the following sections with Figure 1 illustrating the genetic structures of independent and minimal virus systems derived from the tobacco mosaic *tobamovirus.* (TMV) genome.

4.1. Independent-virus vectors

Initial RNA virus vectors were functionally minimal-virus vectors that utilized a "gene replacement" strategy where a foreign gene of interest replaced the capsid protein (CP) gene of a virus [11]. These early vectors expressed foreign genes, but, as with other minimal-virus systems, lacked certain virus functions thereby limiting activities. For example, brome mosaic virus (BMV) CP replacement vectors could not even move from cell to cell in an infected leaf [11], and although TMV-based CP replacement vectors could move from cell to cell, they could not move systemically in inoculated plants [35-37].

With greater understanding of virus function, plant RNA virus vectors were constructed to express a foreign gene product in addition to all required viral proteins [36, 38]. These vectors were the first independent-virus system that expressed recombinant products while moving systemically in a host plant. To construct independent-virus systems for (+) strand RNA viruses, vectors exploit subgenomic mRNA production to express foreign genes by using an additional subgenomic promoter inserted into the virus [38-40]. For viruses that used polyprotein processing, the foreign gene was inserted in translational frame with the existing virus open reading frame (ORF) and peptide sequences that facilitate the proteolytic processing of the fusion protein were present to insure release of the recombinant protein. Some independent-virus vectors are designed to express potential products as fusions to viral proteins, such as the potatovirus X (PVX) CP (reviewed in [41]). Often the fusion methodology employs the foot and mouth virus 2A translational cleavage sequence (see references in [42]). The apparent pausing of the ribosome, and the discontinuity of the peptide bond that results, allows proteins upstream and downstream of the 2A sequence to be differentially targeted, such as a single chain antibody accumulating in the plant apoplast while the CP was sequestered in its normal cytosolic localization [43]. Using these strategies, independent-virus systems have been derived from the genomes of *potexviruses* (including potato virus X; PVX), *tobamoviruses* (including TMV), *comoviruses* (including cowpea mosaic virus), *potyviruses*, *tobraviruses*, *closteroviruses* and others [12, 13, 15].

Most independent-virus vectors are functional in *Nicotiana* or other herbacious species. This follows from their ease of inoculation and the receptivity of species to virus expression

systems. Recently, the adaptation of *closterovirus* vectors to a non-herbacious plant system was accomplished by adaption of Grapevine leafroll-associated virus-2 into an expression vector [44]. This vector showed characteristic phloem-associated expression in inoculated grapevines involving the roots, stems, petioles, leaves and berries. A grapevine-A vector was also shown to induce inhibitory RNA (RNAi) of model genes in micropropogated grapevines [45]. These vectors for use in expression and RNAi-based functional genomics studies open new non-transgenic strategies for researchers in woody plants.

As a more detailed example of an independent-virus system, *Tobamoviruses*-based vectors have been commonly used for recombinant protein expression. These viruses have a (+) sense single stranded RNA genome of ~6400 nucleotides helically encapsidated by many ~2,100 copies of a 17.5 kDa CP in rigid rod shaped particles [12]. The viral replication-associated proteins are directly transcribed from the genomic RNA directly, whereas expression of internal genes is through the production of subgenomic RNAs. Sequences in the *tobamovirus* genome function as subgenomic promoters regulating the production of subgenomic RNAs. The virus movement protein MP and CP are translated from two separate, but co-terminal, subgenomic RNAs, with the CP being among the most abundant protein and RNA produced in the infected cell [46]. In a *tobamovirus* infected plant there are several milligrams of CP produced per gram of infected tissue.

Tobamoviruses-based vectors are readily constructed as independent-virus systems, including cell-to-cell and systemic movement activities mediated by MP and CP, respectively (Figure 1). These vectors benefit from the strength of the viral subgenomic promoter's activity to reprogram the translational activities of infected plant cells such that virus-encoded proteins are synthesized at high levels, often similar to the TMV CP [40]. A foreign gene encoding the protein for overexpression is added in place of the virus CP so it will be expressed from the endogenous virus CP promoter [38, 40]. A second CP promoter from a different *tobamoviruses* strain, of sequence divergent to the first CP promotor, is placed downstream of the heterologous coding region and a virus CP gene is then added 3' terminal to the heterologous subgenomic promoter. *Tobamoviruses*-based vectors infect various species, but most commonly tobacco-related species (genus *Nicotiana*), including *tabacum* and *benthamiana*. For the vector to express foreign proteins, the infectious vector RNA enters plant cells via wounds induced by an abrasive. The virus RNA is released from the CP subunits, translated to produce replication-associated proteins and is replicated in the initial cell. The progeny RNA is moved to adjacent cells in association with the MP to produce infection foci. A proportion of the RNA, complexed with CP, enters the plants vascular system for transport to phloem sink tissues in the aerial leaves. This movement produces the systematic infection and the foreign gene is expressed in all cells that express other virus protein products. Within the cell, the foreign protein is deposited in the site dictated by its protein sequence, either naturally or purposely engineered [12, 46].

Tobamoviruses-based vectors have been used by literally hundreds of researchers to produce a range of human enzymes, antimicrobials, cytokines, subunit vaccine and immunoglobulin proteins. Several reviews have provided surveys of these products [12, 14]. In general, expression results were obtained in *Nicotiana* hosts and proteins were extracted from leaf

tissues using total homogenization and clarification methods or leaf infiltration and isolation of interstitial fluids [46, 47]. Several products have been purified using differential separation and standard chromatographic separations and tested in various model systems of human disease. TMV-based vectors, especially the well-developed GENEWARE® system, have been used to express a large range of recombinant proteins under research and Current Good Manufacturing Practice (cGMP) compliance [14]. One class of products has been successfully tested in human clinical trials, as described below.

Active vaccination of Non-Hodgkin's Lymphoma (NHL) patients with cancer antigens, in this case the idiotypic antibody expressed by the tumorogenic B-cells, has been shown to induce clinical remissions in human clinical trials [48]. However, more efficient and effective vaccines are sought. Full antibodies contain both idiotype-specific elements as well as constant sequences, shared by many antibodies, which may reduce the immunogenicity of the vaccine. In order to provide higher antigen content to vaccines, single chain antibodies (scFvs) were constructed from tumor-derived idiotypic antibodies to provide simpler and more sequence-focused vaccines for clinical testing. This vaccine strategy was shown to be effective in murine models of NHL [49] and a GENEWARE®-based production methodology was developed that could produce >80% of scFvs from human tumor samples [50]. Sixteen patients were enrolled in a Phase I clinical trial under the regulatory oversight of the US Food and Drug Administration [31, 51]. Vaccines were successfully produced for all patients and applied in two dosing groups with and without granulocyte-macrophage colony-stimulating factor adjuvant. The primary endpoint of the study was safety which, as the first parenteral administration of a plant-made vaccine, was an important outcome to monitor. The study results confirmed the safety of plant-derived vaccines, including plant-specific glycoforms present on 15 of the 16 vaccines. The secondary endpoint was determination of the immunogenicity of the vaccines in human subjects. Overall, 70% of the patients developed cellular or humoral immune responses to the scFv vaccines, with the adjuvant improving the frequency of responses, as predicted. The majority of the responses was shown to be vaccine specific and did not cross react with control idiotype proteins. These results demonstrated the flexibility of the TMV-based expression systems as well as the safety and effectiveness of the plant derived products [31, 51].

4.2. Minimal-virus vectors

In contrast to independent-virus vectors, minimal-virus systems are capable of functions supporting RNA replication, yet are lacking in one or more functions necessary for systemic infection. Although this vector was the first type constructed, researchers moved away from this approach in favor of the independent-virus vectors. However, as limitations emerged from independent-virus systems, including the size of genes that can be expressed, host range limitations and problems with systemic movement, researchers revised minimal-virus systems with new energy. The resulting vectors were found to be incapable of systemic movement in inoculated plants, thus they must be delivered to each and every plant leaf to allow cell-to-cell movement activities allow infection of all inoculated leaves. Standard abrasion methods are too tedious to deliver inoculum to each leaf, so new methods were

developed. The most common method is Agro-infiltration of host plants to launch the infection process [33, 52-54]. This process introduces a DNA plasmid, containing the virus vector under the control of an appropriate transcriptional unit within normal Ti plasmid integration sites, into *Agrobacterium tumefaciens* cells to create an inoculum. The *Agrobacterium* strain containing the DNA expression constructs are grown in overnight cultures and diluted for inoculation. Plants are inverted, submerged in inoculum solution and a vacuum is applied removing the air trapped in the leaves. As the vacuum is released, the inoculum replaces the air in the leaf spaces providing the *Agrobacterium* strain access to cells throughout the submerged leaves for invasion. The bacteria then exploits the transfer DNA mechanisms to introduce the DNA copy of the virus expression vector into the nucleus of infected plants. RNA transcription produces infectious RNA or mRNA transcripts that are processed by nuclear enzymes into an intact and capped transcript and exported to the cytoplasm. These transcripts are then translated and replication protein expression initiates similar to independent-virus systems.

The activity of viral movement proteins move the vectors from the initially infected cells to adjacent cells creating a more rapid and synchronous infection of inoculated leaves than independent-virus vectors. This eliminates the delays associated with systemic plant movement and can yield greater amounts of recombinant proteins in a shorter period of time than independent-virus systems [33, 54]. This approach increases the genetic load carried by the minimal-virus systems allowing efficient expression of larger recombinant proteins [55]. Minimal-virus systems have been developed from the genomes of *potexviruses*, *tobamoviruses*, *bromoviruses*, *comoviruses* and *geminiviruses* [12, 13].

Nicotiana benthamiana is an ideal host for minimal-virus expression since it highly susceptible to *Agrobacterium* infection to mediate initial entry and introduction of the viral expression vectors. The expression of a defective form of RNA dependent RNA polymerase in *N. benthamiana* [56] makes it nearly universally susceptible to plant viruses and the great experience with this host has led it to be the common host for independent-virus as well for the expression of many recombinant proteins. The flexibility of Agroinfiltration inoculation procedures allow more than one expression vector into a host plant in a given treatment. The co expression of silencing suppressor proteins has been shown to be a key factor for optimized yields [57-59]. Such methods have been used to produce a range of biopharmaceutical proteins [60-62] and offer strategies to modify the plant enzymatic machinery, producing more stable and "human" like recombinant proteins, including glycan structures [34, 60] which will be discussed later in this review.

Examples of minimum-virus vectors include the systems developed using TMV genomes include those developed by (Figure 1)[63,64]. Two TMV variants were developed – one, actually an independent-virus system, employed the full TMV virus capable of systemic movement [63] and a second – lacked the virus coat protein as minimum-virus system [64]. The minimum-virus system (TRBO vector) produced significantly higher levels of the green fluorescent protein (up to 5.5 mg/g FW). This vector did not require the co-expression of a silencing suppressor and worked with very high inoculum dilution in infiltration medium. The RNA2 of cowpea mosaic virus (CPMV) was also adapted as a minimum-virus system

which overcame the historic limitation of the insert size in CPMV vectors and allows expression hetero-oligomeric proteins from a single vector [65, 66]. However, the system also requires co-expression of silencing suppressors for optimal expression.

DNA viruses have been adapted to minimal virus systems. Both single and bipartite plant geminivirus systems have modified to produce recombinant proteins – usually at the expense of expression of the capsid or key movement or transmission proteins. Maize streak virus is an example of a single component virus which has been converted into an expression vector [67]. Bean yellow dwarf virus (BeYDV) has been developed into a single and dual-component replicon system that permits simultaneous, efficient replication of two DNA replicons and thus high-level accumulation of one or two recombinant proteins in the same plant cell [68, 69]. This system has been used to produce express immunoglobulin proteins and human papilloma virus HPV-16 and the p24 protein of HIV-1 [70]. The system requires co-expression of the silencing suppressors. Some geminivirus systems have been adapted to express recombinant proteins in non-*Nicotiana* species, including lettuce [71].

The most advanced minimum-virus system is magnICON®. This technology has been used to express a large number of recombinant proteins, including cytokines, interferon, bacterial and viral antigens, growth hormone, single chain antibodies (reviewed in [54, 72]). The ability of Agroinfiltration to introduce more than one expression vector into a host plant in the same inoculation allows the use of two magnICON® vectors to produce heteromeric recombinant proteins, such as mAbs. For production of mAbs, two non-competitive virus vectors are used: one based on turnip vein clearing tobamovirus (TVCV) and the second, potatovirus X (PVX [55, 73]). In mAb production, two magnICON® virus expression vectors each contain a separate mAb chain, heavy or light, and are co-delivered by Agroinfiltration. Each vector replicates independently and expresses mAb chains in the same cells that self-assemble functional mAbs at yields up to 1 g/kg fresh weight [33, 54, 55]. These vectors have been used for efficient large scale production of multi-gram batches of mAbs under Current Good Manufacturing Practices that have been tested in several challenge model systems, including non-human primates [14, 74].

5. Synergy of transgenic plants and virus-based expression vectors

Standard integrative plant expression vectors allow transformation of plant lines using *Agrobacterium tumefaciens*-mediated transfer-DNA delivery methods. These methods allows for great flexibility and synergy when mixed with transient, virus-based expression vectors. The mixture of these two approaches allows for the efficient expression of the recombinant biopharmaceutical protein of interest and provision of the required co-factors improving pharmaceutical protein yield and processing. As noted above, the co-expression of silencing suppressor proteins has been shown to be a key factor for optimized yields with some minimal-virus expression systems. Further, entire protein processing systems can be introduced into plants to produce more stable and "human" like recombinant proteins, including glycan structures [60, 34]. Concerns over the potential immunogenicity of plant-specific glycan linkages on recombinant proteins (the presence of β1,2-xylosylation

and core α1,3-fucosylation) have been mitigated by the use of RNAi technologies to down-regulate endogenous beta1,2-xylosyltransferase and alpha1,3-fucosyltransferase genes [75-77] in *N. benthamiana* plant lines. Proteins produced from these lines show almost homogeneous N-glycan species without detectable beta 1,2-xylose and alpha1,3-fucose residues providing a host that produces humanized glycan structures. Indeed, production of antibody products using the magICON® system using the RNAi plants have demonstrated the synergy of the systems – rapid production of high quantity production of antibodies that show humanized glycan structures [14]. Indeed, the lack of fucose glycans on mAb products produced in the *N. benthamiana* producing humanized glycans have shown enhanced potency in the treatment of Ebola infection in a non-human primate model, anti-tumoral investigations of a plant-derived trastuzumab in murine xenograph models and HIV-1 neutralization studies [74, 78, 79]. The mechanism responsible for this enhanced *in vivo* activity appears to be the improved Fcγ and FcγRIII receptors of nonfucosylated, plant produced mAbs. This advantage based on glycan engineering offers great promise for plant derived biosimilar antibody products.

The present of sialic acid terminal sugars on glycan structures of human plasma proteins is correlated with their long half-life and the pharmacokinetic properties of effective recombinant therapeutics which must function in human plasma [80]. The capability to sialylate plant proteins has been demonstrated in transient and transgenic *Arabidopsis* systems by [81-83]. This effort required transformation events providing enzymatic synthesis of the sialic acid metabolic precursor, which is normally not synthesized in plants, in addition to transferase activities. Efforts are now ongoing to introduce this multi-genic modification into *N. benthamiana*. This glyco-modified host in combination with transient virus-based expression of recombinant proteins benefiting from sialylation offers potential single-step production of human plasma enzymes with similar pharmacokinetic properties as the native proteins. By eliminating any need for post-production enzyme modification, successful development and deployment of these plant lines would contribute to both faster speed of delivery and lower cost of goods. From these examples, the synergy between tailored expression hosts, genetically engineered for appropriate protein post-translational modifications, and virus-based expression strategies to provide recombinant products that meet the biological function and production rigors of modern biotechnology.

6. Transient virus-based expression: Transitioning from dicot- to monocotyledonous plants

The focus of the previous material was virus expression systems providing recombinant protein expression primarily in plant dicotyledonous plant species. This emphasis comes from the historical emphasis on dicot expression due to the availability of more facile systems. Yet monocotyledonous species, especially the cereals, are the most important of crops for feeding humans and livestock in many parts of the world. Further, maize, sugarcane, bamboo and other monocot species, are used extensively in the production of biofuels and other industrial products. Monocots possess unique morphological features and seed biology suggesting gene functions not present in dicots. Monocot species have also

developed unique mechanisms for tolerance of adverse environmental conditions like drought and high salinity. In the past, most protein functions were proposed based on homology to better characterized dicot systems, such as *Arabidopsis thaliana*, but functional assays are required to truly ascertain gene function. Overexpression and RNAi gene silencing strategies are key to these direct studies [84]. The construction of transgenic plant lines is not an adequate solution due to the time consuming nature of the approach and the frequency of lethal phenotypes. Therefore, a more rapid, transient strategy is needed. RNA virus expression vectors offer such powerful tools for understanding the biology of these species. In the remaining sections of this review, we will examine the contributions that monocot viral expression systems have made and provide a few detailed examples.

6.1. Use of transient expression vectors as functional gene discovery tools in moncot species

One of the challenges in developing any plant based expression system is to achieve high level expression without triggering the post translational gene silencing (PTGS) and related RNAi mechanisms that plants and other organisms have evolved (reviewed in [18]). These mechanisms have been observed to operate in transgenic plants, and even exploited to generate pathogen-derived resistance to viruses in cases where the silencing of viral transgenes prevents related viruses from infecting the transgenic host [85, 86]. The identification of plant viral proteins that are able to suppress these silencing mechanisms suggested that these mechanisms have evolved in part to prevent or slow viral infection.

Expression of proteins using viral vectors can also trigger PTGS, and is referred to as viral induced gene silencing (VIGS). Using VIGS, an endogenous plant gene can be silenced by inserting only a small portion of the target gene (100-500 nt in length) which creates loss of function phenotypes to study gene function. A wide range of viruses have been developed as VIGS vectors, originally, and most extensively, for dicot hosts [18, 87]. Shortly after the first dicot examples, a barley stripe mosaic virus (BSMV) vector, was used to silence the endogenous *phytoene desaturase (pds)* gene systemically in barley [88].

BSMV, the type member of the hordeivirus family, infects many agriculturally important monocot species such as barley, rice, corn, oat and wheat [89-92]. It is known to be highly seed transmitted in barley, which could be a potential advantage for assessing gene function in seeds and early development. As shown in Figure 2, BSMV has a tripartite (+) sense RNA genome consisting of RNAs α, β and γ [93]. The virus uses well characterized subgenomic promoters for gene expression from each of its genomic RNAs [94].

As a more detailed example of a VIGS vector, BSMV was constructed by inserting fragments of the silencing target so that they would be expressed only as untranslated RNA on a subgenomic promoter following the γb ORF [88]. Although the strongest silencing in barley plants was observed using the barley *pds* fragments, fragments from *pds* taken from maize and rice caused a degree of silencing that was proportional to their sequence similarity with the barley homolog. Further modification of the virus to delete the coat protein enhanced the suppression of the endogenous *pds*. Since this first demonstration of VIGS in barley, BSMV

silencing vectors have been successfully used to demonstrate gene functions related to pathogen resistance, aphid defense, development and mophogenesis in a variety of monocots including oat, rice, and the model grass *Brachypodium distachyon* [87]. BSMV mediated VIGS was recently shown to operate in root, leaf and meiotic tissues of wheat, along with efforts to optimize its efficacy in this important agricultural host [95]. In addition, BSMV mediated VIGS can be inherited and has been observed for up to 6 generations in wheat and barley, which not only enhances the range of phenotypes that can be explored for reasons related to the timing of developmental events, but also because progeny frequently have fewer viral symptoms [87, 96]. Most recently, the β RNA has been modified to incorporate untranslated foreign gene fragments and was used with the modified γ RNA vector described above to silence two endogenous genes simultaneously, or to achieve enhanced silencing of a single gene [97]. In addition to BSMV, a strain of BMV has also been engineered as a VIGS vector and has been used successfully in barley, rice and maize [98, 99].
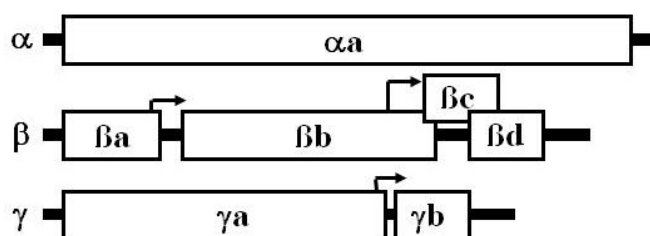
Figure 2. The general structure of BSMV genomic RNAs α, β and γ. Boxes represent the open reading frames on each RNA and are labeled with the the viral protein they encode. Arrows indicate the position of subgenomic promoters used to express the downstream proteins.

Coupling VIGS with high-throughput cloning and sequencing technologies has additionally allowed these viral vectors to be used in functional genomics. In this approach, cDNA libraries are constructed within virus expression vectors and gene function to be assessed by screening infected plant hosts for phenotypic or metabolic changes measured by various input and output focused screening assays. TMV and PVX VIGS vectors were the first to employed in this manner in the dicot *N. benthamiana*, with BSMV used shortly after in the monocot barley [84, 100]. Since these first reports, vector systems for functional genomics have continued to be refined and optimized in an expanding number of hosts [18, 87], including systems amenable for high throughput screens in monocots based on BSMV [101, 102].

6.2. Use of transient virus expression systems for recombinant protein expression in monocot species

As with dicot expression vectors, monocot vectors include both minimal and independent virus vectors [103]. As noted above, minimal type BMV vectors have been constructed by replacing the coat protein ORF with a foreign gene. For example, French *et al.* [11] used this strategy to express the CAT protein in barley protoplasts. In another example, involving BSMV, substitution of the open reading frame (ORF) for the βb triple gene block 1 (TGB1)

movement protein was used to express the luciferase reporter gene [104]. An example of an independent vector is one based on the potyvirus, wheat streak mosaic virus (WSMV). In this system the foreign ORF is inserted in frame within the virus polyprotein and flanked by cleavage sequences that allow for its release after translation [105]. These systems have the limitations of low foreign gene insertion efficiency and the smaller number of hosts that can be infected and tested for recombinant protein expression.

6.3. Using barley stripe mosaic virus vectors for recombinant protein expression

BSMV has been developed to express recombinant proteins as fusions to several individual virus proteins expressed from subgenomic RNAs. To date these systemic vectors have not incorporated any sequences to liberate the foreign protein from the fusion protein. For example Lawrence and Jackson [106] expressed GFP as a fusion to the N terminus of the βb (TGB1) protein at levels sufficient to explore the function of the viral protein in cell-to-cell movement. Higher levels of expression however were achieved with fusions to the C-terminus of the BSMV γb protein. This includes systemic expression of a γb::GFP fusion protein used to study viral movement in barley [107, 108]. More recently, C-terminal γb fusions have been use to test the fungal ToxA protein activity in wheat, barley and *N. benthamiana* [109]. In the latter case, the recombinant virus genome, containing the ToxA gene, was sufficiently stable that Agroinfiltrated *N. benthamiana* was used as the inoculum source for systemic expression in barley and wheat. ToxA phenotypes were replicated in all species. The systemic expression by BSMV of non-fusion recombinant proteins in barley and wheat has been achieved by substituting most of the γb ORF with the gene for either the wild type and mutant ToxA protein [110]. Again, the resulting phenotypes indicate the ToxA proteins were expressed appropriately.

These efforts to develop independent viral expression vectors in monocots are extended by previously unpublished work described here. Our aim in this work was to improve foreign gene stability, increase the level of foreign gene expression, and generate free, non-fusion, foreign proteins that could function and localize independently of viral proteins.

Expression of GFP::γb Fusion Protein from RNA γ

To test whether insert stability was related to the fusion orientation, we constructed a vector with GFP fused (indicated by the "::" in construct name) to N-terminus of the γb protein (γ.GFP::γb, Figure 3A). Co-inoculation of protoplasts and barley leaves with α and β RNAs (BSMV-GFP::γb) resulted GFP fluorescence and fusion protein accumulation to levels that were indistinguishable from co-inoculations with α, β and γ.γb::GFP (BSMV-γb::GFP) as shown in Table 1 (compare #3 and #4). However, as the infection in plants progressed beyond 7 dpi, GFP fluorescence and fusion protein accumulation were observed longer in γ.GFP::γb infected plants than in those infected with γ.γb::GFP. Specifically, GFP::γb expression is regularly observed in leaves 1-4 above the inoculated leaf by BSMV-GFP::γb, compared to 1-2 leaves for BSMV-γb::GFP (data not shown). Thus, the GFP gene is generally maintained and expressed by BSMV-GFP::γb to 18 dpi. We believe that the increased stability of fusions to the N-terminus of γb results from more restrictive requirements for the

deletion of the GFP gene in order to gain a competitive advantage. For example, internal deletions in the GFP ORF have a one in three chance of maintaining the continuity of the ORF with γb, which is critical because γb is an important virulence factor for BSMV [111]. Thus, those deletions which result in a γb frame shift are likely to be less competitive than those viruses which maintained the full GFP ORF. In contrast, for C-terminal fusions of GFP to γb, all deletions within the GFP ORF would maintain γb expression and would presumably be more competitive than the parental virus due the reduced genetic load.
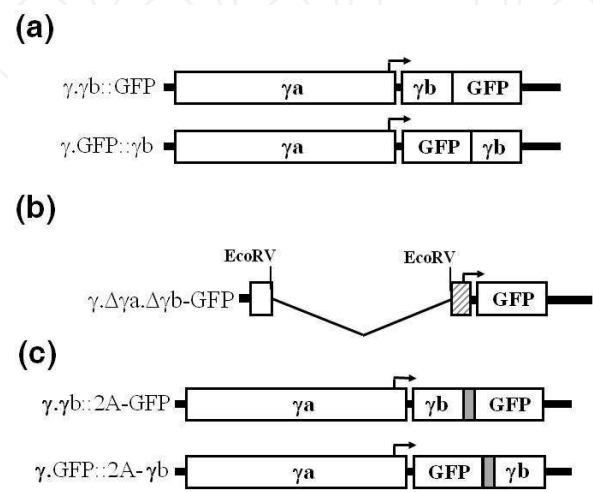
Figure 3. Genomic organization of BSMV γ RNAs engineered to express GFP. Vector components γ.γb::GFP and γ.GFP:: γb as designed to produce a fusion of GFP to the γb protein C and N terminus, respectively (a). The vector component γ.Δγa. Δγb-GFP designed to produce only free GFP (b). Vector components γ.γb::2A-GFP and γ.GFP::2A-γb as designed to produce a free GFP protein following cleavage by the FMDV 2A cleavage sequence from the γb protein C or N terminus, respectively (c). Open boxes indicate ORFs, hatched boxes indicate untranslated ORF sequences, grey boxes indicate FMDV 2A cleavage sequence, arrows indicate subgenomic promoters. EcoRV restriction sites indicate the position of the Δγa sequences that have been deleted from the wild type γ RNA.

| Inoculum					GFP expression		Systemic symptoms of BSMV infection
Inoculation#	Vector RNA components				Tobacco Protoplasts	Barley Plants	
							Barley Plants
1	α	β	none	γ	-	-	yes
2	α	β	TMV-GFP		*****	-	no
3	α	β	γ.γb::GFP		****	systemic	yes
4	α	β	γ.GFP:: γb	γ	****	systemic	yes
5	α	β	γ.Δγa. Δγb.GFP		-	-	no
6	α	β	γ.Δγa. Δγb.GFP	γ	*****	-	yes

Table 1. Comparative expression of GFP in tobacco protoplasts and barley plants using BSMV vector constructs

Expression of native GFP from RNA γ

To develop a BSMV vector able to express a free foreign protein, we tested a variety of novel vectors with the most promising of which described here. In each case, the BSMV γ RNA

was modified to express GFP in combination with wild type BSMV α and β RNA. A three component vector with RNA α, β, and γ.Δγa.Δγb-GFP (a γ RNA containing a deletion of γa, the replicase protein ORF, and GFP in place of the γb ORF, Figure 3C) did not express GFP in protoplasts (#5, Table 1). However, the addition of wild type γ RNA created a four component vector, which was equivalent in GFP expression levels to TMV 30B (TMV-GFP [40]) in protoplasts (compare #6 with #2, Table 1). In contrast to its behavior in protoplasts, the four component vector did not express GFP systemically in barley, even though systemic viral symptoms were observed (#6, Table 1), apparently due to the loss of the GFP bearing component during systemic movement. This system may be amenable to expression in whole plants if delivered using an Agroinoculation strategy, or in transgenic plants expressing the γa and γb proteins to complement RNA α, β, and γ.Δγa.Δγb-GFP.

In a different approach, BSMV vectors expressing N- and C-terminus fusions of GFP to BSMV γb were modified to release GFP *in vivo* after autocleavage of the fusion protein. Cleavage of the fusion protein was achieved by inserting the foot and mouth disease virus (FMDV) 2A co-translational cleavage sequence [112, 113] between the fused ORFs (Figure 3C). Western analysis of γ.GFP::2A::γb and γ.γb::2A::GFP infected tobacco protoplasts and barley plants revealed that the FMDV 2A cleavage sequence was at least >90% effective in both cases (data not shown). Viral spread and GFP fluorescence in barley plants infected with γ.GFP::2A::γb was indistinguishable from those containing γ.GFP::γb. In contrast, the systemic spread of the BSMV-γb::2A::GFP virus was slightly delayed compared to BSMV-γb::GFP (data not shown). Both BSMV-γb::2A::GFP and BSMV-GFP::2A:: γb were able to infect and express GFP in *N. benthamiana* plants in a manner similar to that reported for γ.γb::GFP [108].
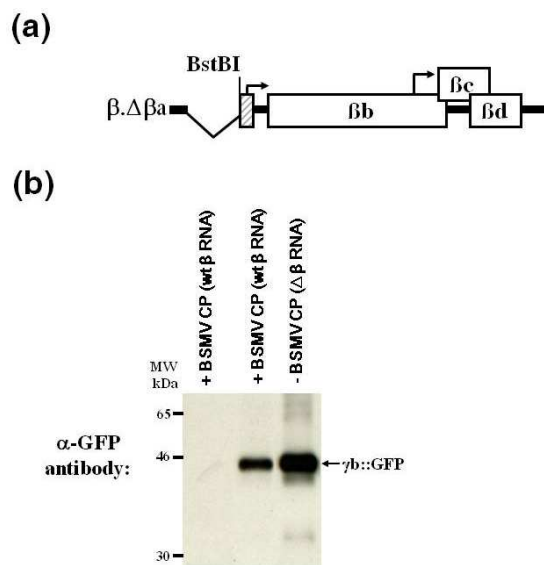
Figure 4. BSMV Coat protein deletion vector and GFP expression. Genomic organization of BSMV β RNA engineered with a deletion of the BSMV coat protein, βa (a). Open boxes indicate ORFs, hatched boxes indicate indicate untranslated ORF sequences, arrows indicate subgenomic promoters. BstB1 restriction site indicates the position of the βa sequences that have been deleted from the wild type β RNA. Western blot analysis of γb::GFP protein in barley leaves (b).
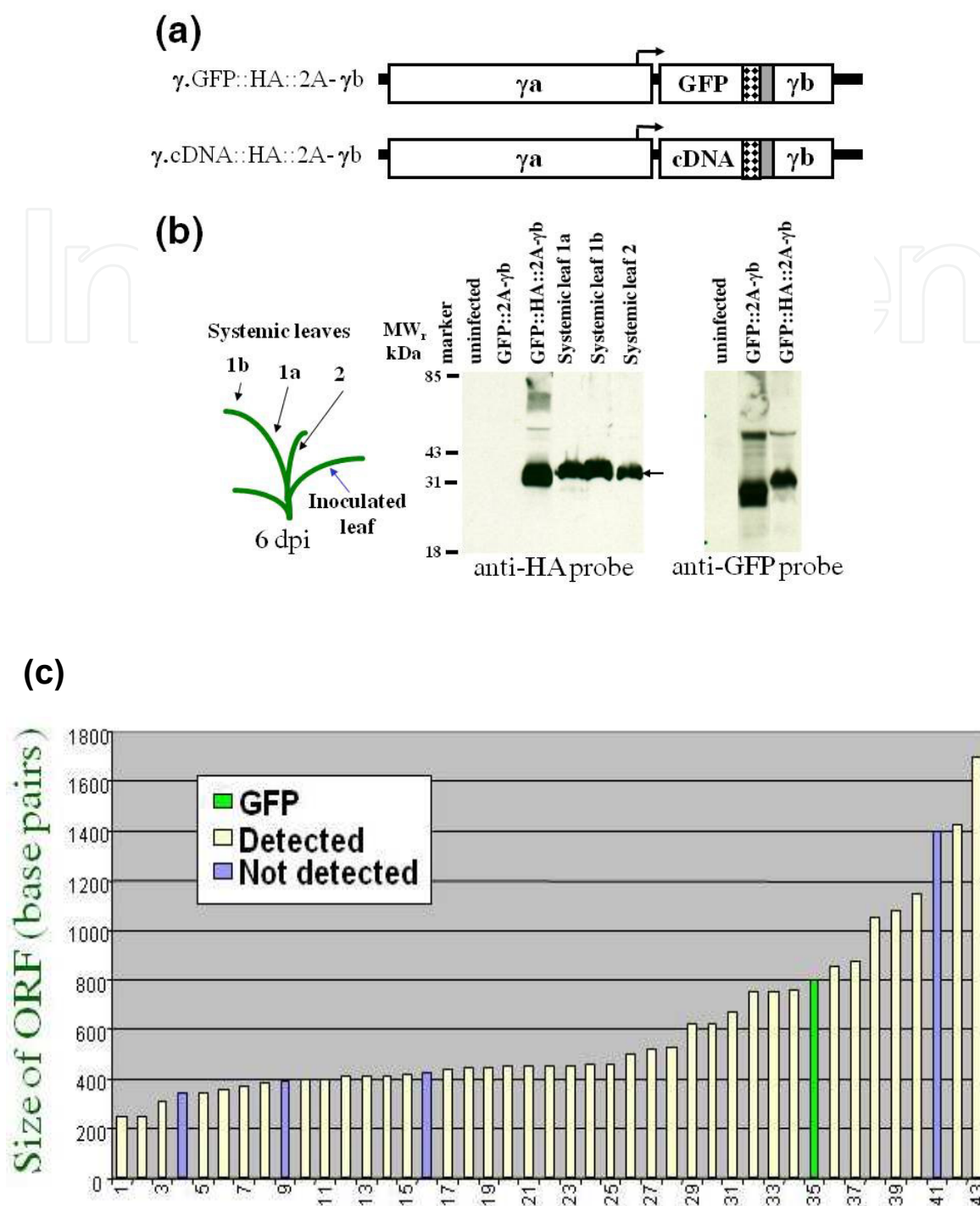
Figure 5. Genomic organization of BSMV γ RNAs and expression of free GFP or heterologous proteins from a cDNA library. Vector components γ.GFP::HA::2A-γb and γ.cDNA::HA::2A-γb as designed to produce a fusion of GFP or a heterologous protein with an HA epitope tag and the FMDV 2A sequence. The 2A sequence is designed to release the γb protein is during translation (a). Open boxes indicate ORFs, checkered boxes indicate the HA epitope sequence, grey boxes indicate FMDV 2A cleavage sequence, arrows indicate subgenomic promoters. Western blot analysis of GFP::HA fusion protein in barley leaves (b). Graph of rice cDNA sequences arranged according to size (c). cDNAs whose predicted proteins were detected by Western blot analysis are unshaded, while blue shaded bars indicate the lack of detectable HA tagged protein. Green bar represents the internal GFP::HA::2A control.

Coat Protein Deletion Enhances Expression from γb Subgenomic Promoter

The coat protein (βa) of BSMV is not required for systemic infection. To determine the impact of βa deletion on βb subgenomic expression, a β genomic RNA containing a deletion of the βa ORF was constructed (β.Δβa, Figure 4A). In tobacco protoplasts, using β.Δβa increased GFP accumulation from the γ RNA 2-5 fold for viruses bearing the γb fusions (γ.γb::GFP, γ.GFP::γb, γ.γb::2A::GFP, and γ.GFP::2A::γb) or GFP substitutions of γb (γ.Δγa.Δγb.GFP) (Figure 4B and data not shown). Using β.Δβa also increased GFP fluorescence and accumulation for γb::GFP, GFP::γb, γb::2A::GFP, and GFP::2A::γb, in systemic barley tissue (data not shown).

Expression of Epitope Tagged Heterologous Proteins from RNA γ Derived Vectors

To detect heterologous proteins, γ.GFP::2A-γb was modified to contain the hemagglutinin (HA) epitope tag fused between GFP and the FMDV 2A cleavage sequence (γ.GFP::HA::2A-γb, Figure 4A). In barley plants infected with BSMV RNAs α, β.Δβa, and γ.and γ.GFP::HA::2A-γb, Western blot analysis detected the HA tagged GFP (Figure 5B). The addition of the HA tag had no effect on the efficiency of the 2A cleavage sequence. These results suggested that the HA epitope tags would allow for the efficient detection of BSMV expressed heterologous proteins.

To test the flexibility of BSMV to express heterologous proteins in barley, plant genes, from a variety of subcellular locations were expressed as N-terminal fusions to HA::2A-γb (γ.cDNA::HA::2A-γb, Figure 5A). A total of 42 full-length rice genes were amplified from sequenced, cDNA library clones and inserted in frame with the HA::2A-γb ORF. All 42 γ.cDNA::HA::2A-γb RNAs were infectious when co-inoculated onto barley plant with BSMV RNAs α and γ.Δβa. For each vector, systemically infected barley tissue was tested in replicate for recombinant protein expression using two anti-HA antibodies. From the 42 genes, 38 genes showed confirmed expression of protein product in each plant tested (Figure 5C). In each case, the size of the protein detected in the Western blot was consistent with the size predicted for the post-cleavage heterologous protein, with the addition of the HA epitope tag and the 2A sequence. The 2A cleavage differed between the different heterologous genes, but cleavage rates of 60-95% were usually observed.

In our survey of 42 full length rice ORFs between 200 and 1800 nucleotides in size. The resulting gene products including proteins of 38, 46, 54 and 64 kDa proteins. Due to the average insert size of the library, proteins of 20-30 kDa were most commonly detected in our study. The flexibility of the BSMV expression vector was further demonstrated is ability to successfully express proteins of cytosolic localization (GFP and BMV CP) and those that are matured through the plant secretory pathway (including a lysozyme, interferon, human growth hormone and protease inhibitor). The maturation of these proteins demonstrates the ability of the 2A cleavage system to deliver proteins to distinct subcellular fates and still retain activity. In addition, these data also demonstrate the ability of the vector to express genes from both plant and animal sources successfully.

In summary, to develop a monocot vector capable of expressing free, heterologous proteins, we have tested a variety of strategies based on the BSMV γ RNA. All of the strategies relied

on expression from the γb subgenomic RNA facilitating expression of >90% (38/42) recombinant proteins tested. The recombinant proteins were of a variety of sizes, ranging from 11 to 64 kDa. These data demonstrate that monocot recombinant expression vectors can be developed that show many of the flexile and attractive features of traditional dicot expression systems. These vectors can be deployed for testing the function of plant genes in both monocot and dicot species as well as express proteins of recombinant proteins of biomedical importance.

Dicot Vectors	Monocot Vectors
Primarily monopartite genomes with some multipartite examples	Primarily multipartite genomes with a monopartite example
Independent and minimal-virus vectors demonstrated for whole plant expression	Independent-virus vectors only demonstrated for whole plant expression; minimal-virus vectors restricted to cell culture expression
Vector delivery as infectious RNA transcripts or Agroinfiltration of DNA-based expression vectors	Vector delivery as infectious RNA transcripts
Successfully used for gene silencing and gene overexpression	Successfully used for gene silencing and gene overexpression
Systemic expression of foreign proteins primarily through non-genetic fusion strategies	Systemic expression of foreign proteins primarily through genetic fusions to virus proteins and inclusion of cleavage sequences
Expression of single gene cistrons	Potential expression of multiple cistrons as β and γ gene fusions
Systemic expression of wide range of gene sizes and classes of proteins	Systemic expression of wide range of gene sizes and classes of proteins
Successful integration into cGMP recombinant protein production environment	Not integrated to date into cGMP production environment

Table 2. Comparison of properties of virus vectors for expression in dicot and monocotyledonous plant species.

7. Conclusion

The last few decades have seen tremendous progress in developing tools and expertise to produce recombinant proteins in plants. Although conceptually straightforward, the technical hurdles included not only improving our understanding of plant biology, development of expression systems, but also the perfection of purification and analytical methods to meet the specifications of research, industrial and medical applications. The successes described in this chapter involved a convergence of economic incentives, market forces and regulatory acceptance, the latter being particularly important for biomedical products. Throughout much of this time, transient viral expression systems have played a significant role. The adaptability of virus systems and their ease of use continue to help push the boundaries of recombinant protein expression in plants. Beginning with a few examples, highlighted in this review, a diverse array of viral vector systems have emerged capable of delivering target genes to a wide array of host species, and compliant with a wide range of

regulatory and technical constraints. As shown in this chapter, expression successes initially observed in dicotyledonous plants have now been extended to monocotyledonous plants through the use of the BSMV genome. A comparison of the properties of virus vectors designed for recombinant protein expression in dicot and monocot plant species is provided in Table 2. Research continues to improve their effectiveness and ease of use. Indeed, the advantages of low development costs, flexible design, and relatively quick turnaround from conception to proof of principle to scale up, will continue to drive innovation and application of viral expression vectors. The synergy of customizing specific plant hosts for post-translational modifications offers a strategy to produce plant-sourced products which match the needs of the proposed end uses. Further, the unexpected discovery of viral vectors as tools to silence specific genes in plants has also been of tremendous value to the plant research community, and has effectively complemented their use in overexpression in a way that has only begun to be appreciated and applied in the last decade. The use of these two complementary approaches to address functional genomics in a high throughput fashion, and on a broad range of hosts, will likely emerge in the coming years.

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