Viral vectors for the expression of proteins in plants

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The use of plant viral vectors for the transient expression of heterologous proteins offers a useful tool for the large-scale production of proteins of industrial importance, such as antibodies and vaccine antigens. In recent years, advances have been made both in the development of first-generation vectors (that employ the 'full virus') and second-generation ('deconstructed virus') vectors. For example, vectors based around the 'full virus' strategy can now be used to express long polypeptides (at least 140 amino acids long) as fusions to the coat protein. In addition, a new generation of vectors was engineered to have a reactogenic amino acid exposed on the surface of the virus, allowing easy chemical conjugation of (separately produced) proteins to the viral surface. This approach is being used to develop new vaccines in the form of antigens coupled to a plant viral surface. Prototypes of industrial processes that require high-yield production, rapid scale-up, and fast manufacturing have been recently developed using the 'deconstructed virus' approach (magnifection). This process, which relies on *Agrobacterium* as a vector to deliver DNA copies of one or more viral RNA replicons to plant cells, has been shown to work with numerous proteins, including full immunoglobulin G antibodies. Other advances in this area have looked at the development of inducible viral systems and the use of viral vectors to produce nanoscale materials for modular assembly.

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Introduction

The development of plants as biofactories for the production of protein products with uses in the pharmaceutical and other industries has flourished in recent years. Although many such proteins are produced through the establishment of transgenic plants, this is a time-consuming and costly process. Plant viral vectors offer an efficient alternative for the expression of heterologous proteins. On introduction to a host plant, a virus engineered to contain a gene of interest will replicate and the protein of interest can be produced in significant quantities. The use of viral vectors has several advantages: the protein product can be produced more quickly and in high yield and, because the gene does not become incorporated into the plant genome, it does not form a heritable trait and is thus contained. During the past three years, the science of plant vectorology has made significant progress. On the positive side, since our last review of the field of plant viral vectors in 2004 [1], several important new processes, improvements and practical solutions have been published. On the negative side, Large Scale Biology Corporation (LSBC; Vacaville, CA), probably the most innovative company trying to commercialize products and processes based on first-generation plant viral vectors, went bankrupt. Somewhat paradoxically, this caused ex-LSBC scientists to publish much of their ongoing early research, thus giving us a unique insight into their strategic approaches regarding viral vector development and novel applications [2••,3,4,5••]. Although this is probably a poor consolation, we would like to start this review by expressing our admiration for the inventiveness of our colleagues.

First-generation viral vectors

First-generation viral vectors are essentially functional viruses that express, in addition to all genes of the wild-type virus, the coding sequence of a protein of interest (the so-called 'full virus' strategy). The protein of interest is expressed from a strong (duplicated) viral promoter, such as the coat protein subgenomic promoter, or as a fusion to the viral coat protein (e.g. for the expression of small protein fragments such as immunogenic epitopes). The gene of interest is delivered to plant cells using infectious nucleic acid copies of the vector or, preferentially, as mature viral particles. The process can be performed on a large scale by spraying plants in the field with a mixture of viral particles and an abrasive such as carborundum. Depending on the efficiency of the vector and its ability to move systemically, two to three weeks are required for most of the tissues of the transfected plants to become infected. An alternative to the method of infection has been presented recently by Azhakanandam *et al.* [6], who used *Agrobacterium* to deliver viral vectors based on potato virus X (PVX) to plants as T-DNA copies via 'agrospray' (e.g. to *Nicotiana benthamiana*) or 'wound-and-agrospray' (e.g. to *Nicotiana tabacum*) methods. The host plants were transgenic for silencing suppression factors. In principle, this new version of 'agroinfection' offers high speed with a very simple protocol. However, the yields of protein obtained so far are quite low (0.04–0.3% of total soluble protein) and the

approach obviously needs further improvement. In addition, both 'agrospray' and magnifection (see below) use live *Agrobacterium* as a vector and, therefore, industrial versions of these processes will have to deal with biological containment of the genetically engineered *Agrobacterium*.

Work on the expression of several interesting product candidates using first-generation viral vectors has revealed both positive and negative features of this approach. On the positive side, many publications have reported the successful expression of different immunogenic epitopes as fusions to viral coat proteins. Furthermore, their immunogenicity, and in some cases a certain level of protection, has been demonstrated in animal models. On the negative side, several groups, including Rybicki's group who focused on plant-based production of the L1 capsid protein of papillomaviruses (human and cottontail rabbit papillomaviruses), have shown that there are potentially some problems with the assembly of virus-like particles in plant cells and, more importantly for our discussion, viral vectors provided yields that were lower than yields obtained with transgenic plants [7,8]. The authors reason that the lower productivity is the result of rapid loss of the transgene from the vector, owing to the large size of the L1 gene (1.5 kb), and provide data to support this conclusion. The limits imposed by 'full virus' vectors on the size of the insert have also been investigated for PVX-based vectors [9]; as found for vectors based on the tobacco mosaic virus (TMV), a negative correlation between insert size and vector stability was observed.

Until recently, only small epitopes (with fewer than 25 amino acids) had been successfully expressed as coat protein fusions, longer epitopes preventing viral particle assembly. Recently, however, Werner *et al.* [10•] found that much longer polypeptides, such as a fully functional fragment of Protein A (133 amino acids), could be displayed on the surface of tobamoviruses as C-terminal fusions to the coat protein, provided a flexible linker is used. These results show that there is no '20–25 amino acid barrier' for coat protein fusions, broadening the field of coat protein fusions.

With new developments in the field of nanobiotechnology, it is becoming more and more obvious that plant viruses provide an attractive source of biopolymers that can be produced cheaply and on a large scale, for use as a feedstock for building new materials. First-generation viral vectors are well suited for making such building blocks, which can be made by reprogramming the virus genetically or by chemical attachment of new functionalities on the surface of the viral particles or even in the inner cavity of the viral particles [11•,12]. We discuss the work done on this subject in more detail in the chapter dedicated to nanoscale materials.

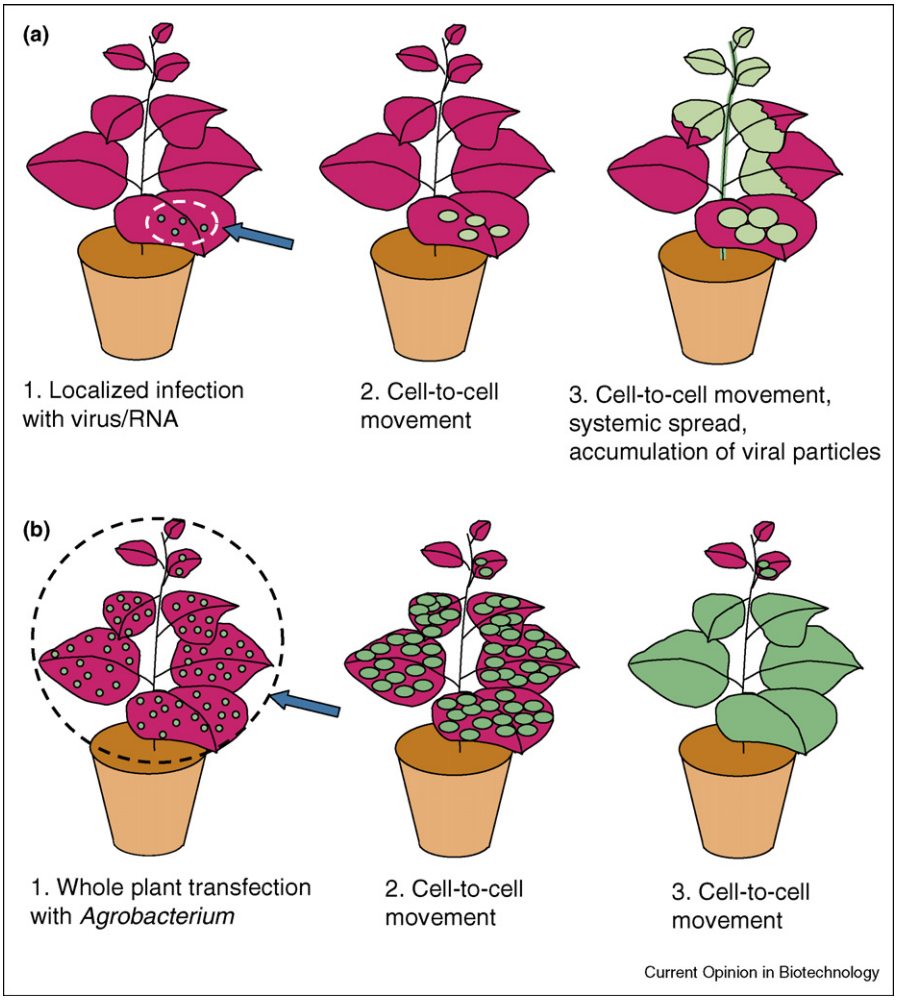
Second-generation vectors: deconstructed vectors and magnifection

The development of an industrial process requires the ability to transfect large numbers of plants and as many leaves/tissues as possible within each plant, in a greenhouse or in the field. A solution to this problem was provided with the 'full virus' strategy by using first-generation vectors capable of systemic spread (Figure 1). The limitations of this approach are obvious, however: inserts larger than 1 kb cannot usually be expressed; until recently, only short epitopes could be effectively expressed as fusions to the coat protein; systemic vectors never infect all harvestable parts of the plant (e.g. the lower leaves); the spread of the virus is asynchronous as infection progresses with different speeds in different leaves; and the vector is usually unstable, thus many infected tissues do not express the protein of interest.

These limitations prompted viral vector engineers to rethink the basic design of vectors. Rather than use complete viral genomes, attempts were made to deconstruct the virus and rebuild an integrated system; only the viral elements required for efficient expression of the sequence of interest were maintained, and the missing functions were provided using non-viral components. Among the different viral components that can be used outside the integrated virus system are the 'molecular machines' that provide for infectivity, amplification/replication, cell-to-cell movement, assembly of viral particles, shutoff of the plant cellular synthesis, silencing suppression, systemic spread, and so on. Some of these machineries are quite inefficient; for example, the ability of the virus to infect the host is low, and is in fact not desirable for biosafety reasons. Also, the ability of the virus to move systemically is usually tissue- and species-specific and is easily impaired as a result of genetic manipulation. Replication/amplification ability, by contrast, is a powerful and relatively robust and species-independent mechanism that is likely to be valuable in most applications as an 'amplifier' of the process of interest.

Two elements of first-generation viral vectors that are limiting are the ability of the vector to move systemically, which is provided by the coat protein, and the low level of expression of the protein of interest, probably because a significant fraction of the cell metabolic resources are devoted to synthesis of a large amount of coat protein. A simple solution was to eliminate the coat protein and replace the systemic movement ability by artificial delivery of the viral vector to the entire plant using *Agrobacterium*. However, for TMV-based vectors, attempts made in 1993 showed that *Agrobacterium* delivery is extremely inefficient, and was estimated at about one infection per 10^8 *Agrobacterium* cells. More recent work with vectors built from different tobamoviral strains was more successful, although still not efficient. Analysis of the early

Figure 1



Schematic description of infection and spread of replicons based on **(a)** first-generation and **(b)** second-generation viral vectors.

infection steps initiated by *Agrobacterium* delivery suggested that the bottleneck for the actual formation of active replicons is the low ability of the primary transcript to leave the nucleus. The RNAs of cytoplasmic RNA viruses such as TMV normally never enter the plant cell nucleus, and therefore have not evolved to be in contact with the nuclear RNA processing machinery. Analysis of the viral genome shows that many regions are unlikely to be recognized as normal coding sequences; thus, a vector that is a simple carbon copy of a wild-type virus is likely to be degraded before it reaches the cytosol. By modifying the vectors in different ways — for example, through the introduction of silent mutations to remove putative cryptic splice sites, changing the codon usage, and adding multiple plant introns — highly active synthetic T-DNA templates have been constructed. When these templates are delivered as DNA precursors using *Agrobacterium*, efficient processing of the DNA information into active replicons occurs in almost all (>93%) cells, a 10^3-fold improvement over non-modified vectors and an up to 10^7-fold improvement over the non-optimized DNA templates reported in the first publications. Improved vectors lead to one successful infection event per 10–20 infiltrated agrobacteria. The infiltration process was also shown to work with several other plant species, although not as efficiently as in benthamiana [13••].

On the basis of these findings, a simple fully scalable protocol for heterologous protein expression in plants was designed that is devoid of stable genetic transformation of a plant, but instead relies on the transient amplification of viral vectors delivered to the entire plant using *Agrobacterium*. The process is in essence an infiltration of whole mature plants or of detached mature leaves with a highly diluted suspension of agrobacteria carrying a proviral replicon on the T-DNA. In this case, infiltration of agrobacteria replaces the conventional viral functions of

primary infection and systemic movement (Figure 1). Amplification within each cell and movement from cell-to-cell is performed by the replicon. Depending on the vector used, the host organism and the initial density of bacteria, the process takes from 4 to 10 days and, depending on the specific gene of interest, can result in the expression of up to 5 g recombinant protein per kg of fresh leaf biomass or over 50% of total soluble protein. Furthermore, as the viral vector does not contain a coat protein gene, it can express longer genes (up to 2.3 kb inserts or up to 80 kDa proteins). The infiltration of plants/detached leaves with bacteria has been achieved in many ways, one simple process being vacuum infiltration after immersing the aerial part of the plant in a bacterial suspension and applying a weak vacuum (approximately –0.8–0.9 bar) for 10–30 s [13••,14,15]. This new expression strategy, called magnifection (Figure 2), combines the advantages of three biological systems: the speed and expression level/yield of the virus, the transfection efficiency of *Agrobacterium*, and the post-translational capabilities and low production cost of plants. A similar approach, based on the use of PVX, has also been described recently [16]. Further development and automization of these protocols could increase the throughput of the process and reduce the amount of manpower required.

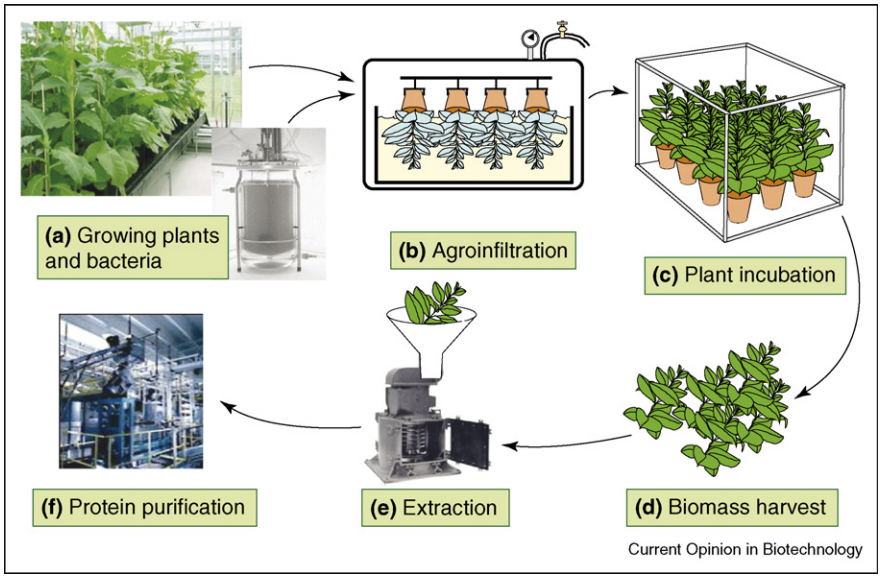
Magnifection has already been used to produce proteins that are fully functional. In particular, biologically active human growth hormone was expressed at high levels (about 1 mg/g of fresh weight) by Gils *et al.* [17]. Similarly, Santi *et al.* [18] expressed two different *Yersinia pestis* (plague) antigens, F1 and V, and an F1–V fusion protein at very high levels (2–3 mg/g). Moreover, they demonstrated in animal models that the plant-made antigens provided high levels of protection against aerosolized plague bacteria. A different group [19] has also achieved expression levels of 0.8 mg/g when expressing the tuberculosis Ag85B antigen. At present, over 50 proteins of different origin and complexity representing all classes of pharmaceutical proteins have been expressed successfully using magnifection [15].

Non-competing vectors for the expression of heterooligomeric proteins

Although the ability of plants to express full-size monoclonal antibodies was discovered 17 years ago, the initial expression protocols suffered from various shortcomings. In particular, although stably transformed plants express correctly folded and functional antibodies of the immunoglobulin A and G (IgG and IgA) classes, yields are generally very low (1–25 mg/kg of plant biomass) [17]. Moreover, the time necessary to generate the first gram(s) of research antibody material is longer than 2 years.

Transient systems, by contrast, allow the much faster production of research quantities of material. However, expression from non-replicating expression cassettes

Figure 2



General scheme for recombinant protein production in plants using magnifection. Depending on the protein of interest, magnifection can produce up to 0.5–5 g recombinant protein per kg of leaf biomass. With a green biomass yield of 100–300 ton/hectare per year, the yield of recombinant protein is expected to be 50–600 kg/hectare/year. The expression time is 6–10 days, making magnifection especially attractive for those applications where rapid industrial manufacturing is required. Components **(a)**, **(e)** and **(f)** are standard existing industrial processes. Components **(b)**, **(c)** and **(d)** are new elements, because large-scale infiltration (b) requires special equipment and the use of bacteria in steps (b), (c) and (d) requires biological containment to prevent the release of genetically engineered bacteria into the open environment.

results in low expression levels [20] and the use of first-generation viral vectors cannot provide high-level co-expression of the two or more polypeptides necessary for the assembly of heterooligomeric proteins such as IgGs. More specifically, expression of two different polypeptides using two separate replicons built from the same virus failed, because of drastically reduced expression [21]. Recently, Giritch *et al.* [22••] designed a rapid and indefinitely scalable process for the high-level expression of functional full-size monoclonal antibodies of the IgG class in plants. The process relies on co-infection of two viral vectors, each expressing one antibody chain. In contrast to the work described above, the two vectors are derived from different non-competing plant viruses, which, unlike vectors derived from the same virus, are able to effectively co-express the heavy and light chains in the same cells in infected tissue throughout the plant. By using vectors built from the backbones of TMV and PVX, one expressing the heavy chain and the other expressing the light chain, high yields of up to 0.5 g/kg fresh biomass of assembled antibody were obtained. This protocol was shown to work with seven different monoclonal antibodies of the IgG1 and IgG2 classes; the molecules obtained were fully functional, and the first gram of material could be produced in less than 2 weeks. The protocol provides a very useful tool for developers of new antibodies and allows the overall development time to be drastically reduced [23].

Expression vectors for industrial-scale protein production: transgenic systems

Magnifection provides protein products with the necessary speed for research and development and also for production. This speed comes at a cost, however: industrial production involving agrobacteria is more expensive and can accommodate only limited volumes of plant material. An alternative strategy to allow large-scale production would be to stably insert the viral replicon on one of the host chromosomes and to activate replicon formation at a time chosen by the operator. With such an approach, development of the product would be slower, as obtaining stable transformation requires at least several months. However, this should not be a limiting factor in most cases. So as not to interfere with plant growth, the stably inserted proreplicon should not by itself be able to trigger amplification, but should require activation; for example, by expression of a recombinase expressed in the same plant under the control of an inducible promoter or by suppression of silencing by the plant. Amplification could also be induced by expression *in trans* of a replicase under the control of a chemically inducible promoter.

Many research groups are working on establishing various versions of such viral-inducible systems. For example, Dohi *et al.* [24] have developed an inducible system that relies on an oestrogen-inducible promoter in BY2 tobacco cell suspensions. Upon induction, a modified tomato mosaic virus expressing green fluorescent protein (GFP) instead of the viral coat protein was shown to amplify and expresses the gene of interest at high levels (10% of total soluble protein). Neither the viral RNA nor GFP were detectable in uninduced cells. Zhang and Mason [25] developed an inducible system based on the bean yellow dwarf geminivirus. In this system, release of a replicon from a stably inserted construct is obtained by expression of the replication initiator protein *in trans*, under control of the ethanol-inducible promoter. Transgenic plants were shown to induce release of the vector and episomal replication to high copy number after ethanol treatment, resulting in up to an 80-fold increase of mRNA levels and up to a 10-fold increase of the translation product.

Vectors for manufacturing 'nanoscale' materials and modular assembly

Owing to their relatively simple macromolecular organization and very high accumulation titers, plant tobamoviruses provide an extremely cheap source of biopolymers of discrete nanoparticle sizes than can be manufactured rapidly and under very simple conditions. TMV, the most well known tobamovirus, is also one of the most extensively studied viruses. It has a positive-sense RNA genome encoded in a single 6.4 kb RNA molecule. The genome encodes four proteins, including the 17.5 kDa coat protein (CP), the most abundant viral product and the only component of the TMV capsid. Over 2100 copies of CP fully protect the single-stranded viral RNA, resulting in rigid rod-shaped viral particles with a length of 300 nm, a diameter of 18 nm and a molecular mass of 40 000 kDa. The genomic RNA is packaged inside a 2 nm wide canal formed by the assembled CP capsid. The CP monomers contribute 95% of the mass of TMV particles. TMV accumulates to levels of up to 10 g/kg of leaf biomass and, therefore, CP represents the most abundant individual protein that can be harvested from plants. The virions can be purified industrially using simple 'low-tech' protocols. Owing to the ability of CP to polymerize *in vivo* and *in vitro*, and because of the high stability and defined size of the assembled virions, CP represents a potentially promising biopolymer feedstock for several applications in nanobiotechnology [26].

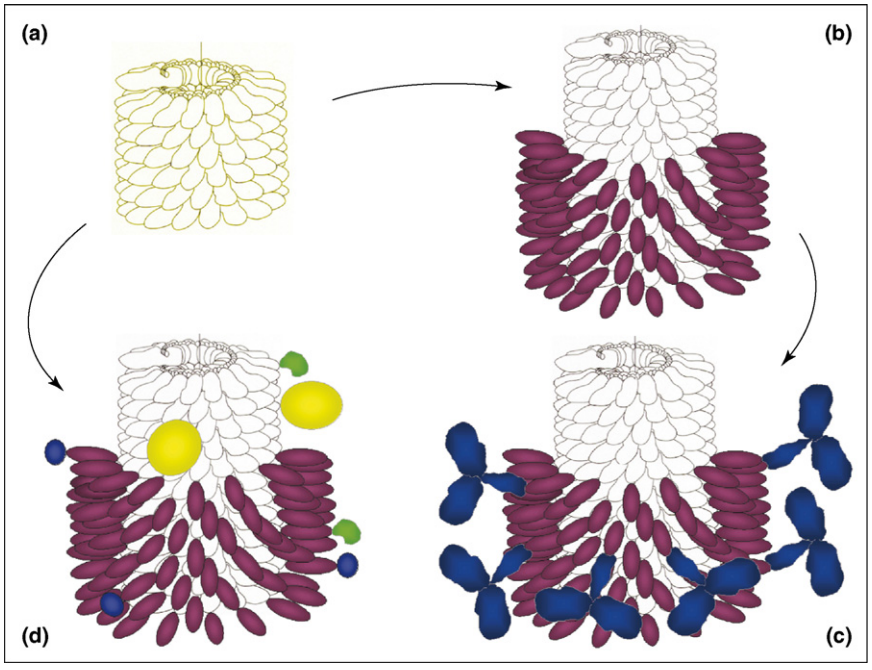
Among the most elegant studies recently published by LSBC researchers are those devoted to the development of modified TMV particles for use as scaffolds for the display of peptide antigens, primarily for vaccine applications [5••]. TMV particles are first engineered to have a reactive group on the surface of the virion surface, which is then used for chemical modification and attachment of a protein or peptide of interest. A randomized library approach was used to introduce a reactive lysine at the externally located N terminus of the coat protein, allowing easy biotinylation of the capsid. A protein–peptide fusion component expressed independently as a strepta-

vidin fusion can then be used to decorate the TMV particles. As an example, TMV particles were decorated with an N-terminal fragment of the canine oral papillomavirus L2 protein; the TMV–antigen complexes were found to be significantly more immunogenic than the uncoupled antigen when tested in mice.

In a separate study [2••], the same authors went on to show that complex multipeptide vaccine formulations can easily be made using chemical conjugation to TMV, providing improved vaccine efficacy. TMV–peptide bivalent fusions were made by conjugating onto TMV particles a peptide antigen (the chicken ovalbumin-derived cytotoxic T-lymphocyte [CTL] peptide, used as a model for a foreign antigen) together with a second peptide designed to increase vaccine efficiency (such as a T-helper epitope or peptides that improve cellular uptake). Such vaccine formulations provided improved vaccine potency in the absence of adjuvant in a mouse model, as demonstrated by enhanced numbers of antigen-reactive T-cells and enhanced tumor protection. In addition, TMV–peptide bivalent fusions containing two different peptide antigens (the mouse melanoma-associated CTL epitopes p15e and tyrosinase-related protein 2, two self-antigens) led to higher protection against tumor compared with single antigen fusions. The data demonstrate the ease of vaccine formulation and qualification, and the utility of combining helper, uptake and additional T-cell targets onto a TMV scaffold. Thus, the flexible nature of the platform allows for simple, rapid and controllable manufacturing of multiepitope vaccine complexes.

An alternative to *in vitro* chemical modification of viral particles is to genetically reprogramme the virus to express protein fusions. As the three-dimensional structure of the coat proteins of many viruses has been determined, fusion proteins can be designed in such a way that the added sequences are predicted to lie at the surface of the assembled viral particle. Despite many attempts by numerous groups, a significant limitation has until now been the small size of epitopes that can be fused to coat proteins without preventing viral particle formation. For example, the limited size of peptides that could be fused to the CP of TMV has restricted these systems to the expression of 20 amino acid or shorter peptide immunogens and of one peptide hormone only. As mentioned above, it has been shown recently that using a flexible linker between the coat protein and the antigen allows the expression of much larger peptides [10•]. In this example, the coat protein was fused to two of the five antibody-binding domains normally present in Protein A. Either one of two different linkers (a flexible and a helical linker) allowed expression of large amounts of the fusion

Figure 3



Nanoparticles based on rod-shaped plant viruses. **(a)** The genetic programming of viruses such as TMV through coat protein fusion allows the construction of **(b)** novel nanoscale materials, such as immunoadsorbent particles containing Protein A fragments (purple). **(c)** These immunoadsorbent particles could be used in processes that require antibody (blue) capture. **(d)** Both genetic programming and chemical conjugation allow the attachment of different molecules to the surface of the virus. Blue, green and yellow elements are additional affinity tags, functional enzymes, enzyme (protease) inhibitors, etc.

protein, with the Protein A fragment retaining functionality. The polymeric nature of these immunoadsorbent nanoparticles allowed the design of a simple protocol for the purification of monoclonal antibodies (mAbs) that provides a recovery yield of 50% with higher than 90% purity. Because of the extremely dense packing of Protein A on the nanoparticles, they have a very high binding capacity of 2 g mAb per g of particles. This characteristic, combined with the high level of expression of the nanoparticles (more than 3 g/kg of leaf biomass), provides a very inexpensive self-assembling matrix that could meet industrial criteria for a single-use immunoadsorbent for antibody purification (Figure 3).

Recombinant RNA viral vectors for vaccine delivery

The use of DNA/RNA vectors as vaccines is still in its early stages. One of the problems of genetic immunization with DNA vaccines is the concern that plasmid DNA may be incorporated into the host genome. One alternative to DNA would be vaccination with RNA, and there are several human and animal viral vectors, such as those derived from Semiliki forest virus (SFV), that show transient persistence. However, manufacturing the virus particles (ideally suitable as a product) could result in replication-competent viruses. Plant virology offers an interesting solution, by *trans*-encapsidating the SFV using TMV coat protein. The resultant vector is safe, easy to manufacture *in vitro*, and facilitates the simple generation of unique nucleic acid/protein antigen compositions [4].

Improved expression hosts

One drawback of using plants as hosts for manufacturing therapeutic proteins is that post-translational modifications of proteins in plants are somewhat different from those in animal/human cells. In particular, plant cells have a different *N*-glycosylation machinery that results in the addition of plant-specific sugars (core-bound xylose and α-1,3-fucose) that might be immunogenic in humans. By contrast, plants do not synthesize animal-specific sugars, such as β-1,4-galactose residues or sialic acid. Fortunately, significant progress has been made in the area of host plant engineering, and two recent papers [27••,28•] show that plants (duckweed and tobacco) can be engineered to have a more human-like glycosylation machinery using either RNA interference or by expressing a human or chimeric β-1,4-galactosyltransferase. It is therefore now possible to make host plants that express recombinant proteins that are essentially devoid of xylose or fucose residues and as a consequence are more human-like and safer. Such hosts could be used both for transient or transgenic expression systems for the expression of glycosylated proteins.

Conclusions

The development of plant viral vectors for transient protein expression has made rapid and impressive progress, and several technical solutions seem to be ripe for industrial application. The next few years will show us whether the processes developed can now be successfully commercialized. The use of vectors based on the 'full virus' strategy seems to be constrained, primarily by the limitations on the number and type of proteins that can be expressed using this technology. The most obvious application for these vectors today seems to be for the production of vaccines as coat protein fusions of short antigenic peptides or through the chemical coupling of antigens to engineered viral particles. Industrialization of the magnifection technology is currently an essential part of the activity of several business and academic groups, and the most serious constraint is the need for a specialized facility that allows handling of plants treated with agrobacteria and that can operate under conditions compliant with current good manufacturing practice. This technology seems to provide a viable alternative to other rapid expression processes (e.g. baculovirus/insect cell systems), where the time 'from DNA to product' has to be very short (e.g. for the production of influenza vaccines, manufacturing for biosecurity situations or the production of individualized vaccines). Finally, engineering of transgenic plants for the chemically induced release of viral replicons from a plant chromosome would provide the ultimate solution for manufacturing proteins for which there are no industrial time constraints. Despite some progress in this field, more work remains to be carried out for this technology to reach its full potential.

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- • of special interest
- •• of outstanding interest

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