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THE ROYAL SOCIETY

Tobacco mosaic virus and the virescence of biotechnology

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There is a growing realization that a modern combination of molecular biology and agriculture will provide a photosynthetic basis for the biosynthesis of an increasing variety of complex and valuable molecules. This 'greening' of biotechnology may impact on the global environment in many beneficial ways, but will perhaps have its most significant impact on human health. In the past decade, the capacity to use plants as an expanded source of therapeutics has grown through the accelerated development of effective viral transfection vectors for gene transfer to cultivated crops. Recombinant vectors based on tobacco mosaic virus (TMV) and other members of the Tobamovirus genus are now used to transfect commercially meaningful quantities of plant biomass cultivated in enclosed greenhouses and multiacre fields. Viral RNA promoters are effectively manipulated for the synthesis of recombinant messenger RNAs in whole plants. Chimeric plant virus and virus-like particles are designed for peptide production and display from recombinant structural protein–gene fusions. Gene functions are assessed and modified by either virus-mediated expression or cytosolic inhibition of expression at the RNA level. Recombinant virus populations, propagated by inoculating plants with infectious RNA transcripts or recombinant virions, have proved to be genetically stable over product-manufacturing cycles. Large volumes of highly purified protein products isolated from transfected foliage conform reproducibly to the specifications required for well-characterized biologics. In some cases, they exceed the specific activities of molecules purified from alternative recombinant and native sources. The resulting products are then formulated according to the developing national regulatory guidelines appropriate for agriculture-based manufacturing. Each of these innovations was first realized by researchers using clones of tobamovirus genes and recombinant genomes. This progress is founded on the heritage of a century of fundamental TMV research.

Keywords: tobacco mosaic virus; recombinant protein production; specified biologics; subunit vaccines; metabolic pathway engineering; gene silencing

1. INTRODUCTION

One of the more valued applications of biotechnology is to provide new solutions to human health-care problems. While safety and efficacy are the primary focus of clinical advances, the costs of drug discovery, development, production and delivery are often only secondary considerations. Medical interventions resulting from this approach can, therefore, be expensive for intended or potential markets. Some examples include the extraction of glycoproteins from human tissues or from recombinant mammalian cell cultures to treat rare heritable metabolic disorders, or the chemical synthesis of peptide epitopes for use as vaccines and immunotherapeutics. The development of many potential applications is presently delayed by the lack of even experimental quantities of high-quality recombinant proteins. Ideally, a wide variety of candidate therapeutics should be available at a cost that would both advance discovery and development and enable wide access to treatments.

Society will increasingly rely on transgenic animals and plants for recombinant proteins because agriculture requires relatively minor capital inputs, while offering flexible and efficient economies of scale with low-cost production and waste-disposal methods. We consider higher plants to provide the most cost-effective, safe and environmentally compatible platform for pharmaceutical production.

Plants and their viruses have coevolved a regulated gating of cell junctions (plasmodesmata) that provides a mechanism for the movement of virus from the site of infection to photosynthetic sink leaves. Viruses such as TMV, which package plus-sense RNA as their infectious genome, are particularly successful and abundant plant pathogens. Viral progeny genomes are hypothesized to be transported along a pathway similar to that of an RNA signal that controls viral replication (Jorgensen *et al.* 1998). Because of the nature of this plant–pathogen interaction, it has long been proposed that genetically modified RNA viruses might be used to functionally amplify and move recombinant sequences throughout the tissues of a plant (Siegel 1983).

Early work with several plant viruses demonstrated their potential as gene vectors. Among the notable achievements were the first transfection of plant protoplasts with infectious RNA transcripts of brome mosaic virus (Ahlquist *et al.* 1984), the synthesis of recombinant marker proteins in cells and inoculated leaves (Brisson *et*

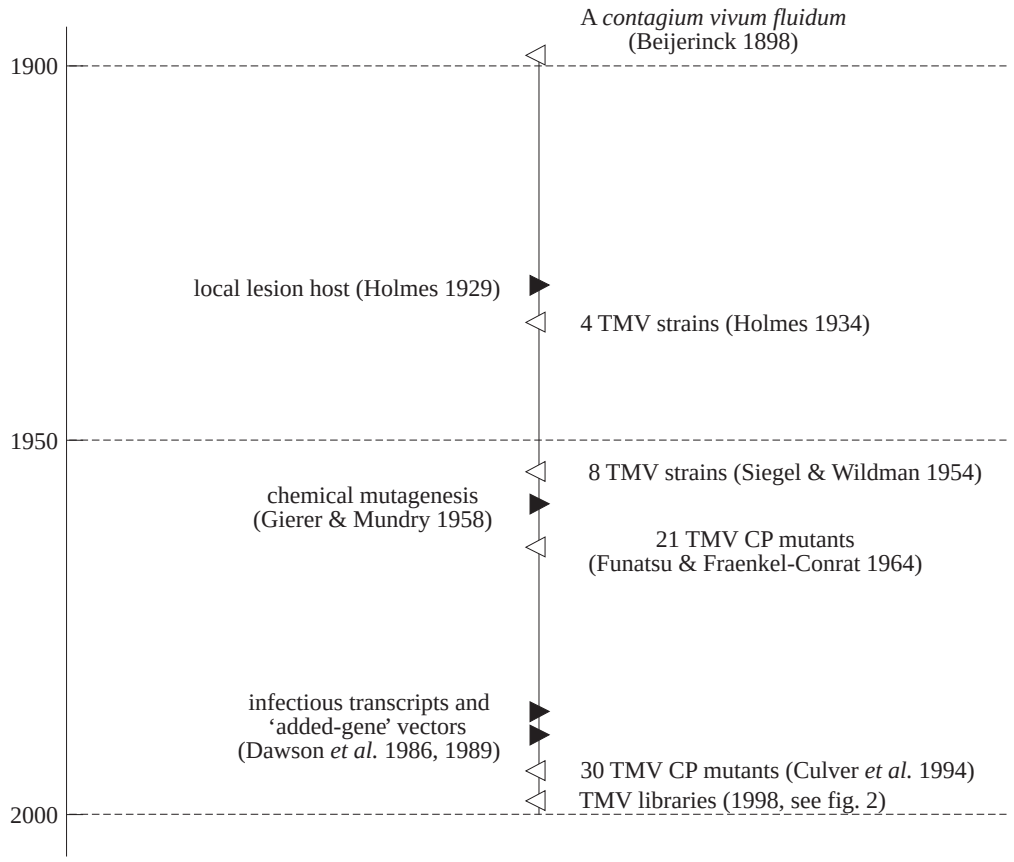

Figure 1. Time-line representing selected studies of TMV strains, mutants and libraries during the 'directed evolution' of the virus (on the right) with the scientific advancements that facilitated these same studies (on the left).

al. 1984; French *et al*. 1986; Takamatsu *et al*. 1987; Dawson *et al*. 1988) and the production of measurable quantities of a cytokine with biological activity (de Zoeten *et al.* 1989). However, the use of recombinant plant viruses as research and production tools for plant biotechnology was not firmly established until tobamovirus vectors were used in a commercial context to transfect whole plants and the resulting products purified on a pilot scale and analysed for their composition and specific activity. The purpose of this review is to present an account of the development of these concepts and their impact in increasing applications of the new botanical biotechnology.

2. A HISTORICAL OVERVIEW

In a molecular biologist's view of life, understanding increases in proportion to the number of defined mutants isolated and evaluated. A time-line representing selected examples of genetic variation evaluated in individual TMV studies in the past 100 years since the beginning of virology (Beijerinck 1898) is displayed in figure 1 and discussed elsewhere in this issue. The purpose here is simply to illustrate, by a few examples, how a steady increase in numbers of defined phenotypes and sequences of TMV that are under investigation in a given study is made possible by the legacy of the preceding work. Thus, the description of the local lesion response (Holmes 1929) preceded the use of four tobamovirus strains in a study of the inheritance of the same localizing trait (Holmes 1934). Twenty years later, eight strains were grouped by their physical and chemical properties (Siegel & Wildman 1954). Determination of the amino acid (AA) sequence of 21 coat protein mutants (CP mutants) (Funatsu & Fraenkel-Conrat 1964) was preceded by chemical mutagenesis of the TMV genome (Gierer & Mundry 1958) to increase the range of variants that could then be isolated by transfer from a single lesion and passaged for further study. The first successful 'added-gene' vectors (Dawson *et al.* 1989) were made possible by positioning promoter sequences at specific sites in full-length cDNA copies of the viral genome, from which it was possible to synthesize RNA transcripts *in vitro* that were infectious when inoculated on to leaves (Dawson *et al.* 1986; Meshi *et al.* 1986). The nature of the viral coat protein elicitor of the host hypersensitive response (HR) was then thoroughly dissected in atomic detail using a series of 30 mutants created by specific site-directed mutagenesis of the coat protein open reading frame (ORF) (Culver *et al.* 1994).

Within a few years of 1986, hundreds of genes and mutations were recombined in chimeric tobamoviral vectors. The genetic variability defined and sampled in individual experiments increased dramatically as sequence libraries were introduced. Now, one can direct the evolution of a population of genes to provide information relative to specific products or research phenomena (figure 2). The complexity of the population required depends on the difficulty of the question asked, the

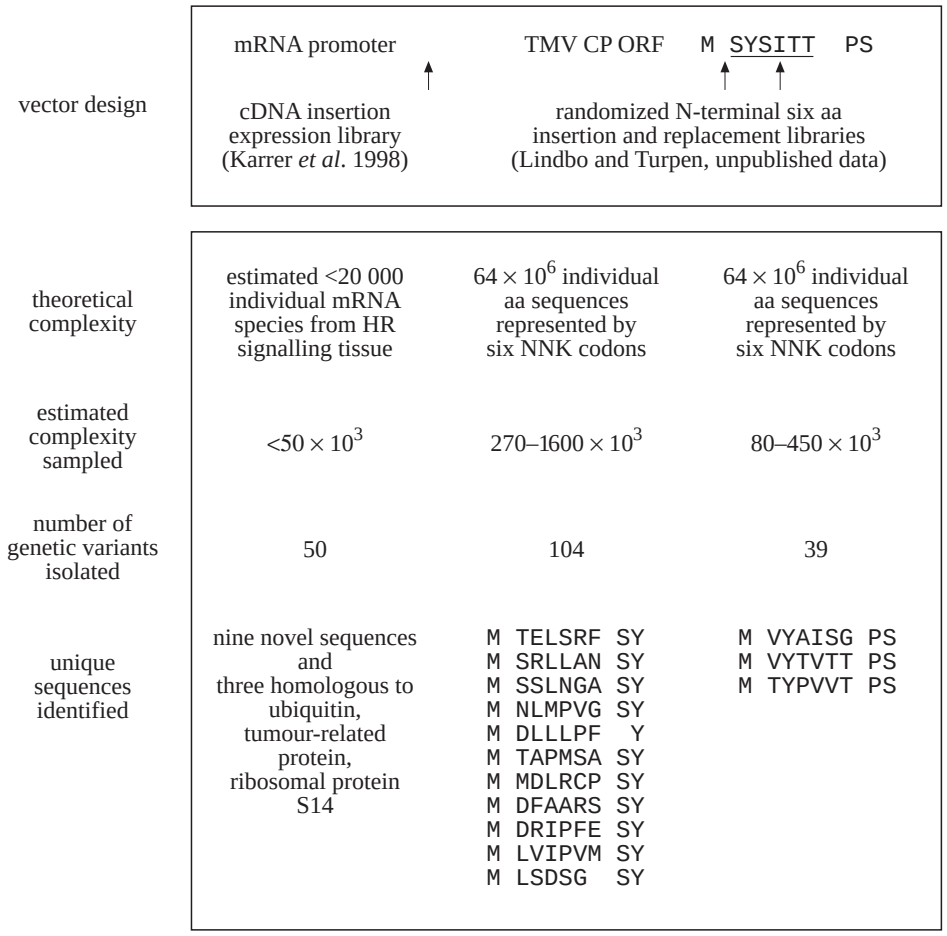

Figure 2. The first TMV-based libraries illustrate the variety of information potentially accessed in future experiments. In the cDNA expression library of Karrer *et al*. (1988), there may be a theoretical complexity of $<20\,000$ different mRNA species expressed. The sampled complexity is estimated by counting the lesion-forming units on *N. tabacum* cv. Xanthi nc. The actual complexity sampled is unknown because different mRNAs are expected to be represented in this library in proportion to their relative abundance in the messenger pool. Of 50 000 individual clones, 50 induced necrosis on the susceptible cultivar Xanthi and were studied further. The peptide libraries of Lindbo & Turpen incorporate randomized nucleic acid sequences (see Appendix A). The theoretical complexity is calculated from the permutations of possible amino acid (aa) sequences encoded by the library ($20^6 = 24 \times 10^6$). The complexity sampled is estimated as a range of values based on either the number of independently transfected protoplasts expressing coat protein antigen detected in an immunoblot of lysed protoplasts (low range) or measured in control transfections using virus marked with green fluorescent protein (high range). During the course of two experiments totals of 104 and 39 lesions were collected and 14 unique sequences identified. The aa sequences presented are predicted from nucleic acid sequence data. The electrophoretic mobility of each of these CP mutants is retarded relative to wild-type TMVCP as analysed by SDS-PAGE (data not shown). Viruses from a total of 66 lesions were individually passaged on the susceptible tobacco cultivars Xanthi and/or MD609 and symptoms on inoculated and systemic leaves were recorded over a three-week period. In 30% of the inoculated plants a typical mosaic symptom developed. In 30% the symptom was attenuated, delayed, or included mild veinal chlorosis. In 10% of the inoculated plants no symptoms were observed. The remaining 30% of the inoculated plants developed novel and distinct symptoms on inoculated and/or systemically infected leaves. These symptoms included a range of necrosis, general leaf distortion, mild to severe chlorosis, veinal chlorosis, etch, vein banding, stunting and epinasty.

confidence level desired for an answer and the time-frame and equipment allotted to sample the variation. Thus, a cDNA library derived from tobacco leaves undergoing the HR was cloned into a TMV-based expression vector and used to identify a series of 12 tobacco gene sequences that elicit the HR (Karrer *et al*. 1998). The sequences of nine of the clones had not been previously reported, and five induced expression of PR2, a gene known to be specifically activated in the HR signalling cascade.

The N-terminal amino-acid sequence in the TMV coat protein (TMVCP) was randomized using libraries of replacement or insertion sequences and screened for the population subset that retained the ability to assemble, disassemble, and that did not arrest the virus replication cycle (J. A. Lindbo and T. H. Turpen, unpublished data). The library was transfected into tobacco protoplasts and progeny virus was passaged through single lesions on to the tobacco (*Nicotiana tabacum*) cultivar Xanthi nc. The first three independent sequences selected from a library of 64×10^6 potential amino acid (aa) sequences, designed to replace the N-terminal six aa of the coat protein, all contained tyrosine in position 2 as predicted from structural principles. A variety of N-terminal extensions were tolerated, but individual variants induced a great variety of symptom types in susceptible cultivars of tobacco. Therefore, it appears that the typical mosaic symptom

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phenotype is easily influenced by specific properties of the virion surface or the equilibrium pools of coat protein subunits and assembly intermediates.

The initial TMV libraries summarized in figure 2 were created and evaluated manually. The population complexities currently being evaluated in our laboratories require extensive automation. Thus, because TMV is being 'evolved' and propagated by humans for a variety of purposes, it occupies a uniquely successful and expanding evolutionary niche. Imagine the use of this genetic complexity for discovery and prototyping applications. What factors brought TMV to be used in this context, and what consequences are foreseen for this technology?

(a) *'Tulip-breaking' virology*

In the 1980s, while working in a plant molecular biology group at the Zoëcon Research Institute (Palo Alto, CA), I became aware of the few examples of plant viruses being used for crop improvement. An often-cited precedent for the utility of plant viruses is the virus-induced variegation of tulip petals cultivated from the 16th century onwards in Europe (Grill 1983; Fraser 1992). Variegated lines containing a potyvirus, tulip-breaking virus, were rare and highly valued for their beauty. More recently, pollen sterility became a modern trait used in the production of hybrid seed. Our group began to characterize and clone novel seed-transmitted viruses and virus-like agents that were associated with cytoplasmic or maternally inherited male sterility in crops, and cloned other important viruses to develop pathogen-derived resistance by transgenic methods (Grill *et al*. 1983; Turpen *et al*. 1987, 1988; Turpen 1989). For many reasons, these agents were typically much more difficult to work with than TMV. The underlying concept that plant viruses could become useful as gene vectors was developed from these experiences and embodies the following principles.

1. Plant viruses are plant parts. In infected plants, viruses are regulated components of plant anatomy and physiology.
2. Plant viruses are consumer products. They are ubiquitous components of plant matter from flower petals to the food chain. They are found in roasted sunflower seeds, cured tobacco leaves and the organic farmers' market produce.
3. Plants are valued for their traits. Genes are useful if they add value. The mode of inheritance, whether a trait is vegetatively transmitted as in bulbs of broken tulips or more reliably conferred by graft transmission, need not concern a consumer. Buyer preferences are most often based on the characteristic value enhancement of the product itself and not the method of production.
4. The value of a plant (or plant-derived product) is determined by supply and demand with relatively free trade in many societies. In others, such as that found in the seventeenth-century in The Netherlands, the government will attempt to regulate price and availability with variable success.
5. To modify plants genetically for commercial purposes, it may not be necessary to produce, market and sell transgenic seed. Transfected tissues will have many advantages.

At the time Biosource initiated a long-term collaboration with the laboratory of Dr W. O. Dawson to develop and commercialize plant viral vectors, our team realized the great potential for these new genetic tools and appreciated the advantages afforded by the existing body of knowledge about TMV.

(b) *Choosing TMV*

Many of the characteristics that made TMV and tobacco an attractive experimental system over the past century have facilitated the development of commercial gene vectors in the past decade. Virus particles, as well as genomic RNA, can initiate infections efficiently from inoculated wounds, producing a tremendous amplification of RNA and accumulation of viral proteins and particles in a brief period of time while minimizing damage to the host. TMV seems a simple genetic system only because of the details known about the four or five reading frames described elsewhere in this issue. However, there is nothing simple about balancing the equilibrium of self-assembling and disassembling complexes required for movement and encapsidation of the genome. There is nothing simple about regulating translation, mRNA synthesis and genomic RNA replication. As knowledge of these processes increases, so will the opportunity to create improved vectors for more reliable expression of a wider range of sequences, in a larger number of plant species and organs.

Functional components of the genome of TMV and other viruses came to be viewed as a 'tool-box' for genetic engineering (Wilson 1989). For example, the 5′ non-coding region of TMV known as the omega fragment was discovered to be a *cis*-active translational enhancer (Gallie *et al*. 1987). A disease-resistance gene was invented from the TMVCP sequence (Powell Abel *et al*. 1986). Pseudovirus particles were created by adding coat protein *in vitro* to heterologous RNAs that were linked genetically to the TMV origin-of-assembly nucleotide sequence (Sleat *et al*. 1986). The first indication that plant viruses might 'carry' foreign sequences and retain biological activity came when Haynes *et al*. (1986), at Connaught Laboratories Ltd, genetically embedded a poliovirus epitope into the TMVCP and assembled particles with and without genomic RNA using recombinant protein made in *Escherichia coli*. The principle that chimeric virus particles had potential as protective immunogens was thus established with TMV, as well as with the more commonly known hepatitis B surface antigen particles (Valenzuela *et al*. 1985). After it became possible to synthesize infectious RNA from cloned cDNA copies of plant viral genomes, chimeric virus particles derived from TMV and other viruses were shown to present foreign peptides on their surface without significantly inhibiting the ability of the viruses to replicate and to infect whole plants systemically (Porta *et al*. 1994; Turpen *et al*. 1995; Fitchen *et al*. 1995).

The versatility of this single ORF or coat protein fusion strategy for gene expression has steadily increased, with the inclusion of additional expression elements such as leaky stop codons (Skuzeski *et al*. 1991; Hamamoto *et al*. 1993) or the 2 Å sequence from foot-and-mouth disease

virus that interrupts peptide bond formation during translation (Ryan *et al*. 1991; Santa Cruz *et al*. 1996). Using these designs, it is now possible to synthesize multiple proteins from a single mRNA. Together with the improved ability to design and use RNA subgenomic promoters (Donson *et al*. 1991) for the expression of added sequences, a family of powerful gene vectors has evolved. These concepts have spread rapidly to influence the development of gene vectors from other plant and animal RNA viruses (Bredenbeck & Rice 1992; Scholthof *et al*. 1996).

Although TMV has no known biological vector, the presence of TMV in tobacco products results in a global distribution of the virus in the environment. Yet the epidemiology and control of disease spread are also well understood (Gooding 1986). In the United States, Biosource, together with the Federal and State Departments of Agriculture, encouraged a broad public participation in the decision-making process to test recombinant viruses in the field. We found widespread support for the concept, including the support of tobacco growers. Since 1991, we have conducted a total of eight field trials of genetically modified TMV in three States. As expected from the existing cultural practices used to manage TMV, we observed no transmission to adjacent non-inoculated rows or weed species (Grill 1992). After two seasons of crop rotation, tobacco was cultivated again on test sites with no carry-over of infectivity.

3. DEVELOPMENT

Biosource was founded on the assumption that plants would be a preferred and expanding source for many industrial and pharmaceutical materials. We believed that plant viruses were required for efficient genetic experimentation and that TMV would be an ideal first choice for commercial development. Validation of these assumptions is now evident in the performance of the vectors and the quality of the resulting products and information.

(a) *Recombinant peptide and protein production*

The abundance of the TMVCP in infected plants and the potential purification advantages presented by a particulate structure prompted us and others to fuse peptides directly to coat protein domains at the virion surface. The overall peptide yield and simplicity of downstream processing provide a significant cost advantage over chemical synthesis of peptides or alternative recombinant methods. As potential sites for insertion of the peptides in the TMVCP subunit, there are three surface-exposed regions in its four-helix bundle structure; both the N- and C-termini and a surface loop area corresponding to aa 59–65 (Namba *et al*. 1989). Each of these regions may accept peptide fusions without a total loss of the ability of the resulting virus to infect plants systemically.

The virus TMV291 contains a 12-amino-acid insertion of a malarial epitope from the *Plasmodium* circumsporozoite protein, replacing aa 64 and 65 of the central TMVCP loop region (Turpen *et al*. 1995). The chimeric virus particles promoted a potent antigen-specific response in mice, but the surface presentation needs further optimization to improve protection against disease as is often the case in carrier/epitope immunizations.

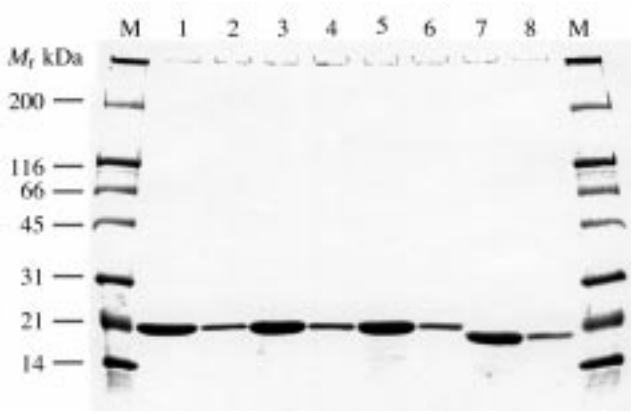

Figure 3. Large-scale purification of chimeric viral particles. Purified coat protein was analysed by SDS-PAGE on 10–20% polyacrylamide gels and stained with Coomassie Brilliant Blue. Lanes 1–8 contain, alternately, 10 and 1 μg each of protein. Lane M contains protein molecular weight standards with the relative molecular mass (M_r) indicated in kDa. The samples in lanes 1–6 are from three independent lots of TMV291 purified from field-grown tobacco cultivars MD609 (lanes 1 and 2), TN86 (lanes 3 and 4) and KY8959 (lanes 5 and 6). The samples in lanes 7 and 8 are from wild-type TMV purified from field-grown tobacco cultivar MD609. These virus particles conform to specifications with respect to genetic stability, elemental analysis, nicotine and endotoxin contamination, mass and AA composition of the protein.

Before we continued vaccine development, we confirmed the expected economical bioprocessing performance for a carrier of this design. Even for a virus reaching only a tenth of the concentration of wild-type TMV, kilogram quantities of particles can be manufactured at costs that can provide a subunit vaccine at a cents-per-dose target price. Additionally, we showed that TMV291 (figure 3), as well as other chimeric virus particles, can be manufactured with lot-to-lot consistency.

Many costly recombinant proteins require protein folding and glycosylation, mediated by the endoplasmic reticulum and Golgi apparatus, as well as other post-translational modifications. Human α-galactosidase A (Gal-A) provided a challenging opportunity to assess the capacity of virus-transfected leaves to accumulate active products because the mature enzyme requires signal peptide cleavage, disulphide-mediated folding, N-linked glycan site occupancy and homo-dimerization of 50 kDa subunits for activity. An inexpensive source of this lysosomal enzyme is required to develop replacement therapy for a rare hereditary storage disorder known as Fabry disease (Desnick *et al*. 1995). The catalytic properties of the enzyme also provided a convenient and quantitative measure of recombinant product fidelity across heterologous expression systems as indirectly observed previously for other enzymes in the development of TMV vectors (Kumagai *et al*. 1993).

Metabolic storage disorders are the most common group of hereditary abnormalities. Many of these conditions, particularly those caused by dysfunctional lysosomal enzymes, are likely to be treatable by supplementation with exogenously produced enzymes. Yet, for any particular molecular defect, a relatively small number of individuals are affected and, therefore, projected R & D and treatment costs are very high. Even 30 years after the

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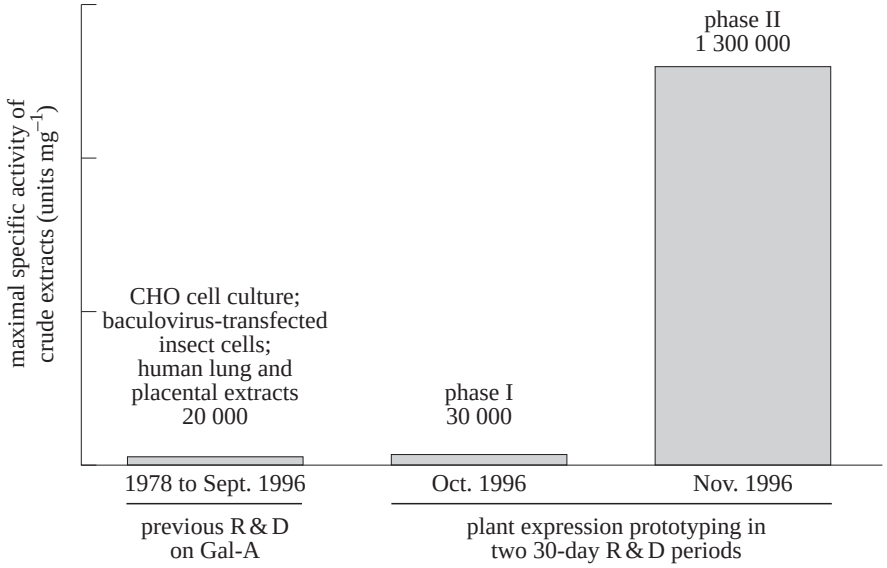

Figure 4. Use of TMV to prototype gene expression in plants. The specific activity (units mg^{-1} protein) of extracts from transfected leaves containing the native human cDNA for Gal-A are compared to previous sources (Coppola *et al*. 1994; Desnick *et al*. 1995) and to the results obtained after a series of carboxy-terminal modifications were analysed for expression and subcellular compartmentalization in leaves. Units of enzymatic activity are based on the cleavage of the fluorescent analogue substrate 4-methylumbelliferyl α-D-galactopyranoside expressed in nmoles cleaved per hour at 37 °C.

specific enzymatic defect in Fabry disease was described (Brady *et al*. 1967), no new technologies have been successfully applied to supply enzymes to these patients.

Initial expression of Gal-A using chimeric TMV vectors proved marginally superior to other technology (figure 4). However, after a month of experimentation, we obtained crude extracts with a greater than 50-fold increase in specific activity (Pogue *et al*. 1997). We used the TMV vector as an information tool to quickly analyse a series of carboxy-modified sequences, and obtained evidence for three pools of Gal-A in transfected leaves. If localized to the endoplasmic reticulum, Gal-A was found to be active, but it accumulated only transiently. Gal-A accumulated in an inactivated form, without evidence of extensive proteolysis, in a post-Golgi compartment, whereas highly active Gal-A accumulated in the interstitial fluid or apoplast of the leaf. The apoplast is a compartment compatible with the stable accumulation of this product without contamination by the majority of other cellular proteins. Finally, Gal-A purified from plant interstitial fluid without homogenization of the leaf has a specific activity more than 10% higher than previously reported for the enzyme from other native or recombinant sources (figure 5). It is perhaps not surprising that the enzyme isolated as a by-product of the biosynthetic growth of leaves during a two-week transfection would be of better quality than enzyme previously purified from the waste products of animal cell fermentation or scavenged from human tissues.

Highly purified, large-scale preparations of Gal-A have the batch consistency required for pharmaceutical product development (figure 5). To our knowledge this is the first recombinant glycoprotein to be purified from the leaf apoplast. We have confirmed by NMR analysis (A. E. Manzi, personal commununication) that the predominant glycan chain present on Gal-A isolated from the leaf interstitial fluid is of the same plant complex type, containing the pentasaccharide core previously observed on plant proteins isolated from seeds or plant cell cultures (Lerouge & Faye 1996). We conclude that, for the production of

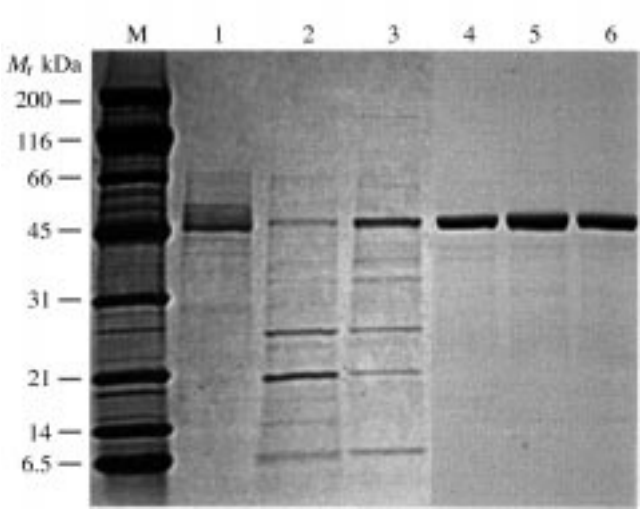

Figure 5. Milking leaves for recombinant secretory protein. Large-scale purification of human Gal-A. Purified protein was analysed by SDS-PAGE on 10–20% polyacrylamide gels and stained with Coomassie Brilliant Blue. Lanes 1–6 each contain 2 µg of protein. Lane M contains protein molecular weight standards with the relative molecular mass (M_r) indicated in kDa. Lane 1 contains Gal-A standard purified from human placenta. Lane 2 is the crude protein extract from leaf interstitial fluid. Lane 3 is protein eluted from the Butyl Streamline® (Pharmacia Biotech) chromatography step. Lanes 4–6 are three lots of enzyme finished with separate chromatography conditions. The highest specific enzyme activity reported previously for Gal-A was 5.0×10^6 units mg^{-1} (Calhoun *et al*. 1985) for enzyme isolated from human lung tissue. Gal-A purified from transfected leaves routinely has a specific activity of over 5.5×10^6 units mg^{-1} protein.

highly active and relatively homogeneous glycoforms of Gal-A, none of the key post-translational modifications required was saturated during the rapid burst of viral protein synthesis nor were host components induced that damaged the product, accelerated turnover, or prevented purification from TMV-transfected foliage.

Another delay in the greening of biotechnology has been due to the lack of investment in large-scale industrial bioprocessing to purify recombinant products from plants. In 1995, the advantages of TMV vectors compelled Biosource to purchase and adapt a processing facility at Owensboro, Kentucky, for pilot-scale extractions of plant biomass. In 1998, we completed construction of a new facility for the production of specified biologics. This facility is designed to meet current Good Manufacturing Practices (cGMP) standards and will be validated in 1999. We have used over 200 tonnes of field grown, TMV-transfected biomass per year to implement a series of scaleable unit processes. As examples, the chimeric virions described above are purified by homogenization of tobacco, selective denaturation and removal of contaminants, followed by ultrafiltration and precipitation of the particles (Turpen *et al*. 1997). For Gal-A, interstitial fluid is extracted by vacuum infiltration of the tissue and industrial basket centrifugation. This dilute feedstream is filtered and then recombinant protein is trapped by expanded bed chromatography and further purified by conventional chromatographic methods (Cameron *et al*. 1998).

(b) *Metabolic pathways and gene function*

These experiments in protein production as well as additional nucleic acid sequencing studies (Kearney *et al*. 1993) demonstrate the high-fidelity transient expression of many sequences in TMV-based vectors. The ability to rapidly amplify and propagate genetic information in differentiated tissues of a higher eukaryote has broad implications for general studies of gene function, the regulation of metabolism and the production of secondary metabolites.

The carotenoid pathway is convenient for study because the phenotypes induced by viral transfection can be recognized by eye. These pigments function in the plastid during light-harvesting and photoprotection. TMV-based overexpression of tomato phytoene synthase in *N. benthamiana* produced an orange phenotype and accumulated tenfold higher levels of phytoene (Kumagai *et al*. 1995). Similarly, ectopic expression of pepper capsanthin–capsorubin synthase resulted in an orange phenotype and channelled 36% of the total carotenoid content to the non-native pigment capsanthin (Kumagai *et al*. 1998). Significantly, this chromoplast pigment was incorporated into photosystem II of the chloroplast thylakoid membrane, thereby displacing native xanthophylls stoichiometrically. This experiment revealed a compensatory regulatory mechanism operating in the leaf carotenoid biosynthetic pathway and simultaneously identified a subcellular location for the deposition of a non-native pigment.

TMV-based vectors can be used to inhibit gene expression from the cytosol. High levels of phytoene also accumulated in photobleached, transfected leaves of *N. benthamiana* by silencing the expression of the downstream enzyme phytoene desaturase using fragments of a gene derived from tomato and cloned in either the sense or antisense orientation (Kumagai *et al*. 1995). Therefore, the virus-induced gene silencing originally observed to target viral-derived transgenes (Lindbo *et al*. 1993) can be widely and conveniently applied in functional genomics studies.

4. CONCLUSION

TMV-based vectors can be used to discover, prototype, produce and even deliver novel molecules (Turpen & Reinl 1997; Pogue *et al*. 1998). This integrated approach has many immediate commercial applications. One such cost-sensitive opportunity is in subunit vaccine development. In many cases antigen expression in transfected plants will be superior to the use of transgenic plants. TMV can be used to present foreign epitopes on its particle surface, or to direct the synthesis of other heterologous self-assembling polymeric carriers such as the hepatitis B surface antigen particle (Reinl *et al*. 1994), or particles based on the alfalfa mosaic virus coat protein (Yusibov *et al*. 1997). Immunomodulatory adjuvants, for example, cytokines, can be produced with high biological activity (K. Hanley and G. P. Pogue, personal communication). It is possible that some of these materials may be conveniently formulated for oral delivery of efficacious subunit vaccines (Modelska *et al*. 1998).

The fact that complex enzymes of high specific activity can be reproducibly purified from transfected leaves implies that relatively homogeneous and high-quality folding, and post-translational modifications, occur in the plant secretory pathway. This is a critical safety and efficacy issue in vaccine production because microheterogeneity in subunit vaccine preparations can reduce potency, and in very rare instances, prove hazardous, by potentiating disease. The efficacy as a subunit vaccine of a soluble antigen purified from the leaf interstitial fluid was recently confirmed in preclinical trials (McCormick *et al*. 1999). Cancer vaccines for certain B-cell lymphomas can be designed from the idiotype of the surface-exposed antibody because this complementarity-determining region of the antibody is a tumour-specific antigen. TMV-based vectors are the system of choice for rapid production and optimization of these high-quality, single-chain Fv antibody fragments in a time-frame relevant to individualized patient therapy. It is very likely that such an immunotherapeutic product will bring plant-derived recombinant biologics into the mainstream of health-care practices.

As often seen in the past 100 years, TMV remains of contemporary importance. The virus continues to be an integral part of the scientific advancement of the times. Today, biotechnology is becoming green. Studies with TMV have provided some of the means and the fundamental knowledge required to customize the biosynthetic potential of plants for an expanding range of applications.

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chromatography assistance. Some of this work was supported by grants from the National Science Foundation and the National Institutes of Health.

APPENDIX A

The peptide libraries described in figure 2 are biased by the design and chemical coupling efficiencies of the randomized sequence. These libraries are comprised of codons having a sequence NNK where N = A, C, G, or T and K = G or T for a complexity of $[(4)(4)(2)]^6 = (32)^6 = 1 \times 10^9$ different nucleotide sequence permutations. Triplet codons are calculated to occur in this library with the following coding frequencies: {R, L, S} = 0.094; {A, G, P, T, V} = 0.062; {Q, N, D, C, E, H, I, K, M, F, W Y, Amber Stop} = 0.031.

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