Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Biotechnology

Innovation in plant-based transient protein expression for infectious disease prevention and preparedness

Frank Sainsbury[1,2]

Addressing new challenges in global health and biosecurity requires responsive and accessible platforms for the manufacture of preventative or therapeutic interventions. Transient protein expression in plants has evolved into a technology that offers a unique combination of rapid expression, inherent scalability, and flexibility in gene stacking with the capability to produce complex proteins and protein assemblies. Technical developments that have driven the progress of transient expression in plants include advanced expression systems, protein engineering and synthetic biology approaches to transiently, or stably, modify host plants. The plasticity of transient expression in plants, speed of scalability and relatively low capital costs, highlight the great potential of this technology in the future of human and animal health.

Addresses
[1] Centre for Cell Factories and Biopolymers, Griffith Institute for Drug Discovery, Griffith University, Nathan, QLD 4111, Australia
[2] Synthetic Biology Future Science Platform, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Brisbane, QLD 4001, Australia

Corresponding author: Sainsbury, Frank (f.sainsbury@griffith.edu.au)

Current Opinion in Biotechnology 2020, **61**:110–115

This review comes from a themed issue on **Plant biotechnology**

Edited by **Ralf Reski**, **Ed Rybicki** and **Gary Foster**

For a complete overview see the Issue and the Editorial

Available online 6th December 2019

https://doi.org/10.1016/j.copbio.2019.11.002

Introduction

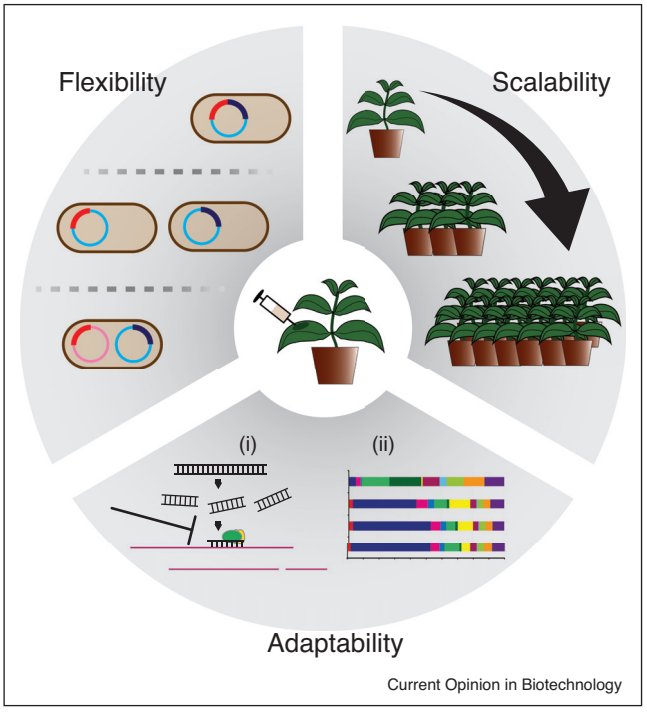
Globalisation and climate change have resulted in changing patterns of the emergence and global spread of infectious diseases. The majority of emerging infectious diseases are zoonotic or vector-borne with over 85 such diseases discovered since 1980 [1]. Notable cases, including highly pathogenic avian influenza (H5N1) and severe acute respiratory syndrome (SARS) among others, have highlighted the difficulty in predicting zoonotic disease outbreaks. Vaccination is a crucial tool in combatting zoonosis and the spread of circulating pathogens, and is central to the One Health paradigm that acknowledges the interaction between human and animal health, as well as the influence of the environmental and social context [2]. Addressing infectious disease within this framework requires, above all, responsive protein production. The maturation of plant-based transient expression has demonstrated that this technology is able to meet this requirement for both human and animal health [3,4].

Around the turn of the century, transient transformation of plant tissues emerged as an alternative mode of transgenesis [5] that has created new opportunities for plant biotechnology in biologics production [3], metabolic engineering [6] and synthetic biology [7]. Introducing disarmed *Agrobacteria tumefaciens* strains carrying binary vectors into the leaves of whole plants, known as agroinfiltration, results in T-DNA delivery to all cells in the area infiltrated with bacterial suspension. This approach has three considerable benefits for recombinant protein production in infectious disease prevention and preparedness: (i) it enables easily scalable and rapid upstream processes, (ii) it allows straightforward multiplexing of gene constructs (gene stacking), and (iii) it decouples host cell engineering from plant viability and biomass accumulation (Figure 1). The implications of these advantages with regard to responsive vaccine production, and recent technological advances complementing these features, are the subject of this review.

Transient expression technologies: speed and flexibility

Among the enabling technologies that have turned plants into viable hosts for pharmaceutical and therapeutic protein production, transient expression in the leaves of whole plants has been the most transformative. Driving the uptake of this technology in academic and commercial settings was the development of expression systems derived from the regulatory sequences, replication elements, and inhibitors of post-transcriptional gene silencing of various plant viruses [8•]. More recently, taking advantage of existing genome-wide RNA stability data, a systematic analysis of diverse viral and plant 3′ untranslated regions (UTRs)/terminator sequences demonstrated the additive potential of native, chimeric or duplicated regulatory sequences in directing high-level transient expression [9••]. Further opportunities for increasing expression also now come from the demonstrated that entirely synthetic, and highly effective, UTRs can be designed from first principles [10••]. Determining the optimal combination of regulatory elements for a given recombinant protein remains empirical, though the ease and speed with which combinations can be rapidly screened using transient expression is a significant advantage.

Figure 1



Salient features of transient expression in plants as a vaccine production host.
When performing transient protein expression in plants each plant is a disposable and biodegradable bioreactor [14••]. Therefore, scaling up production simply requires agroinfiltration of a greater number of plants. Expression characteristics remain the same, meaning that both upstream decisions and processes, such as the co-expression from multiple constructs and/or the inclusion of cell physiology modifying accessory proteins are translatable to all scales of production. The flexibility afforded by transient expression is such that there are multiple ways in which constructs can be combined for co-expression; in multicistronic vectors, by mixing *A. tumefaciens* cultures, or by using multiple compatible plasmids. Furthermore, the use of mature plants in which the biomass has already accumulated, permits the modification of host cell physiology to an extant not compatible with viable growth and development, such as strong suppression of post-transcriptional gene silencing (i) and modifiers that lead to wholesale modification of the host proteome (ii).

In a landmark study, Medicago Inc. demonstrated that transient expression could be used to produce a virus-like particle (VLP) vaccine against Influenza H1N1 within three weeks of identification of the circulating pandemic strain [11]. This represents a fourfold to eightfold reduction of the time it takes to make Influenza vaccines by the traditional egg-based method. The responsiveness of plant-based transient expression for the production of vaccines represents a competitive advantage [12] that has attracted industry and government agencies to explore the capabilities of this approach. In 2007, the Defense Advanced Research Projects Agency (DARPA) of the USA began investing in the development and proof-of-concept operation of commercial scale facilities for the manufacture of vaccines via agroinfiltration in plants. Holtz *et al.* provide a detailed insight into the design and operation of one such facility [13••], scaled to produced tens of millions of vaccine doses per month [3,13••]. The commissioning of these exemplar facilities highlighted how rapidly manufacturing sites for transient plant-based expression can be established as well as how smooth the scaling of upstream processes is, demonstrating the responsiveness of plant-based vaccine production. The key to the scale-up advantages of plant-based expression lies in the fact that each plant is a single-use bioreactor [14••]. Expression conditions remain the same at all scales, allowing for direct translation of constructs and combinations of constructs tested at small scale.

The co-delivery of multiple expression constructs by agroinfiltration is remarkably simple. The simplest approach is to mix *A. tumefaciens* cultures containing individual constructs. This provides great flexibility in testing combinations of genes and expression cassettes for the assembly of protein complexes or co-expression of host cell engineering constructs to enhance expression. However, it has been shown that co-expression is more efficient when using a multicistronic construct delivered by a single *A. tumefaciens* culture, ensuring co-expression in all transformed cells [15]. An alternative approach is the delivery of T-DNA from binary vectors with compatible replication origins from a single culture [16]. This has the advantage of minimising the size of each construct when multiple expression cassettes are required, potentially simplifying cloning steps.

In some cases, it may be necessary to modulate expression levels of individual constructs to optimise yields. For example, some multicomponent VLPs require coat proteins in varying stoichiometry. The most straightforward method to regulate expression from individual constructs is via relative dilution of the *A. tumefaciens* suspension carrying that construct. However, at a certain dilution not all cells are transformed by each strain, which limits co-expression from multiple constructs [15]. Protein expression from individual expression cassettes can be controlled at a translational level via point mutations in the 5′ UTR [17] or via engineered mRNA stability using mutant or chimeric 3′ UTRs [9••,18]. Ideally, the capacity to tune expression levels is predictable. Since the impact of different regulatory sequences is protein coding sequence-dependent, intimate knowledge of elements within an expression cassette can be a distinct advantage. In a particularly elegant example, the levels of individual proteins among four Bluetongue virus coat proteins were controlled by single mutation to the 5′ untranslated region to modulate translation and optimise yield of the complete VLP [19]. An alternate approach is to use protein engineering to achieve the same effect. This was demonstrated by the production of African horse sickness virus VLPs where mutations to increase the stability of 1 out of 4 structural proteins resulted in increased VLP yields [20].

Structurally authentic vaccines

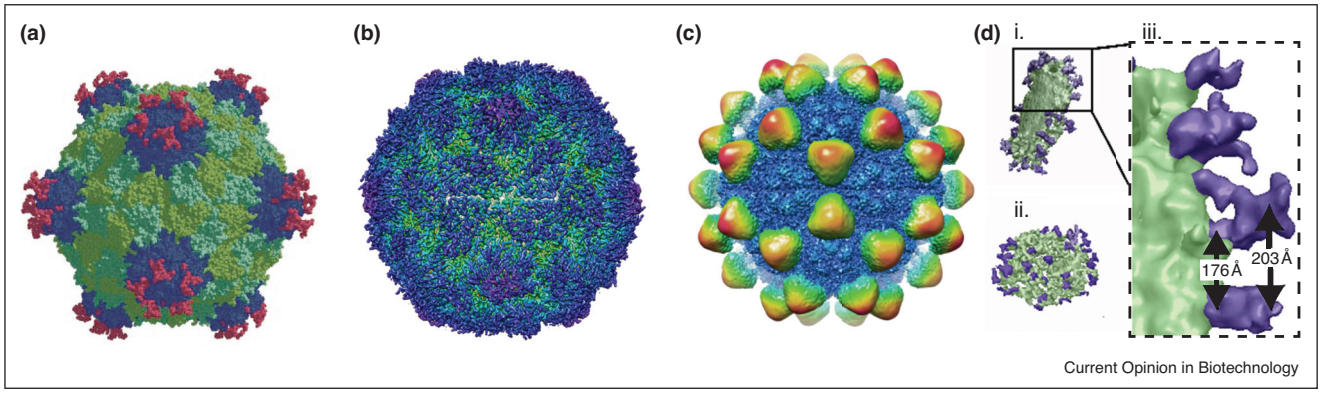
Transient expression in plants is particularly good for the production of complex protein assemblies, such as VLPs [21,22]. The design, production and analysis of VLP vaccines produced in plants have been facilitated by high resolution biophysical characterisation, such as that provided by X-ray crystallography and cryo-electron microscopy (cryo-EM) (Figure 2). Cowpea mosaic virus was the first plant virus used for epitope display [23] and as early as 1996, X-ray crystallography was used to probe the conformation of Human rhinovirus 14 epitopes presented using this platform (Figure 2a). The structure revealed that the epitope was linearised *in vivo*, probably explaining why the epitope-specific antibodies raised by the particles were not neutralising [24].

Cryo-EM and cryo-electron tomography require far less material and simplified sample preparation than what is required for crystallography, and these techniques are now being used to show the precision with which authentic VLPs of human and animal viruses are assembled following transient expression in plants. Single particle analysis was used to demonstrate the structural authenticity of Poliovirus type 3 VLPs made transiently in plants and engineered to be more stable than wild-type VLPs (Figure 2b) [25••]. Stability is a significant challenge, especially in resource-poor settings where there may be difficulties maintaining the cold-chain during vaccine distribution, and the protective immunity afforded in mice by these plant-made Poliovirus VLPs is promising [25••]. Cryo-EM has also been used to determine the structure of a plant-made VLP vaccine against Atlantic Cod Nervous necrosis virus that confers protective immunity in sea bass (Figure 2c) [26]. Tomographic imaging of Influenza hemagglutinin-based VLPs transiently expressed in plants also showed that these enveloped virus VLPs are morphologically similar to Influenza virions, although the distance between hemagglutinin trimers was slightly greater on the VLP (Figure 2d). The authors hint at a relationship between this distance and immunogenicity, and show that the VLPs preserve native interactions with immune cells [27]. Similar plant-made particles have completed Phase II clinical trials [28]. Given the structural basis for immunogenicity of subunit vaccines including VLPs, cryo-EM is sure to become a more commonplace approach in the quality control and structure-based development of vaccines transiently expressed in plants.

Heterologous modifiers of host cell physiology

Host plant cells can be considered as a production chassis, in which physiological conditions can be optimised to favour recombinant protein quality and quantity. Transiently expressed modifiers of physiology are a means to synthetically recondition the production chassis without the lengthy process of generating recombinant plant lines. Various biomolecules have been employed to transiently engineer host plants including protein, RNA species leading to RNAi, and plant hormones. An advantage of transient modification of cells in mature plants is that it allows for tailoring of host cell physiology that would otherwise be unsustainable in transgenic plants. For example, strong suppressors of post-transcriptional

Figure 2



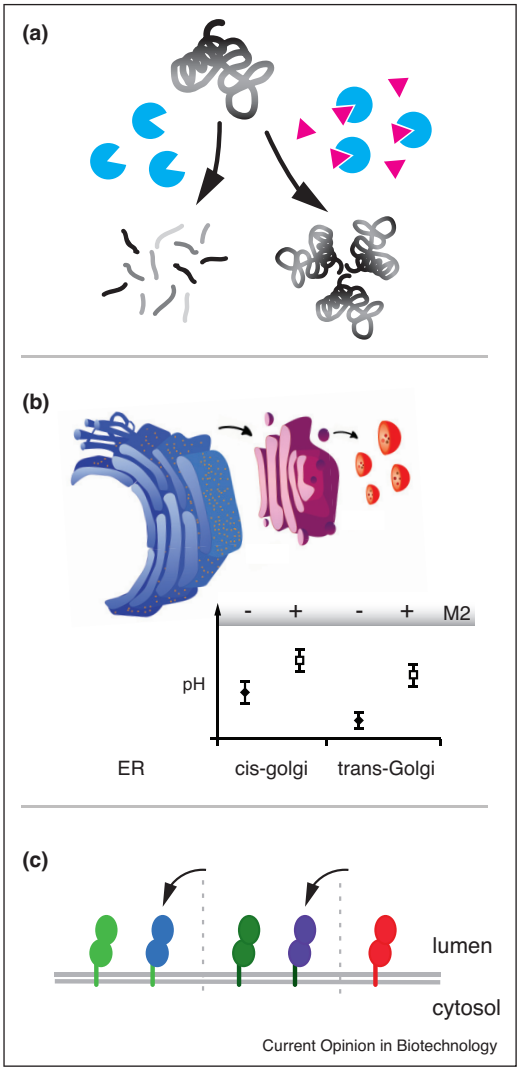
High resolution biophysical characterisation of VLPs transiently expressed in plants.
(a) X-ray crystallography structure of the chimeric Cowpea mosaic virus displaying an epitope from Human rhinovirus 14 in red [24].
(b) Cryo-EM reconstruction of the stabilised mutant of Poliovirus type 3 VLP to 3.6 Å [25••].
(c) Cryo-EM reconstruction of the Atlantic Cod Nervous necrosis virus VLP to 3.7 Å for shell domain (in blue) and lower resolution for the trimeric protrusions flexible P domain [26].
(d) Influenza reconstructed by cryo-electron tomography generated from a tilt series of images of individual particles showing trimeric hemagglutinin in purple [27]. (i) Discoid particles comprising >65% of particles in the preparation, (ii) spherical particles comprising ~30% of particles in the preparation (a small number of star-shaped hemagglutinin rosettes were also observed), (iii) close-up of discoid particle showing hemagglutinin trimers are separated by approximately 200 nm, which is around two times the spacing found on the native virion.

gene silencing are developmentally lethal in their wild type form, yet commonly used to improve transient recombinant protein expression [29,30].

Many recombinant candidate vaccines, such as viral glycoproteins and secretory immunoglobulins are expressed in and targeted to the secretory pathway of plants [31]. These complex proteins have specific requirements for protein maturation and stability and various parameters contributing to their accumulation have been targeted for improvement by transient modification (Figure 3). Unintended proteolysis is often cited as a concern for plant-based expression and the identification of specific proteolytic activities in the plant secretory pathway allowed rational inhibition of these activities by the co-expression of protease inhibitors (Figure 3a) [32]. This strategy can result in a 1.4-fold increase in recombinant antibody production [33] and consideration of inhibitors regulated in response to plant–pathogen interactions is also uncovering effective protease inhibitors for the protection of recombinant proteins [34•]. RNAi constructs have also been used to knock down proteolytic activities [35], which has led to a 1.6-fold increase in interleukin 10 accumulation in whole plants [36]. The continued identification of specific proteases directly involved in the degradation of recombinant proteins in the secretory pathway increases the potential impact of transient or stable knock-down approaches [37]. An alternative approach to improving protein stability is to modulate the pH of the secretory pathway via the expression of proton channels (Figure 3b). The co-expression of Influenza M2 ion channel significantly raises the pH in the late stages of the secretory pathway of plants, mirroring the strategy of the influenza virus to prevent premature conformational change of the hemagglutinin [38]. This approach led to increased accumulation of acid-labile isotypes of hemagglutinin, and it was subsequently shown that M2 expression results in a broad remodelling of protease activities in the secretory pathway [39]. Another important aspect of protein maturation in the secretory pathway is glycosylation. Transient glycolengineering is the process by which additional glycosyltransferases are co-expressed (Figure 3c) [40,41,42•]. This strategy has been used to generate numerous human-like N-linked glycan profiles including branched glycans [43] and O-linked glycans [44] that do not normally form in plants. In theory, it also includes transient knock-down of endogenous glycosyltransferases, which has been achieved in numerous stable plant lines [42•].

In addition to transiently tuning host cell physiology by the recombinant expression of modifiers, innovations in chemically regulating plant development or modifying the response to agroinfiltration have been reported. For example, the selected application of plant hormones has been used to rebalance the proteome from an antibacterial response to favour recombinant protein accumulation [45]. Hormones have also been used to modify plant architecture, increasing biomass and recombinant protein yield [46]. The over expression of recombinant proteins puts enormous stress on the cell and the co-expression of

Figure 3



Transient host cell modifications to improve the yield and quality of recombinant secretory proteins.
(a) Prevention of recombinant protein degradation by endogenous proteases (cyan) through the co-expression of protease inhibitors (magenta).
(b) Modulation of the pH in the secretory pathway via the co-expression of the Influenza M2 ion channel. The pH gradient of the secretory systems changes from around neutral in the endoplasmic reticulum (blue) and gradually decreases through to the *trans*-Golgi (red) before generally raising again in the unbuffered apoplast. Insert shows the pH change in the *cis*-Golgi and *trans*-Golgi mediated by M2 co-expression [38].
(c) Glycoengineering through the co-expression of glycosyltransferases designed to insert into certain locations within the secretory pathway using chimeric or plant-derived transmembrane domains (green), diverting the synthesis of glycans towards the desired profile.

stress-tolerance proteins or the inclusion of an antioxidant in the infiltration media can also increase yields of recombinant proteins from transient expression [30]. As expression levels are reaching a greater proportion of soluble protein, attention is turning towards the improvement of host cell chassis within whole plants to consistently produce not only high yields, but also high quality recombinant proteins.

Conclusions

In the years since transient expression systems changed the way whole plant hosts were used to make recombinant protein, the capability of transient expression in plants to produce complex antigens in high yields and with short timelines has been unequivocally demonstrated. The flexibility of this technology as discussed here, together with the speed and ease of scale-up, presents a platform uniquely positioned to respond to emerging infectious diseases. While even the highest yielding systems have a large production footprint [12], the construction of production facilities require relatively low capital expense [14••] and can be constructed relatively fast [13••]. This has been recognised by governments and governmental agencies, which has in turn increased commercial viability. In addition to preparedness for human health and biosecurity [47], there is also a commercial case for the production of veterinary vaccines and therapeutics [12,48]. Attention is turning to the improvement of whole plant hosts and much of this review discusses the transient modifications that can be made to improve protein expression. However, there is also considerable effort to improve plants specifically for recombinant expression through the use of gene-editing techniques, for example, in glycoengineering [49,50]. The technical and commercial maturation of expression technologies, along with product-specific host cell tailoring, demonstrates the responsive nature of transient vaccine expression in plants, to the potential benefit of global health and biosecurity.

Conflict of interest statement

Nothing declared.

Acknowledgement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Gebreyes WA, Dupouy-Camet J, Newport MJ, Oliveira CJB, Schlesinger LS, Saif YM, Kariuki S, Saif LJ, Saville W, Wittum T *et al.*: **The global one health paradigm: challenges and opportunities for tackling infectious diseases at the human, animal, and environment interface in low-resource settings**. *PLoS Negl Trop Dis* 2014, **8**.

2. Monath TP: **Vaccines against diseases transmitted from animals to humans: a one health paradigm**. *Vaccine* 2013, **31**:5321-5338.

3. Lomonossoff GP, D'Aoust MA: **Plant-produced biopharmaceuticals: a case of technical developments driving clinical deployment**. *Science* 2016, **353**:1237-1240.

4. Rybicki EP: **Plant-made vaccines and reagents for the one health initiative**. *Hum Vaccin Immunother* 2017, **13**:2912-2917.

5. Kapila J, DeRycke R, VanMontagu M, Angenon G: **An agrobacterium-mediated transient gene expression system for intact leaves**. *Plant Sci* 1997, **122**:101-108.

6. Reed J, Osbourn A: **Engineering terpenoid production through transient expression in *Nicotiana benthamiana***. *Plant Cell Rep* 2018, **37**:1431-1441.

7. Sainsbury F, Lomonossoff GP: **Transient expressions of synthetic biology in plants**. *Curr Opin Plant Biol* 2014, **19**:1-7.

8. • Peyret H, Lomonossoff GP: **When plant virology met agrobacterium: the rise of the deconstructed clones**. *Plant Biotechnol J* 2015, **13**:1121-1135.
Excellent review that tells the story of how the most effective transient expression systems came to be.

9. •• Diamos AG, Mason HS: **Chimeric 3' flanking regions strongly enhance gene expression in plants**. *Plant Biotechnol J* 2018, **16**:1971-1982.
This paper is described the first use of transcriptomic data sets to mine for regulatory sequences specifically to enhance transient expression. Demonstrates the complexity of choosing the right 3'UTR and sets a new standard for expression levels in the absence of replicating elements.

10. •• Peyret H, Brown JKM, Lomonossoff GP: **Improving plant transient expression through the rational design of synthetic 5' and 3' untranslated regions**. *Plant Methods* 2019, **15**:108.
First example of rational design of synthetic UTRs for high-level transient expression based on parameters known to impact protein expression such length, GC content, low secondary structure and sequence motifs.

11. D'Aoust MA, Couture MMJ, Charland N, Trepanier S, Landry N, Ors F, Vezina LP: **The production of hemagglutinin-based virus-like particles in plants: a rapid, efficient and safe response to pandemic influenza**. *Plant Biotechnol J* 2010, **8**:607-619.

12. Schillberg S, Raven N, Spiegel H, Rasche S, Buntru M: **Critical analysis of the commercial potential of plants for the production of recombinant proteins**. *Front Plant Sci* 2019, **10**.

13. •• Holtz BR, Berquist BR, Bennett LD, Kommineni VJ, Munigunti RK, White EL, Wilkerson DC, Wong KY, Ly LH, Marcel S: **Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals**. *Plant Biotechnol J* 2015, **13**:1180-1190.
This account provides fascinating insight into the design, construction, and operation of a large scale facility for the production of pharmaceutical grade material by transient expression in plants.

14. •• Buyel JF: **Plant molecular farming integration and exploitation of side streams to achieve sustainable biomanufacturing**. *Front Plant Sci* 2019, **9**.
This critical review of the literature highlights the benefits of plants as single use bioreactors for the production of recombinant proteins and also points to addition products that could arise from process waste streams.

15. Saxena P, Thuenemann EC, Sainsbury F, Lomonossoff GP: **Virus-derived vectors for the expression of multiple proteins in plants**. *Methods Mol Biol* 2016, **1385**:39-54.

16. Pasin F, Bedoya LC, Bernabe-Orts JM, Gallo A, Simon-Mateo C, Orzaez D, Garcia JA: **Multiple T-DNA delivery to plants using novel mini binary vectors with compatible replication origins**. *ACS Synth Biol* 2017, **6**:1962-1968.

17. Sainsbury F, Lomonossoff GP: **Extremely high-level and rapid transient protein production in plants without the use of viral replication**. *Plant Physiol* 2008, **148**:1212-1218.

18. Meshcheriakova YA, Saxena P, Lomonossoff GP: **Fine-tuning levels of heterologous gene expression in plants by orthogonal variation of the untranslated regions of a nonreplicating transient expression system**. *Plant Biotechnol J* 2014, **12**:718-727.

19. Thuenemann EC, Meyers AE, Verwey J, Rybicki EP, Lomonossoff GP: **A method for rapid production of heteromultimeric protein complexes in plants: assembly of protective bluetongue virus-like particles**. *Plant Biotechnol J* 2013, **11**:839-846.

20. Dennis SJ, Meyers AE, Guthrie AJ, Hitzeroth II, Rybicki EP: **Immunogenicity of plant-produced African horse sickness virus-like particles: implications for a novel vaccine**. *Plant Biotechnol J* 2018, **16**:442-450.

21. Marsian J, Lomonossoff GP: **Molecular pharming - VLPs made in plants**. *Curr Opin Biotechnol* 2016, **37**:201-206.

22. Rybicki EP: **Plant molecular farming of virus-like nanoparticles as vaccines and reagents**. *Wiley Interdiscip Rev Nanomed Nanobiotechnol* 2019:e1587.

23. Sainsbury F, Canizares MC, Lomonossoff GP: **Cowpea mosaic virus: the plant virus-based biotechnology workhorse**. *Annu Rev Phytopathol* 2010, **48**:437-455.

24. Lin TW, Porta C, Lomonossoff G, Johnson JE: **Structure-based design of peptide presentation on a viral surface: the crystal structure of a plant/animal virus chimera at 2.8 angstrom resolution**. *Fold Des* 1996, **1**:179-187.

25. •• Marsian J, Fox H, Bahar MW, Kotecha A, Fry EE, Stuart DI, Macadam AJ, Rowlands DJ, Lomonossoff GP: **Plant-made polio type 3 stabilized VLPs-a candidate synthetic polio vaccine**. *Nat Commun* 2017, **8**.

This paper presents a definitive demonstration that transient expression in plants can be used to create structurally authentic vaccines for human health as confirmed by high-resolution cryo-EM of a plant-made Polio-virus VLP.

26. Marsian J, Hurdiss DL, Ranson NA, Ritala A, Paley R, Cano I, Lomonossoff GP: **Plant-made nervous necrosis virus-like particles protect fish against disease**. *Front Plant Sci* 2019, **10**.

27. Lindsay BJ, Bonar MM, Costas-Cancelas IN, Hunt K, Makarkov AI, Chierzi S, Krawczyk CM, Landry N, Ward BJ, Rouiller I: **Morphological characterization of a plant-made virus-like particle vaccine bearing influenza virus hemagglutinins by electron microscopy**. *Vaccine* 2018, **36**:2147-2154.

28. Pillet S, Couillard J, Trepanier S, Poulin JF, Yassine-Diab B, Guy B, Ward BJ, Landry N: **Immunogenicity and safety of a quadrivalent plant-derived virus like particle influenza vaccine candidate-two randomized phase II clinical trials in 18 to 49 and >= 50 years old adults**. *PLoS One* 2019, **14**.

29. Saxena P, Hsieh YC, Alvarado VY, Sainsbury F, Saunders K, Lomonossoff GP, Scholthof HB: **Improved foreign gene expression in plants using a virus-encoded suppressor of RNA silencing modified to be developmentally harmless**. *Plant Biotechnol J* 2011, **9**:703-712.

30. Norkunas K, Harding R, Dale J, Dugdale B: **Improving agroinfiltration-based transient gene expression in *Nicotiana benthamiana***. *Plant Methods* 2018, **14**.

31. Margolin E, Chapman R, Williamson AL, Rybicki EP, Meyers AE: **Production of complex viral glycoproteins in plants as vaccine immunogens**. *Plant Biotechnol J* 2018, **16**:1531-1545.

32. Goulet C, Khalf M, Sainsbury F, D'Aoust MA, Michaud D: **A protease activity-depleted environment for heterologous proteins migrating towards the leaf cell apoplast**. *Plant Biotechnol J* 2012, **10**:83-94.

33. Robert S, Khalf M, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D: **Protection of recombinant mammalian antibodies from development-dependent proteolysis in leaves of *Nicotiana benthamiana***. *PLoS One* 2013, **8**.

34. • Grosse-Holz F, Madeira L, Zahid MA, Songer M, Kourelis J, Fesenko M, Ninck S, Kaschani F, Kaiser M, van der Hoorn RAL: **Three unrelated protease inhibitors enhance accumulation of pharmaceutical recombinant proteins in *Nicotiana benthamiana***. *Plant Biotechnol J* 2018, **16**:1797-1810.

This work uncovered novel protease inhibitors effective in protecting secreted proteins by screening candidate protease inhibitors identified via their stable/strong activity, or by involvement in plant–pathogen interactions, from both plants and pathogens.

35. Mandal MK, Ahvari H, Schillberg S, Schiermeyer A: **Tackling unwanted proteolysis in plant production hosts used for molecular farming**. *Front Plant Sci* 2016, **7**.

36. Duwadi K, Chen L, Menassa R, Dhaubhadel S: **Identification, characterization and down-regulation of cysteine protease genes in tobacco for use in recombinant protein production**. *PLoS One* 2015, **10**.

37. Jutras PV, Grosse-Holz F, Kaschani F, Kaiser M, Michaud D, van der Hoorn RAL: **Activity-based proteomics reveals nine target proteases for the recombinant protein-stabilizing inhibitor SlCYS8 in *Nicotiana benthamiana***. *Plant Biotechnol J* 2019, **17**:1670-1678.

38. Jutras PV, D'Aoust MA, Couture MMJ, Vezina LP, Goulet MC, Michaud D, Sainsbury F: **Modulating secretory pathway pH by proton channel co-expression can increase recombinant protein stability in plants**. *Biotechnol J* 2015, **10**:1478-1486.

39. Jutras PV, Goulet MC, Lavoie PO, D'Aoust MA, Sainsbury F, Michaud D: **Recombinant protein susceptibility to proteolysis in the plant cell secretory pathway is pH-dependent**. *Plant Biotechnol J* 2018, **16**:1928-1938.

40. Castilho A, Strasser R, Stadlmann J, Grass J, Jez J, Gattinger P, Kunert R, Quendler H, Pabst M, Leonard R *et al.*: **In planta protein sialylation through overexpression of the respective mammalian pathway**. *J Biol Chem* 2010, **285**:15923-15930.

41. Vezina LP, Faye L, Lerouge P, D'Aoust MA, Marquet-Blouin E, Burel C, Lavoie PO, Bardor M, Gomord V: **Transient co-expression for fast and high-yield production of antibodies with human-like N-glycans in plants**. *Plant Biotechnol J* 2009, **7**:442-455.

42. • Montero-Morales L, Steinkellner H: **Advanced plant-based glycan engineernig**. *Front Bioeng Biotechnol* 2018, **6**:81.

Excellent review of the possibilities for glyco-engineering in plants from the knock-down of plant glycans to the augmentation of the capacity for glycosylation and the introduction of heterologous glycotypes.

43. Castilho A, Gattinger P, Grass J, Jez J, Pabst M, Altmann F, Gorfer M, Strasser R, Steinkellner H: **N-Glycosylation engineering of plants for the biosynthesis of glycoproteins with bisected and branched complex N-glycans**. *Glycobiology* 2011, **21**:813-823.

44. Dicker M, Tschofen M, Maresch D, Konig J, Juarez P, Orzaez D, Altmann F, Steinkellner H, Strasser R: **Transient glyco-engineering to produce recombinant iga1 with defined N- and O-glycans in plants**. *Front Plant Sci* 2016, **7**.

45. Robert S, Goulet MC, D'Aoust MA, Sainsbury F, Michaud D: **Leaf proteome rebalancing in *Nicotiana benthamiana* for upstream enrichment of a transiently expressed recombinant protein**. *Plant Biotechnol J* 2015, **13**:1169-1179.

46. Goulet MC, Gaudreau L, Gagne M, Maltais AM, Laliberte AC, Ethier G, Bechtold N, Martel M, D'Aoust MA, Gosselin A *et al.*: **Production of biopharmaceuticals in *Nicotiana benthamiana*-axillary stem growth as a key determinant of total protein yield**. *Front Plant Sci* 2019, **10**.

47. Streatfield SJ, Kushnir N, Yusibov V: **Plant-produced candidate countermeasures against emerging and reemerging infections and bioterror agents**. *Plant Biotechnol J* 2015, **13**:1136-1159.

48. Topp E, Irwin R, McAllister T, Lessard M, Joensuu JJ, Kolotilin I, Conrad U, Stoger E, Mor T, Warzecha H *et al.*: **The case for plant-made veterinary immunotherapeutics**. *Biotechnol Adv* 2016, **34**:597-604.

49. Jansing J, Sack M, Augustine SM, Fischer R, Bortesi L: **CRISPR/Cas9-mediated knockout of six glycosyltransferase genes in *Nicotiana benthamiana* for the production of recombinant proteins lacking beta-1,2-xylose and core alpha-1,3-fucose**. *Plant Biotechnol J* 2019, **17**:350-361.

50. Li J, Stoddard TJ, Demorest ZL, Lavoie PO, Luo S, Clasen BM, Cedrone F, Ray EE, Coffman AP, Daulhac A *et al.*: **Multiplexed, targeted gene editing in *Nicotiana benthamiana* for glyco-engineering and monoclonal antibody production**. *Plant Biotechnol J* 2016, **14**:533-542.