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

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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.

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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 biologies 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.
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 produce 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 sickle virus VLPs where mutations to increase the stability of 1 out of 4 structural proteins resulted in increased VLP yields [20].

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
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
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.

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