

Plantibodies: applications, advantages and bottlenecks

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Various strategies have been developed to exploit plants as bioreactors for the production of pharmaceutical antibodies, to engineer antibody-mediated pathogen resistance or to alter the plant phenotype by immunomodulation. Recent research developments focus on the fine-tuning of expression systems and the detailed characterisation of recombinant products, including the implications of plant-specific glycosylation.

Meanwhile, the first of these plant-derived antibody products has successfully completed early phase clinical trials.

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Abbreviations

ER	endoplasmic reticulum
Ig	immunoglobulin
rAbs	recombinant antibodies
scFv	single-chain Fv fragment
sIgA	secretory immunoglobulin A

Introduction

Antibodies are bioactive molecules that, owing to their individual and specific binding properties, allow a large diversity of potential applications. These include medical diagnosis and therapy, the sensitive detection and removal of environmental contaminants, control of pathogens, and industrial purification processes. Antibodies provide an invaluable tool in fundamental research, because of their ability to interfere with metabolic processes within an organism.

The concept of using plants as heterologous expression hosts for recombinant antibodies (plantibodies) is more than a decade old [1]. The combination of antibody and plant engineering, two rapidly advancing technologies, has resulted in the expression of a diversity of molecular forms in different plant species [2].

As we move closer to specific applications involving recombinant antibodies (rAbs), the focus of recent research activity has shifted towards strategies and decision making for achieving well-defined objectives (commercial or otherwise) involving specific forms of rAbs. Major targets include the improvement and comparison of different expression systems in terms of efficacy and feasibility. These encompass an assessment of the quality of the product and the use of antibody molecules with improved characteristics (e.g. fusion proteins with enhanced or novel functions).

Contemporary applications in agronomic research include immunomodulation of physiological processes and engineering of antibody-mediated resistance to pathogen infection. The most advanced application, however, is the utilisation of plants as bioreactors to produce antibodies required for medical use or industrial processes.

In this review, we concentrate on recent advances in expression technology and highlight emerging applications and constraints in the biopharming of plantibodies.

Advances in transformation and expression technology

Both *Agrobacterium*-mediated transformation and particle bombardment have been used to introduce antibody genes into plants [3]. Particle bombardment allows the simultaneous introduction of multiple constructs, thereby expediting the recovery of transgenic lines expressing multimeric antibodies such as secretory immunoglobulin A (sIgA) [4•].

The recombinant protein can be deposited throughout the plant or in specific organs. The deposition and storage of antibody molecules in seeds of various crop plants has been demonstrated [3,5,6]. As a recent example, high accumulation of a single-chain Fv fragment (scFv) antibody in pea seeds was reported using the seed-specific USP promoter [7]. The high stability of an scFv antibody was again confirmed in tobacco seeds over a period of 1.5 years [8].

Plant cell or organ culture in bioreactors is more expensive than agricultural production, but offers advantages as rAbs can be produced in containment and under controlled conditions. Recently, expression of a murine immunoglobulin (Ig) G1 in hairy root cultures [9–11], leading to secretion of the rAb into the medium, was reported.

An alternative to nuclear gene transfer is the transformation of organelles. Recent advances in chloroplast transformation methodology resulted in the plastidial expression of a multimeric vaccine [12]. Similarly, an rAb has also been expressed in chloroplasts [13]. Expression of recombinant proteins in the chloroplast genome has some advantages compared with nuclear gene transfer (e.g. high levels of expression and containment).

Transient expression systems involving viral vectors [14–16] or agroinfiltration [17,18] are effective means for obtaining moderate quantities of recombinant product within a very short time frame (a few weeks). Such systems may prove to have advantages compared with routine small-scale bacterial expression systems for obtaining correctly folded, soluble proteins.

Table 1**Examples of pharmaceutical antibodies produced in transgenic plants.**

Antigen	Plant	Antibody form	Application	Company/reference
Streptococcus surface antigen SAI/II	Tobacco	slgA/G (CaroRX)	Therapeutic (topical)	Planet Biotechnology, CA [4*,34]
Herpes simplex virus	Soybean, rice	IgG	Therapeutic (topical)	EPiocyte [35,51]
Non-Hodgkins lymphoma idiotypes	Tobacco, virus vector	scFv	Vaccine	Large Scale Biology Corporation, CA [14]
Human IgG	Alfalfa	IgG	Diagnostic	[52]
CEA	Tobacco, rice wheat, pea, tomato	scFv	Therapeutic/diagnostic	[17,38]
CEA	Tobacco (transient and stable)	Diabody	Therapeutic/diagnostic	[18]
Respiratory syncytial virus,	Corn	IgG	Therapeutic (inhaled)	EPiocyte (www.epiocyte.com)
<i>Clostridium difficile</i>	Corn	IgG	Therapeutic (oral)	EPiocyte (www.epiocyte.com)
Sperm	Corn	IgG	Contraceptive (topical)	EPiocyte (www.epiocyte.com)
Various	Corn	IgG	Therapeutic/diagnostic	Integrated Protein Technologies c/o Monsanto, MO [40]
Colon cancer antigen	Tobacco	IgG	Therapeutic/diagnostic	[53]

CEA, carcinoembryonic antigen.

Stability of transgene expression over multiple generations is a prerequisite for any commercial application involving plant-based expression systems. Efforts to understand and eliminate factors responsible for transgene silencing are therefore useful [19]. Homology-based, dosage-dependent post-transcriptional gene silencing has recently been reported in *Arabidopsis thaliana* lines expressing an Fab fragment [20]; however, given the large volume of literature referring to stable antibody production, such problems seem rather sporadic, although interesting.

To direct expression and deposition of a plantibody and/or to increase its accumulation, the use of specific targeting signals is often useful. Most scFv antibodies accumulate to higher levels when expression is targeted to the apoplast or the endoplasmic reticulum (ER), rather than to the cytosol. The ability of some scFv antibodies to accumulate in the cytosol appears to be dependent on their intrinsic properties. An *in vivo* assay based on the yeast two-hybrid system has been developed to evaluate candidate scFv fragments in the reducing intracellular environment of the cytosol [21*], and efforts to identify and engineer stable scFv scaffolds hold great promise [22*]. Accumulation of rAbs in the cytosol is attractive for several intracellular antibody applications, such as engineering viral resistance.

Assembly and post-translational modifications in the ER are important for the synthesis of full-size antibodies and Fab fragments, and these molecular forms must be directed to the secretory pathway. Peeters and colleagues [23*] demonstrated that an Fab fragment was efficiently secreted to the apoplast of roots and leaves of *Arabidopsis*. Results obtained with a hybrid immunoglobulin (IgA/G), however,

suggest that the sorting of complex molecules within the secretory pathway is not only dependent on particular signal sequences, but also on the protein itself [24*]. Tissue-specific differences in protein deposition have been observed with an scFv containing an ER retention signal. This scFv was detected in ER-derived protein bodies and also in protein storage vacuoles of transgenic rice endosperm cells [25]. Association of an assembled IgG to the plasma membrane was demonstrated by the addition of a transmembrane anchor to the heavy chain [26].

Recent applications

Disease resistance and metabolic traits in plants

Applications that rely on modulating antigen levels *in vivo* are dependent on the precise expression and accumulation of antibodies in specific subcellular compartments and specific tissues.

Passive immunisation of plants has been described to reduce infection and symptoms caused by viruses and mollicutes, and significant progress has been made towards engineering resistance against insects [27,28*]. Although antibody-mediated fungal resistance in plants remains to be demonstrated, monoclonal antibodies have been identified that inhibit fungal growth [29].

Immunomodulation is a powerful tool for studying or altering the function of an antigen *in vivo*. To this end, an antigen, which may be an enzyme or metabolite, can either be stabilised or blocked in its action [28*,30]. Physiological and morphological changes were observed *in planta* when an artificial abscisic acid (ABA) sink was created by the production of an ABA-specific scFv in the ER of tobacco and potato plants [30–32].

Plants as bioreactors

Many of the antibodies currently produced in plant-based expression systems are high-value products for pharmaceutical use. Indeed, plants represent cost-effective systems for the large-scale production of pharmaceuticals and provide additional levels of safety compared with mammalian production systems [2,5,33]. For several complex molecular forms including sIgA, plants offer the only commercially viable system for large-scale production [5]. The most advanced product is CaroRX, a dental caries-preventing sIgA produced in tobacco that has already been subjected to clinical studies in humans with favourable results [4[•],34]. Another plantibody that is likely to result in a product for human medical applications is a humanised antibody against herpes simplex virus (HSV) glycoprotein B. This antibody was expressed in soybean and shown to be effective in a model study using mice [35]. More recently, agroinfiltration of tobacco was used to produce a diabody against carcinoembryonic antigen (CEA). Binding of the purified antibody to human colon carcinoma cells was demonstrated *in vitro* [18].

Further examples of pharmaceutical antibodies produced from plants have been extensively reviewed previously [33] and are listed in Table 1. These examples highlight the potential of plant-produced pharmaceutical antibodies for commercial use. Other than applications in human healthcare, plantibodies may prove useful as feed additives [36] or for phytoremediation [37].

Remaining challenges in biopharming

Product quantity and quality

A critical factor determining the economic viability of any production system is the level of product that is accumulated per unit biomass and feasibility of scaling up production. Equally importantly, the quality of the end product in terms of functionality and homogeneity needs to be fully assessed.

The level to which an rAb accumulates in a particular expression system can be enhanced by appropriate regulatory elements in the expression construct, by optimising codon usage, and by enhancing the stability of the antibody. Over the past few years, a range of different plant systems has been developed for use as bioreactors to produce recombinant proteins, including rAbs. The choice of system to use for large-scale production will depend on the efficiency of the expression system *per se* and its suitability for scale-up, storage and downstream processing. Considerations such as the anticipated production scale, the value and use of the product, the geographical production area, the proximity of processing facilities, intellectual property, safety considerations (self-pollination, containment levels) and economic considerations are of relevance in this context [38]. Several companies have chosen seed crops, particularly corn, as a vehicle for the commercial production of pharmaceutical antibodies (Table 1).

To meet the quality requirements for a pharmaceutical product, antibodies need to be functional, homogeneous, stable, non-immunogenic and free from contaminants. Protein degradation will reduce levels of functional product in plant tissues, even if initially the molecules are correctly synthesised and assembled. Apart from lowering the efficiency of the system, the presence of inactive protein fragments, which may be difficult to separate during purification, will compromise the quality of the product. Degradation that occurs during extraction can be minimised by the addition of protein stabilisers and proteinase inhibitors, whereas proteolysis *in planta* represents a more serious challenge [10,11]. It has been proposed that fragmentation of IgG occurs mainly in the apoplast of shoot and root tissues, but also within the cell [11]. Stevens *et al.* [39] recently suggested that proteolytic degradation in leaves is, in part, linked to the natural process of senescence. This suggests that the physiological state of the plant host might have an impact on antibody integrity, and that different plants, tissues and conditions may be more or less suitable for the production of high-quality antibodies.

Purification

Ease of purification is the major cost factor for biopharmaceutical production and influences directly the choice of expression system. In many cases, the initial processing steps of plant material will benefit from technology and equipment commonly used for food processing, whereas the final steps usually consist of standard chromatography procedures. Significant progress has been reported for rAbs expressed in tobacco [4[•]] and corn [40]. Expression in seeds assures excellent storage properties and thus added flexibility in processing management and batch production. The limited range of endogenous proteins in seeds is an advantage in separation and allows the formulation of strategies to minimise native protein extraction [40]. Alternative methods including the use of oleosin- or polymer-fusions to facilitate purification of recombinant proteins have been discussed recently [33] and may also be applicable to antibody molecules.

Glycosylation

Glycosylation of antibodies varies according to the production system [41^{••}]. Differences in the glycosylation patterns of proteins produced in plants and humans have raised much concern regarding the potential immunogenicity of plant-specific complex N-glycans, which are present on the heavy chain of plant-derived antibodies [42]. Although a plantibody injected into mice did not provoke a significant serum immune response [43], differential glycosylation remains a major constraint for applications in human healthcare. A strategy that has been widely adopted in cases where glycosylation-dependent effector functions are not needed, is the removal of relevant peptide recognition sequences for N-glycosylation. Golgi-mediated modifications may be avoided by ER retention of a recombinant antibody via the addition of a C-terminal Lys-Asp-Glu-Leu (KDEL; in single-letter

amino acid code) sequence. In an elegant approach towards the humanisation of plant glycans, human β -1,4-galactosyltransferase was stably expressed in tobacco plants [44**]. Upon crossing these plants with those expressing a murine antibody, a plantibody with partially galactosylated N-glycans was obtained.

The glycosylation profile of endogenous proteins and of a recombinant immunoglobulin in tobacco leaves was also affected by senescence [45*], highlighting the role of physiological factors that may cause subtle qualitative differences.

Timelines and regulatory issues

In terms of timelines for protein production, plants are comparable with animal systems (e.g. goat and chicken): it takes about 20 months to good manufacturing practice (GMP)-quality clinical supply production [6,40]. In corn, the first commercial lot (~1 kg of antibody) is projected to be available 36 months after initiation of gene transfer. From then onwards each generation provides a $100\times$ scale-up [40]. Species such as tobacco, with an extremely high number of seeds per plant, may allow an even faster scale-up; however, other constraints imposed by the use of a system rich in toxic secondary metabolites may pose other problems.

The regulatory framework for plant-derived recombinant pharmaceuticals remains to be fully established. GMP regulations will apply, but these may need refinement to make them appropriate and relevant to plant-based expression systems [46]. Although contamination risk with mammalian viral pathogens and prions is no longer an issue, regulatory guidelines unique to plants will have to address the potential presence of herbicide and pesticide residues [47]. Environmental impact is also an issue of some concern and general procedures for labelling and containment need to be established to preclude the unintended entry of transgenic crops expressing recombinant pharmaceutical proteins into the food chain. Strategies for containment include, amongst others, counter-selectable markers, use of self-pollinating crops, and male sterility [48,49].

Conclusions

From a technical viewpoint, the production of a wide range of antibodies in plants is now feasible. Engineering of increased pathogen resistance and alteration of phenotypes by immunomodulation have been demonstrated. Furthermore, the importance of antibodies as an *in vitro* research tool has been extended to *in vivo* applications in functional studies of proteins and other compounds. Various strategies have been developed to exploit plants as bioreactors for the production of pharmaceutical antibodies; recent developments focus on the detailed characterisation of recombinant products, including the implications of glycosylation. Recent indications that tissue-specific and physiological factors may have an impact on the quality and glycosylation pattern of a plantibody will perhaps lead to new insights and production strategies.

Although significant progress has been reported, purification remains the most significant cost factor for biopharmaceutical production. In this respect it may not just be coincidence that the two products closest to market at this point, CaroRX and genital herpes antiviral formulation, are designed for ectopic application.

Although some plant-derived antibody products have successfully completed early phase clinical trials, several issues including regulatory guidelines and public acceptance must still be resolved.

Currently, more than 200 novel antibody-based potential products are in clinical trials worldwide, and market demand will certainly strain the capabilities of existing production systems. Plants are highly competitive in terms of productivity, safety, cost and timelines [50*]. Consequently, they represent a viable alternative to mammalian and prokaryotic expression systems. Easy scale-up of production is a major advantage of transgenic plant systems, thus in terms of cost-effectiveness the full potential of plants may be realised best at higher production requirements. Long-term targets for plant bioreactors may therefore encompass high-volume, low-cost antibodies, which do not require extensive purification.

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