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Manufacturing antibodies in the plant cell

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Keywords:	Molecular farming, Plant-made antibodies, Plantibodies, sIgA, Agroinfiltration

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Manufacturing antibodies in the plant cell

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Abbreviations:

BiP, Immunoglobulin Heavy Chain Binding Protein; **CHO**, Chinese Hamster Ovary; **CDR**, Complementarity-Determining Regions; **ER**, Endoplasmic Reticulum; **UPR**, Unfolded Protein Response; **ERAD**, Endoplasmic Reticulum Associated Protein Degradation; **TMV**, Tobacco Mosaic Virus; **TSP**, Total Soluble Protein;

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Abstract:

Plants have long been considered advantageous platforms for large-scale production of antibodies due to their low cost, scalability, and the low chances of pathogen contamination. Much effort has therefore been devoted to efficiently produce mAbs (from nanobodies to secretory antibodies) in plant cells. Several technical difficulties have been encountered and are being coped with. Improvements in production levels have been achieved by manipulation of gene expression and, more efficiently, of cell targeting and protein folding and assembly. Differences in mAb glycosylation patterns between animal and plant cells are being successfully addressed by the elimination and introduction of the appropriate enzyme activities in plant cells. Another relevant battlefield is the dichotomy between production capacity and speed. Classically, stably transformed plant lines have been proposed for large scale mAb production, whereas the use of transient expression systems has always provided production speed at the cost of scalability. However, recent advances in transient expression techniques have brought impressive yield improvements, turning speed and scalability highly compatible assets. In the era of personalized medicines, the combination of yield and speed, and the advances in glyco-engineering have made the plant cell a serious contender in the field of recombinant antibody production.

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1 Introduction

Recombinant gene technology is called to be one of the main ways to respond to the increasing manufacturing needs for novel molecular entities. Since the production of human insulin in *E. coli*, more than one hundred recombinant therapeutic drugs have been approved for commercialization and a tremendous amount of recombinant proteins are being used in diagnosis or research [1]. Fermentation in prokaryotes and yeast accounts for the majority of recombinant products; however, the increasing complexity of products is promoting the use of eukaryotes other than yeast as production systems. Such is the case of Chinese Hamster Ovary (CHO) cells, baculovirus-infected insect cells, and transgenic/transplactomic plants or plant cells. All these platforms have dramatically improved their performance in recent years to cope with to the expected shortage of recombinant protein manufacturing capacity [2, 3]. For instance, the modifications introduced in prokaryotic systems have made them increasingly able to produce and display complex proteins [4], or have facilitated the introduction of whole metabolic pathways [5]; similarly, yeast strains have been engineered to display nearly-human glycosylation profiles [6]; CHO cells have multiplied their yields, reaching g/L levels [7]; alternative animal systems as chicken eggs have arisen [8], and baculovirus-based systems have been adapted to the production of multi-subunit protein complexes [9].

Monoclonal antibodies (mAbs) have attracted much of the effort in the field of recombinant protein technology. mAbs combine two types of bioactivities in the same molecule: the high binding specificity of their variable region, and the well-defined structure and effector functions of their constant regions. In addition, bioengineers have taken full advantage of this "natural" structure for the design of an array of antibody-

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related structures. As a consequence, recombinant antibodies and their manmade derivatives have become a sort of magic bullet of modern biotechnology [10]. They are widely used as diagnostic reagents, both in health and research, but also more recently as therapeutic drugs, particularly for cancer treatments, where they have shown high efficacy [11].

Human recombinant mAbs have entered the anti-tumor therapy mainstream through several technological waves. Initially, when just murine hybridoma technology was available the only mAbs were of mice origin. Therapeutic antibodies were then "humanized" by grafting the murine regions into human IgG constant frames. "Humanization" of murine mAbs was further improved by exchanging mouse scaffolds in the Complementarity-Determining Regions (CDR) for sequences of human origin. Finally, when phage display technology was developed for the screening of antibody-binding regions, human antibody libraries became available that allowed reconstitution of fully-human recombinant antibodies for therapy [11, 12].

Manufacturing technology was developing in parallel with display technologies. Initially there was not a definitive platform for the production of recombinant antibodies. Unlike single chain (scFv) antibody fragments, which could be produced as periplasmic proteins in bacterial fermentors, the more complex full-size recombinant antibodies required eukaryotic cells as biofactories. Plants, among others, were postulated from the beginning as a suitable production platform [13]. Transgenic plants' technology was however too slow and rudimentary to compete, and CHO cells finally became the standard for the antibody industry. To this date, all commercial therapeutic antibodies are produced in CHO cell fermentors. However monopoly of CHO cells is likely bound to end in the next few years, as laboratories and private companies demand new capabilities, such as speed, scalability and freedom to operate.

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It is not surprising that plants were initially envisaged by some as the most promising system for recombinant protein –and particularly antibody– production. Plant-made antibodies offer several advantages, since they are (i) cheap, as plant cultivation guarantees low cost of goods; (ii) abundant, due to the enormous scaling up capacity of agronomical practices; and (iii) safe, since their evolutionary distance to humans reduces the chances of contamination with human pathogens (e.g. retrovirus, prions) (see also [14-17] for recent reviews on this issue)

Here we review the recent advances that have revitalized the field of plant-made antibody production, focusing in those modifications –both in the antibody and in the plant– that might facilitate the manufacturing of mAbs in the plant cell.

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2 Different formats for different purposes: from nanobodies to secretory IgA

Recombinant antibody-based molecular designs range from those most faithfully imitating nature, as IgG, to those aimed at maximum simplicity, as nanobodies, or those more complex or innovative designs, as immune-complexes or multivalent structures. Each design, having its particular biosynthetic requirements (i.e. folding, secretion, glycosylation), may suit for certain production platforms and not others. Here we review those antibody formats that have been produced in plants and their adequacy to the plant production systems.

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2.1 Full size antibodies

Human IgG (Fig. 1) is probably the most wanted mAb isotype for plant production, because of its widespread use as an anti-tumor agent. In addition, downstream purification methods for IgG are well established and protein A affinity purification is a recognized safe standard for posterior therapeutic applications. Full size antibodies in

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stably transformed plants are normally produced using dual gene constructs encoding the heavy (H) and light (L) chains, each of them under the control of a promoter and a transcriptional terminator. Human immunoglobulin native signal peptides are well recognized in the plant cell, although certain authors prefer the use of plant native signal peptides [18]. Both expression cassettes are often combined within the same binary vector frame (also referred to as "co-transformation in cis"). The goal is to provide coordinated expression of both antibody chains, which has been often considered a pre-requisite for stable IgG expression [19]. These complex constructs are often difficult to engineer, extending the antibody development process. Alternative methods are "sequential co-transformation", using different selection markers, and "co-transformation in trans", with H and L chains (and any additional polypeptide) inserted in different expression cassettes and transformed simultaneously using a single selection marker. This latter approach has been reported for cereals in combination with biolistics [20], yielding high levels of co-transformation. The use of biolistics facilitates construct manipulation due to the elimination of the requirement for large binary vectors used in *Agrobacterium*-based transformation. In consequence, the development phase for recombinant mAb production is accelerated. Nevertheless, form the pionner work of Hiatt et al [13] to the most recent production of anti-HIV prophylactic IgG in corn [21, 22], the lengthy process of establishing a homozygous transgenic plant line with stable and homogeneous IgG production has been the Achilles' heel of plant-made antibodies. More recently, the use of transient expression systems has contributed to ease these limitations, paying a toll in scalability. The use of transient expression systems for IgG production will be discussed in the section below.

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2.2 Antibody fragments

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The observation that H chains retain some antigen-binding capacity even in the absence of L chains led to attempts to develop an even smaller antigen-binding unit in a miniaturized format. The smallest format found to keep full binding activity was the so-called single-chain fragment (scFv), in which the two variable regions, VH and VL, are artificially linked by a flexible polypeptide (Fig. 1). There are several examples in the literature showing scFv expression in different plant species (tobacco, tomato, pea) and plant organs (leaves, fruits, seeds) [14]. The reported production levels are also highly variable: in general scFvs are better expressed as ER-retained proteins, from almost undetectable levels to up to 6% TSP [23].

By serendipity, it was discovered that part of the humoral immune response of camels and llamas is based largely on H-chain antibodies where the L chain is missing. These unique antibody isotypes interact with the antigen through a single variable domain, referred to as VHH [24] (Fig. 1). Single domain antibodies have been produced in plants for immunomodulation of enzyme activity [25]. Plants may also be used as production platforms of single domain antibodies, although in certain circumstances VHHs may result toxic when produced in leaves at high levels (Orzaez et al, unpublished results).

It has to be kept in mind that scFv and VHH do not require glycosylation and can be assembled in prokaryotic systems like *E. coli* fermentors, which constitute therefore a strong competitor for plants. For the same reason, transplastomic plants are also suitable for the production of recombinant antibody fragments [17].

In the production of miniaturized antibodies, plants offer selective advantages compared to other platforms: the almost unlimited scaling up capacity and their use for food and animal feed makes plants an ideal platform for the development of veterinary vaccines [26]. This was the case for the pea seed production platform for anti-

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coccidiosis antibody fragments once pursued by german-based company NovoPlant as a prophylactic device agent against chicken coccidiosis [27, 28]. This and similar approaches may still be considered as highly convenient once the negative European public perception on biofarming eases its halt in the development of local companies specialized in antibody production.

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2.3 Innovative antibody-based structures

A number of plant-made antibody-based structures has been proposed for improving stability, downstream processing or immune functions. Taking advantage of the stability conferred by IgG constant regions during expression in plants, chimeric molecules containing an antigenic (HIV-1 p24) region fused to Cγ1-2 regions of IgG H chain have been shown to stabilize antigen production [29]. In another example, antibody fusion with elastin like proteins was shown to increase anti-HIV mAb yields and to facilitate downstream processing, without affecting neither their properties nor their glycosylation patterns [30, 31]. Particularly challenging structures for plant expression are recombinant immune complexes [32], which consist of complex arrangements where a certain antibody is fused against its own antigen creating a structure with potential immunomodulating activity. It is foreseeable that, as we learn more about immunological concepts like adjuvancy, immunomodulation and tolerance, newly designed antibody-based molecular structures with improved biological activity are likely to appear that will require a suitable platform (i.e plants) for large scale production.

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2.4 Secretory antibodies

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A particularly interesting antibody isotype for plant-base production is the secretory immunoglobulin A (sIgA). In mammals, sIgA is the most important antibody isotype for mucosal protection. SIgA is formed in two stages (Fig. 2): First, B-lymphocytes produce dimeric IgA molecules in the lamina propria. Dimerization is regulated by the J-chain. Later, IgA dimers (dIgAs) bind to the polymeric immunoglobulin receptor (pIgR) at the basolateral surface of mucosal epithelium cells and are subsequently transcytosed to the intestinal lumen. Here the extracellular portion of pIgR, known as secretory component (SC), is proteolytically cleaved and remains attached to the IgA polymer rendering the heteromultimeric structure known as sIgA. This antibody isotype constitutes a highly stable complex that covers mucosal surfaces, acting as a first barrier against infection [33].

Bulk production of sIgA for passive immunotherapy requires efficient, low cost manufacturing systems. Ma and co-workers demonstrated that plant cells can assemble mammalian antibodies in a secretory form: tobacco plants transformed with all four genes encoding chimeric sIgA/G produced fully functional secretory antibodies [34]. The secretory version of this antibody accumulated at high levels in the plant, retained specificity against *Streptococcus mutans,* and conferred protection against dental caries [35]. In fact, a milestone for plant molecular farming was the first EU approval for the commercialization of a plant-made recombinant product, which was awarded to California based company Planet-Biotech for the production of sIgG/A as a medical device for human health. Human sIgA has also been assembled in rice and corn endosperm [20, 36] and recombinant bird sIgA complexes against *Eimera* have been transiently assembled in tobacco plants [37]. sIgA has a great potential as a prophylactic molecule for the protection against pathogens that infect mucosal surfaces. In this sense, edible plant organs seem specially suited for recombinant sIgAs: they can be produced

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in large amounts as required for passive prophylactic agents; moreover, partially purified, mucosal compatible plant-derived formulations containing sIgA could become an affordable vehicle for treatment and/or prevention of infection in oral or topical routes [38].

3. Potential molecular targets for the activation of antibody production in the plant cell

How to make a plant cell resemble a lymphocyte in terms of antibody production is a challenging biotechnology work. A close look at how mAbs are made in natural mAb biofactories, the mammalian plasma cells, can give clues on how to improve current technology in plants.

Lymphocytes differentiate into plasma cells upon co-activation by antigen and helper T-cells. The hallmarks of this differentiation are a cell division arrest, size increase and the expansion of the endomembrane system that ensures high levels of protein secretion. In agreement with the expansion of the secretion machinery, differentiating plasma cells up-regulate their levels of ER chaperones and other ER-resident enzymes as BiP and Disulfide Isomerase. This phenomenon resembles the Unfolded Protein Response (UPR), a conserved mechanism activated in response to ER stress [39]. In the current model for UPR, three proteins act as proximal transducers: the Inositol-Requiring Enzyme 1 (IRE1), the Activating Transcription Factor 6 (ATF6) and the Protein kinase-like Endoplasmic Reticulum Kinase (PERK). All three elements are expressed constitutively and bound to BiP in basal conditions. Their release from BiP, either due to stress or as a response of a developmental program, unleashes a transcriptional response mediated by the X-box Binding Protein 1 (XBP1) transcription

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factor that results in the activation of several chaperones, but also apoptosis if certain injury threshold is reached [40].

The understanding of the molecular mechanisms for plasma cell differentiation has opened the way for the rational engineering of artificial cells through the genetic up-regulation of a complete pathway instead of discrete genes. This was recently accomplished by overexpression of truncated version of XBP1 in CHO cells, which successfully induced a constitutive UPR that turned out in a significant increase in mAb production [41].

Is there a lesson to be learned from plasma cell differentiation in plant-made antibodies? Could plant mAb production be enhanced by activation of UPR? A closer look at the way the plant cell endomembrane system copes with an increase in the demands for protein secretion could give some clues on how this could be accomplished.

There are at least two circumstances in which plant cells activate their endomembrane system in a way that resembles plasma cell differentiation: during seed development and in response to pathogen attack. In both situations plant cells activate a developmental program that is equivalent to the yeast and mammalian UPR, and can be experimentally induced by the treatment with ER-stress inducers such as tunicamycin, an inhibitor of N-glycosylation [42, 43].

During the establishment of Systemic Acquired Resistance (SAR) in response to pathogen attack, Pathogenesis Response (PR) proteins are produced and sent to the vacuole and apoplast in high amounts. NPR1, a well-known master regulator of SAR, has also been shown to control the expression of protein secretory pathway genes [44], unveiling an interesting link between SAR and plant UPR. Indeed a certain parallelism can be drawn between lymphocyte activation in the vertebrate immune system and the

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activation of SAR in the plant immune system: in vertebrates, the presence of non-self peptides or pathogen antigens indirectly activates, with the concurrence of T-helper cells and cytokines, the differentiation of plasma cells and/or the activation of cytotoxic T cells, natural secretory factories of antibodies. In a similar fashion, plant cells which are distant from the site of infection undergo the activation of a defense response by diffusible compounds like salicylic acid.

Once the link between UPR and SAR is established, it is interesting to look for plant regulators of UPR as potential candidates for activating the secretory pathway in plants. IRE1 homologues have been identified in plants; however their involvement in plant UPR has not been established. Although plants lack clear orthologues of XBP1 or ATF6, the promoters of ER chaperones, such as BiP, do contain regulatory elements responsible for ER-stress response. In *Arabidopsis*, at least two bZIP transcription factors, bZIP60 and bZIP28, were found to regulate ER-stress [45]. Interestingly, bZIP60 is translocated to the nucleus in a truncated form, similar to the activation mechanism of mammalian ATF6 [46]. In agreement with a model that unifies ER-stress response and developmentally activated secretion, the cleaved form of bZIP60 was seen to accumulate in anther tapetal cells, which are known to secrete large amounts of proteins and lipids and whose ER is hypertrophied, as observed in ultrastructural analysis. These results suggest that bZIP60 and bZIP28 could be potential candidates for manipulating secretory functions in plant cells.

The second physiological situation under which plant cells resembles plasma cell differentiation is during seed development. In this case, plant cells activate storage protein production in ER-derived protein bodies or protein storage vacuoles. Among other changes, the preparation for active protein production includes an up-regulation of BiP and other chaperones. This activation of the ER may be interpreted as a

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developmentally-programmed event rather than a stress response triggered by cargo proteins, since it precedes the actual up-regulation of storage proteins. In maize, the activation of storage protein biosynthesis during seed development is activated by opaque-2, also a bZIP transcription factor. Opaque-2 binds the promoter region of storage zein genes, and up-regulates a number of enzymes involved in nitrogen and carbon metabolism, favoring the new role of endosperm cells as "biofactory" [47, 48].

The influence of mAb production upon the plant endomembrane system has not been studied in detail. Nevertheless, all the observations indicate that plant-made antibodies recruit plant chaperones in an analogous way as they do in animal cells. The presence of immunoglobulin chains in plants is associated with an increase in BiP and PDI expression, and plant BiP has been shown to mediate folding of IgG. BiP binds preferentially unfolded H chains which are then displaced by the presence of L chains [49]. These indications suggest that the activation of the secretory functions in the plant biofactories could positively influence mAb production. However the experience obtained from mammalian systems anticipates the requirement for coordinated upregulation of all downstream processing events including folding and assembly steps. Besides, it may not be such a straightforward strategy: while BiP overexpression seems to help to cope with drought stress in soybean [50], it has been reported to have detrimental effects on the accumulation of a recombinant T-cell pollen allergen in rice [51]. A more systemic approach for the activation of plant secretion, perhaps involving regulatory elements shared with plant UPR may help in boosting antibody production.

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5 Two edges sword: delivery versus disposal

Although extracellular secretion is the "natural" route for antibody production, it is not unusual to see mAbs accumulating in intracellular compartments such us the vacuole or

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the ER. Interestingly, these are the compartments naturally used by plants cells (e.g. seed endosperm) to store proteins in the form of ER-derived protein bodies (PBs) and protein storage vacuoles (PSV). This rises the possibility to engineer these compartments as final destinations for plant-made mAbs. An interesting insight on this direction was provided by comparing the accumulation in tobacco leaves of anti-caries Guy´s 13 antibody in two isotypes: IgG and a sIgG/A hybrid form. Murine IgG antibody, which was expressed in tobacco leaves at reasonable 1% TSP levels was efficiently secreted to the apoplast. Surprisingly, sIgA/G complex accumulated at levels up to 8% TSP. Although the differences in accumulation levels were initially attributed to the stability provided by SC, it was later observed that it was the accumulation in the vacuole and ER rather than the apoplast what could account in part for the higher level of sIgG/A production. Mistargeting to the vacuole was found to be provoked by a criptic vacuolar sorting signal located at the tailpiece of IgG/A constant region [52, 53]. Thus, intracellular targeting and accumulation could be regarded as a potential mechanism to increase mAb productivity in plants. Unfortunately intracellular retention was accompanied by discrete degradation of IgG/A probably as a result of proteolytic activity in the vegetative (lytic) vacuole.

One of the peculiar characteristics of the plant secretory pathway is the presence of vacuoles, multipurpose organelles that often resemble the extracellular milieu in terms of protein trafficking. Recently, it has become established that targeting to the vacuole can follow two different routes. In the classical route, ER cargo is loaded to the Golgi apparatus and from there delivered into the vacuole in the presence of a vacuolar targeting signal. This classical route is followed by many hydrolases delivered to the lytic vacuole, as well as by seed storage proteins delivered to PSVs. Proteins following this route are decorated with complex glycans, a hallmark of their transit through the

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Golgi apparatus. It has been repeatedly observed, however, that many proteins, including recombinant antibodies engineered for secretion, ended up accumulating in the vacuole without the characteristic glycosylation patterns of the Golgi apparatus. This observation suggests the existence of an alternative shortcut to the classical route [54]. The determinants for secretion or vacuolar delivery are not yet fully understood, but the direct route from ER to the lytic vacuole may represent in some cases a sort of quality control mechanism for the disposal of improperly folded proteins. An extreme example of how complicated the subcellular delivery of a recombinant antibody may be is provided by the detailed localization studies of sIgA produced in rice endosperm. Here, subcellular targeting turned out to be dependent on the folded status of the polypeptides, with non-assembled chains accumulating within ER-derived protein bodies, while the assembled antibody accumulated specifically in protein storage vacuoles [20].

This and similar results raise the possibility of designing specific targeting to subcellular compartments, namely vacuole or ER-derived bodies, for the accumulation of recombinant mAbs, following the path of seed storage proteins [55]. Retention in the ER can be easily induced by adding retention signals KDEL or HDEL. In fact, ER-retention has been repeatedly reported to enhance accumulation of recombinant antibodies in plants, most especially in the case of antibody fragments highly dependent on disulfide bridges for proper folding [56]. For certain proteins, ER milieu seems to provide a certain degree of stability, which is lost once the protein is delivered to the Golgi apparatus towards secretion. Another often claimed advantage of mAb retention in the ER is the absence of Golgi-derived complex glycosylation that could potentially cause immunogenicity in target organisms. mAbs free of complex glycans can be also targeted to the vacuole by engineering cleavable membrane anchored fusions to integral

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proteins present in the Golgi-independent pathway to the vacuole, as recently proposed [57].

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6 Cut into pieces: stability, proteolysis and subcellular localization

Literature on plant-made mAb is loaded with successful stories that may lead to the conclusion that all antibodies are well expressed in plants. However this is probably an observational selection effect; it is common experience to find large differences in expression levels among different mAbs and, although not often reported, a number of difficult-to-express mAbs yield unexpectedly poor expression levels. These striking differences were often attributed to positional effects of the transgenes. However with the use of transient methodologies (that will be discussed below), where positional effects can be discarded, it has become evident that stability differences among mAb do exist. This effect is even more striking when antibody variable regions are imported from combinatorial libraries (e.g. phage display selection). In an experience where 10 full-size chicken antibodies derived from phage display where transiently expressed in *N. benthamiana* leaves, only six of them showed detectable accumulation levels in western analysis [37]. An explanation for this low success rate is that Ig variable domains selected by phage display (in contrast to hybridoma-selected lines) have never encountered ER-folding and quality control systems and may therefore be inadequately adapted to it. In mammalian cells, improperly folded Ig domains are retained longer in the ER bound to BiP, and are finally translocated to the cytoplasm by the ER-quality control mechanism (ERAD) and ultimately degraded via the proteosome. Plant ERAD-like systems are active in degrading misfolded proteins. Recently it has been demonstrated that allelic forms of barley MLO protein which differ in single amino-acid substitutions are specifically degraded via ERAD in a process that requires proteasome

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function [58]. Interestingly, this situation resembles that of variable antibody chains where idiotype differences are often determined by a few amino-acid changes. Plant ERAD-like mechanism is assumed to play a role in the degradation of improperly folded antibody chains; however this remains to be experimentally established.

Another often misreported problem is the presence of small antibody fragments, probably resulting from discrete proteolytic degradation of HC and LC. mAb fragments are especially difficult to remove by affinity purification and may represent a mayor drawback in the optimization process. Generation of antibody fragments smaller than the intact molecule is a common observation in plant expression systems [19, 59, 60]. A detailed analysis of the antibody fragments produced by a secreted IgG in a root expression system showed that they were not caused by antibody degradation during protein extraction. Two smaller fragments of 50- and 80-kDa were identified as the products of protease activity in the apoplast, whereas two additional fragments of 120- and 135-kDa were most likely the products of proteolytic degradation along the secretory pathway outside the endoplasmic reticulum (ER); the carbohydrate residues of the 135-kDa antibody suggested that this fragment was formed between the ER and Golgi [61]. A deeper understanding of the plant protease activities responsible for antibody degradation is needed to minimize the detrimental effect of proteolysis in plant mAb production.

Another particularly interesting subcellular localization for plant molecular farming is the plastid, particularly as a result of transplastomic approach due to their capacity to produce high yields of recombinant proteins. However plastids, lacking endomembrane system, are unable to synthesize fully assembled, glycosylated antibodies. In an attempt to produce an antibody structure resembling full-size mAb in algae chloroplasts, a single chain IgA-like molecule was engineered consisting of a complete human αHC

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covalently linked to a human LC variable region. The chimeric single chain IgA was adequately folded in the plastid and displayed IgA-like binding activity [62]. However it should be noted that in the absence of glycosylation such antibodies are unable to bind Fc receptors and activate effector functions, and this poses a limitation in the range of potential applications of plastid-derived antibodies.

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7 Antibody decoration: advances in plant glyco-engineering

Antibodies are highly glycosylated in their constant regions. As much as variable regions determine mAb binding activity, glycosylation of constant regions determine many other important antibody features such as resistance to protease degradation, half life in serum and, most importantly for mAb-based immunotherapy, effector's function by binding Fc receptors. Plant-made antibodies are glycosylated following the basic principles of the eukaryotic secretion machinery (Fig. 3). ER-translocated nascent peptides are decorated in certain Asn residues with an oligosaccharide rich in mannose. Later in the process, mannose rich oligosaccharides are trimmed by ER-resident mannosidase and subsequently engaged by N-acetyl-glucosamine transferase, which adds terminal N-acetylglucosamine residues forming a typical biantennary complex that enters the early Golgi . Up to this point, plant glycosylation does not differ from that in animals. Differences however do occur when proteins enter the Golgi apparatus, where the formation of complex N-glycans takes place. In humans, biantennary complexes are specifically decorated with α 1-6 fucose, β 1-4 galactose, and syalic acid. All three residues are absent in plants, which instead decorate with α 1-3 fucose, β 1-2 xylose, and sometimes a terminal structure known as Lewis a (β (1,3)Gal[α(1,4)Fuc]) (see [63-65] for recent reviews on this issue).

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It is arguable to what extent "humanization" of plant-made mAb glycosylation patterns is strictly necessary, or if this will depend on the intended application. The data of possible allergenicity of plant specific glycans is controversial. Although no glycan-specific antibodies were generated in mice when exposed to plant-derived antibodies [66], this effect can not be generalized as it seems to be species-specific [63, 64, 67]. As a precautionary principle, especially for recombinant antibodies indented for systemic use, the current tendency is to imitate human glycosylation in plants. To this end, a two-step design has been followed. The first one is the *knock out* of those plant enzymes adding plant-specific residues, namely α 1-3 fucosyl transferase (FT) and β 1-2 xylosyl transferase (XT). ΔFT/XT plants have been engineered either using null mutants or RNAi technology in several species as *Arabidopsis*, tobacco and *Lemma minor*. In this latter system, North Carolina-based company Biolex reported the production of homogenously glycosilated antibodies devoid of plant specific glycans [68]. Nevertheless, the presence of β 1-4 galactose and specially syalic acid as terminal residues in animal glycosylation patterns strongly determines the half life of the antibody in the serum, and therefore the engineering of plants with human specific transferases is also a desirable step. First attempts to *knock in* β 1-4 galactosyl transferase (GalT) were only partially successful as they rendered very heterogeneous decorations [69] [70], but the result was much better when GalT was targeted to the late Golgi. The crossing of GalT *knocked in* plants with ΔFT/XT mutants generated a tobacco line with a humanized glycosylation pattern. These plants were used, in combination with Icon´s magnifection system (see below), for the production of highly homogeneous humanized antibodies [71]. Interestingly, antibodies produced in ΔFT/ΔXT/GalT plants were more effective than their CHO-made counterparts in neutralizing HIV infection, apparently due to their homogeneous glycosylation pattern

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that resembles that of human circulating IgG. This provides an example of how the tuning of N-glycans in plants can in some cases improve the therapeutic value of mAbs.

The only human modification that remains to be achieved in plants is the addition of terminal syalic residues. However this requires the introduction of a whole metabolic pathway in plants to synthesize, activate, transport and translocate N-acetylneuraminic acid into the late Golgi. This has been already accomplished with baculovirus-infected insect cells [72] and, despite the technical difficulties, the first steps in this direction have been also taken in plants [73] a proof of the raising interest in the use of plants as antibody production platforms.

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8 Little and fast or lots and slow: transient versus stable transformation technologies

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Scalability was traditionally considered a main advantage of plant platforms. Unexpectedly, some of the most promising plant-based expression systems currently propose speed rather than scalability as their main advantage. Initially, transient expression technologies were based solely on recombinant viral vectors, which spread recombinant protein production as they infect the plant (see [74] for a review on viral expression systems). However, the relatively big size of the antibody chains imposes a handicap in viral replication and movement, and clonal exclusion precludes the co-infection in a single cell with two viral vectors containing heavy and light chains. Particularly interesting was the work made with Tobacco Mosaic Virus (TMV) in Large Scale Biology Corporation (LSB). This company developed a rapid TMV-based expression system for the production of anti-idiotypic scFv antibodies against non-Hodgkin's lymphoma [75] LSB even successfully began clinical trials, but failed to

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maintain itself in the market. In a certain way, LSC anticipated the current boom of transient expression technologies in plants.

Most current transient technologies are based on *Agrobaterium tumefaciens* and its high efficiency to transfer DNA to plant cells in leaf tissues and other organs like roots or fruits [76]. With this technique, *Agrobacterium* suspension cultures are infiltrated by pressure into the intercellular space of leaf mesophyll, efficiently transferring its T-DNA to the plant cell nucleus (a technique known as agroinfiltration). Recombinant gene constructs are actively transcribed and translated in a temporal window that begins approximately two days post-inoculation and ceases approximately 3-5 days later. It has been shown that silencing suppressors may burst expression at its maximum and delay the decline in expression, indicating that transient expression is being limited by silencing rather than by T-DNA degradation [77]. One of the most attractive features of agroinfiltration is that the efficiency of co-delivery is very high, allowing easy co-transformation of H- and L-chain constructs for antibody production. Moreover, transgene expression levels obtained by agroinfiltration are in the range of the best stable transformed lines. This provides acceptable production levels that have become the basis of some of the technology employed by companies as Canada-based Medicago Inc [78].

A major breakthrough in transient technologies was developed by Icon Genetics group and their magnifection technique. Magnifection takes the best of two worlds by combining the amplification power of viral vectors with the highly contained co-transformation efficiency of agroinfiltration. This methodology is based on a viral replicon derived from TMV, which is devoid of the capsid protein gene and therefore lacks its capacity to move systemically. The replicative unit was *deconstructed* in two pieces, with one of them designed to harbor a gene of interest. Each viral segment,

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which is flanked by a homologous recombination site, is inserted in a separate expression cassette and introduced in a separate binary vector. Both vectors, together with a third one containing a recombinase, are co-delivered into plant cells using agroinfiltration. The above mentioned high co-tranformation rate of this methodology ensures that a number of cells will become transformed with all three constructs simultaneously. In the event of triple co-transformation, recombinase activity ensures that a complete viral replicon will be *reconstructed* within the cell. The reassembled RNA unit contains, together with the gene of interest, a RNA-dependent-RNA-polymerase, that ensures gene amplification, and a movement protein (MP), which ensures cell-to-cell spreading of the replicon throughout the entire agroinfiltrated leaf [79, 80]. Since RNA viruses do not enter the nucleus in its natural life cycle, Icon's vector was elegantly tailored for its artificial nucleus-to cytoplasm passage by addition of introns and removal of cryptic splicing sites [81]. As a result, its performance was significantly improved reaching levels of up to 40% TSP for certain recombinant proteins.

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Despite its impressive performance, Icon´s magnifection technology faced the same problem as other viral systems for mAb production. Co-infection within a single cell of viral replicons containing H and L chains is a rare event due to clonal exclusion. The problem was solved by introducing a second, non-competing virus in the game. It was known for long in plant virology that viral co-infection may occur provided that both players do not compete for the same cell resources as is the case for Potato Virus X (PVX) and TMV (Fig. 4). Using complementary vectors derived from these viruses, H and L chains were successfully co-expressed in *N. benthamiana* leaves, reaching commercially viable levels of up to 10% TSP [82]. It is noteworthy that the cell secretion machinery is apparently able to cope with this vast amount of newly

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circulating cargo without collapsing, and it is tempting to speculate that UPR-like adaptations discussed above could be taking place in response to the presence of viral proteins in the cell. A profile of PR expression could indicate if a UPR-like reaction is helping the ER to cope with mAb cargo in maginfected leaves. Icon Genetics was acquired by Bayer Innovation and it is currently attempting to bring to the market the concept of personalized vaccines against non-Hodgkin's lymphoma once ventured by LSB.

The use of transient methodologies is gaining pace and new and improved methodologies are continuously emerging. Recently, Sainsbury and Lomonossof reported a strikingly simple methodology based on Cowpea Mosaic Virus (CPMV) [83, 84]. The system, which also makes use of *Agrobacterium* as shuttle system, achieves extremely high levels of protein production without viral replication. It is based on the ability of a mutated version of 5´UTR of CPMV RNA2 to drive high levels of transcription. The absence of viral replication excludes clonal exclusion allowing high levels of co-transformation and reaching IgG expression levels of 10% TSP with a very simplified method. This and other transient rapid methodologies will surely have a role to play in responding to the increasing needs for rapid, tailor-made recombinant proteins.

9. Summary and perspectives

As plant biotechnology develops, the restrictions initially hampering the development of plant-made recombinant proteins are being eased. This has brought a renewed interest of companies and research groups in plant-made antibodies. Transgenic technology has improved during the last 20 years, with many more species being routinely transformed and simpler and more efficient methods in place [85, 86].

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Downstream processing of plant extracts, which was initially neglected by plant biotechnologists, and turned to be a nightmare for the commercialization of the so-called "plantibodies", has been conveniently addressed by several companies, which have substantially improved and scaled up the purification protocols [87].

While some old concerns are gradually being addressed, new opportunities arise from previously unforeseen technologies. Transient expression protocols provide the required speed for many modern manufacturing processes, as those related to custom production for individualized therapies. The full "humanization" of plant glycosylation patterns is underway, and those steps accomplished so far have produced recombinant mAbs with superior neutralizing activities when compared with competing platforms [71, 88]. Finally, as plant recombinant products are gradually available, current restrictions in the regulatory framework may well be relaxed, opening new opportunities. Hopefully, this and other regulatory changes, like the recent clearance for commercialization of partially purified botanical mixtures [89], may pave the way for large scale production of cheap prophylactic products based on plant-made mAbs, one of the big promises of this technology.

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10 Acknowledgements

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11 Figure Legends

Figure 1. Antibody formats manufactured in the plant cell. (A) Schematic representation of a typical human IgG antibody, showing constant heavy (CH1-3), constant light (CL), variable heavy (VH) and variable light (VL) domains. The structure of IgG-derived antibody fragments is also shown. (B) Representation of a camelid antibody comprising only heavy chains but full antigen binding capacity. (C) Comparison of full length antibody with different antibody fragments and fusions expressed in plants. ScFv: single chain antibody fragment; VHH: single chain camelid antibody (nanobody); scFv-Fc single chain full-size antibody proposed for plastid expression; scFv-elastin: single chain-elastin fusions.

Figure 2. Natural biosynthesis of secretory IgA in mucosal surfaces. Polymeric IgA (pIgA) (composed of IgA and J chain) is secreted by plasma cells at the basolateral surface of mucosal epithelium. The Poly-immunoglogulin receptor (pIgR) mediates the transcitosis of IgA throughout the epithelium cell. At the luminal side, pIgR is cleaved releasing pIgA bound to its extracellular portion, the Secretory Component (SC). The final molecular complex, composed of IgA, Jchain and SC is known as secretory IgA (sIgA).

Figure 3. N-Glycosylation patterns in plants and animals. Schematic representation of the formation of typical complex glycans in the Golgi apparatus of animals (upper panel) and plants (lower panel).

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Figure 4. Simplified scheme of magnifection for the production of full-size mAbs. (A) Each chain of the mAb is inserted in a different, compatible viral vector, to allow coinfection of plant cells. Both vectors have been engineered to prevent systemic infection. In this example, the TMV-derived vector expresses the RNA-dependent polymerase (Pol), the movement protein (MP) and the mAb heavy chain (HC) from an actin promoter; and the PVX-derived vector lacks the coat protein but contains the RNA-dependent polymerase (Pol) and the mAb light chain (LC) genes expressed from the 35S promoter. RB and LB are the T-DNA right and left borders, respectively. (B) *Nicotiana* plants are vacuum- -infiltrated with low-density *Agrobacterium* cultures harboring the TMV and PVX vectors, resulting in a high proportion of cells being co-infected with HC (blue) or LC (red)-expressing constructs. Expression of the TMV movement protein allows cell-to-cell movement of TMV and PVX RNAs, thus spreading the co-expression of LC and HC in the same leaf areas (depicted as purple sectors resuting from red and blue color mixes).

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In their natural biofactories, the lymphocytes, antibodies are synthesized through the cell endomembrane system. Antibody synthesis requires coordinated expression, folding and assembly of H and L chains. First, the corresponding mRNAs are matured and translated in the rough ER; then their nascent polypeptides are translocated into the ER and the N-terminal signal peptide is subsequently cleaved; folding is assisted by ER resident chaperones as the Immunoglobulin Heavy Chain Binding Protein (BiP) [37]. Besides, the Endoplasmic Reticulum Associated Protein Degradation (ERAD) quality control mechanism ensures that unfolded antibody chains are removed from the ER and degraded via the proteosome [38]. Finally, antibody chains are linked together by disulfide bonds established by ER-resident protein disulfide isomerase (PDI) [39].

In artificial systems such as CHO cells, several genetic strategies have been followed to increase MAb production. This includes engineering of anti-apoptotic factors to inhibit cell death in high density cultures, an approach that can be directly translated to plant cell suspension systems but possibly not to transgenic plants. A second obvious strategy consists in increasing gene expression by different means, like raising recombinant gene copy number, using more active promoters or targeting recombinant DNA to active transcriptional sites. These approaches, which do have a clear parallel in plants, have been only partially successful. It is generally accepted that MAb yield is not always a reflection of the abundance of its transcript in the cell. Instead, folding and assembly have been considered as major bottlenecks in antibody production. Consequently, the engineering of mammalian cells as artificial factories has often tried to remove folding and assembly bottlenecks, i.e. by upregulating discrete steps in the process, as PDI and BiP. However these discrete approaches have not been particularly successful. Protein

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folding and assembly can be considered as a chain of potential bottlenecks, therefore it is not surprising that removal of a single bottleneck will only marginally alleviate constrains imposed in the system -reviewed by [40].

A more comprehensive approach recently undertaken to improve MAb production in artificial mammalian systems relies in a better understanding of plasma cell differentiation. Plasma cells are natural antibody factories:

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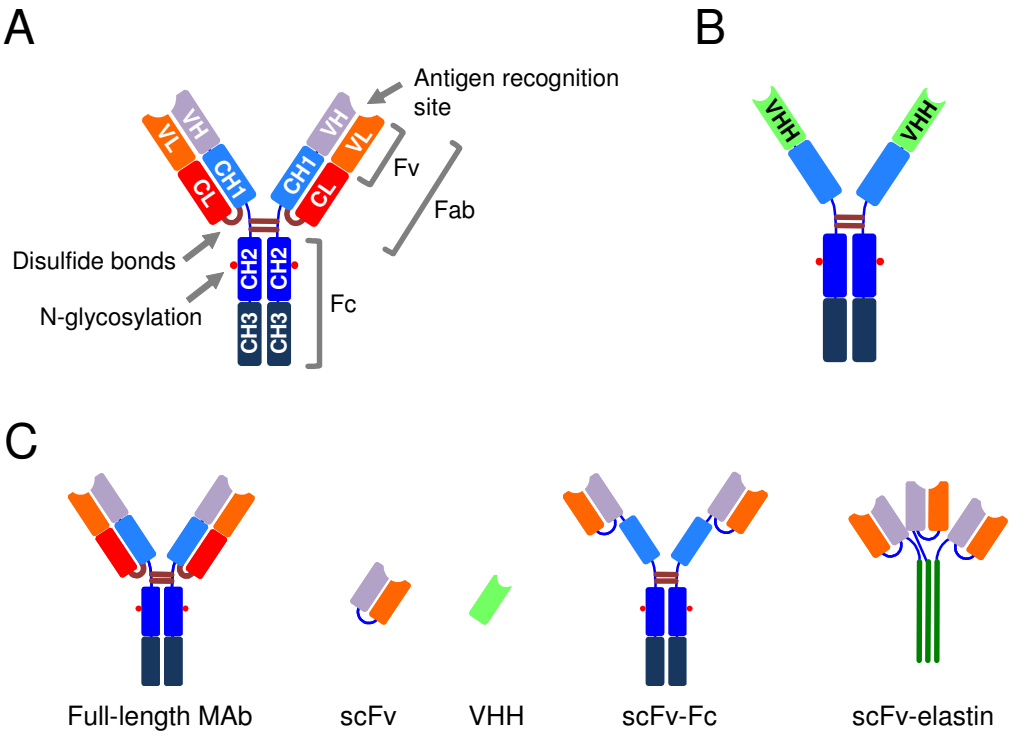
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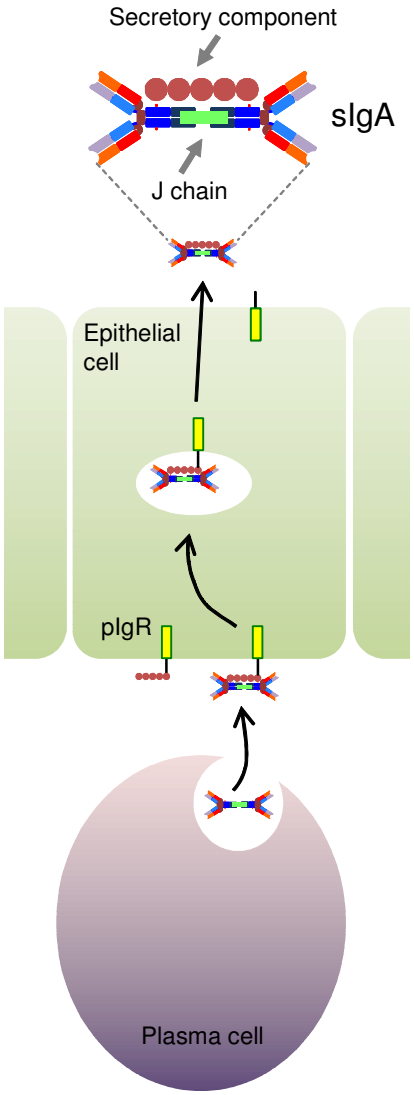
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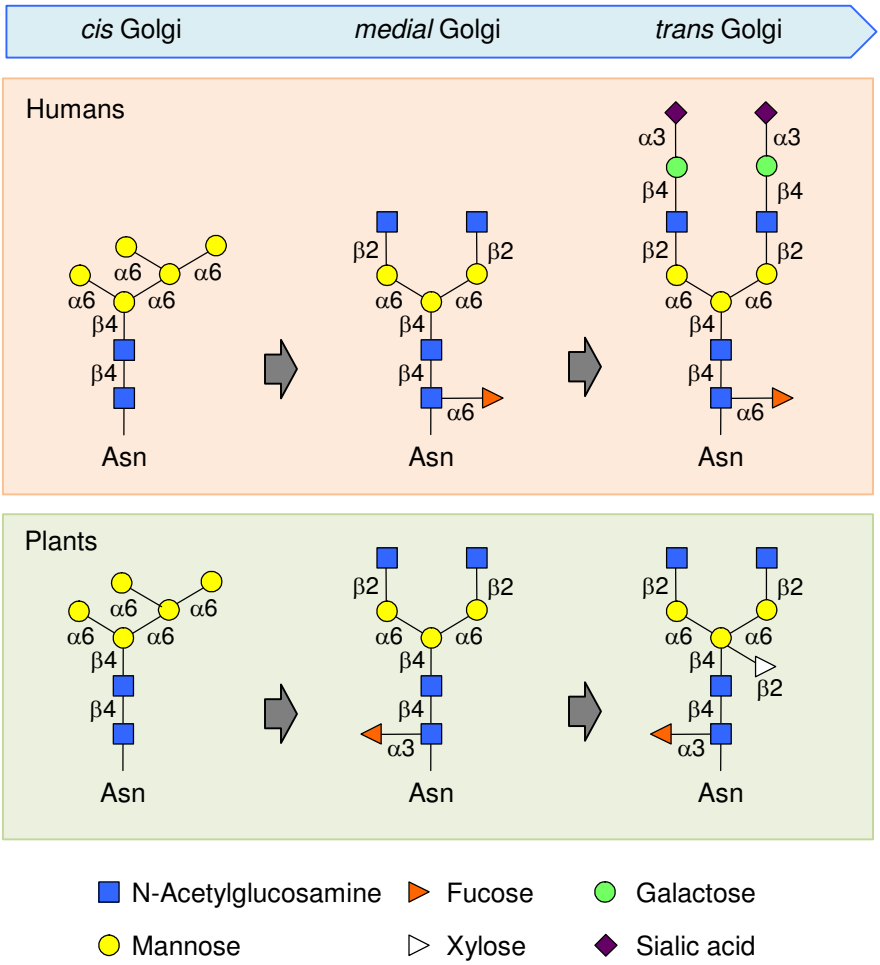
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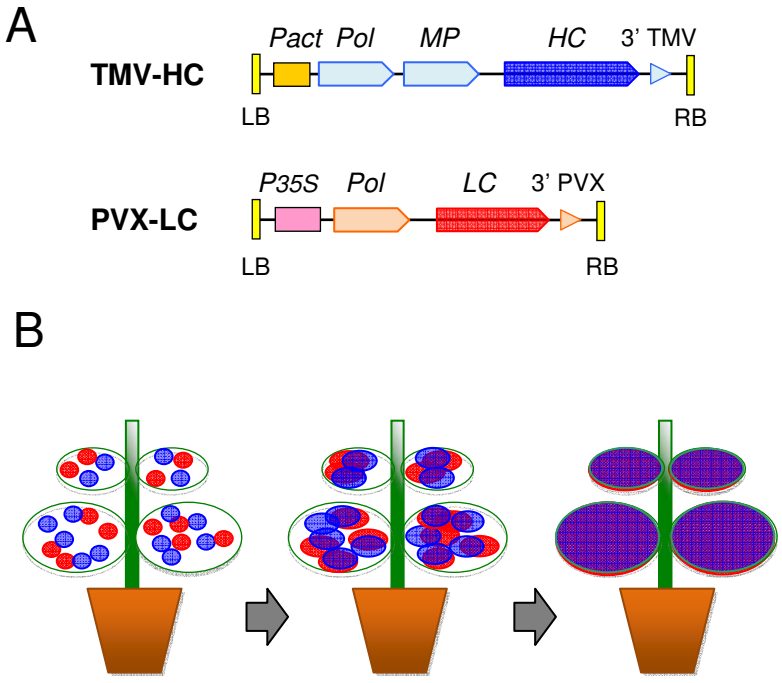
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