

**Title**                    **Antibodies from plants for bionanomaterials**

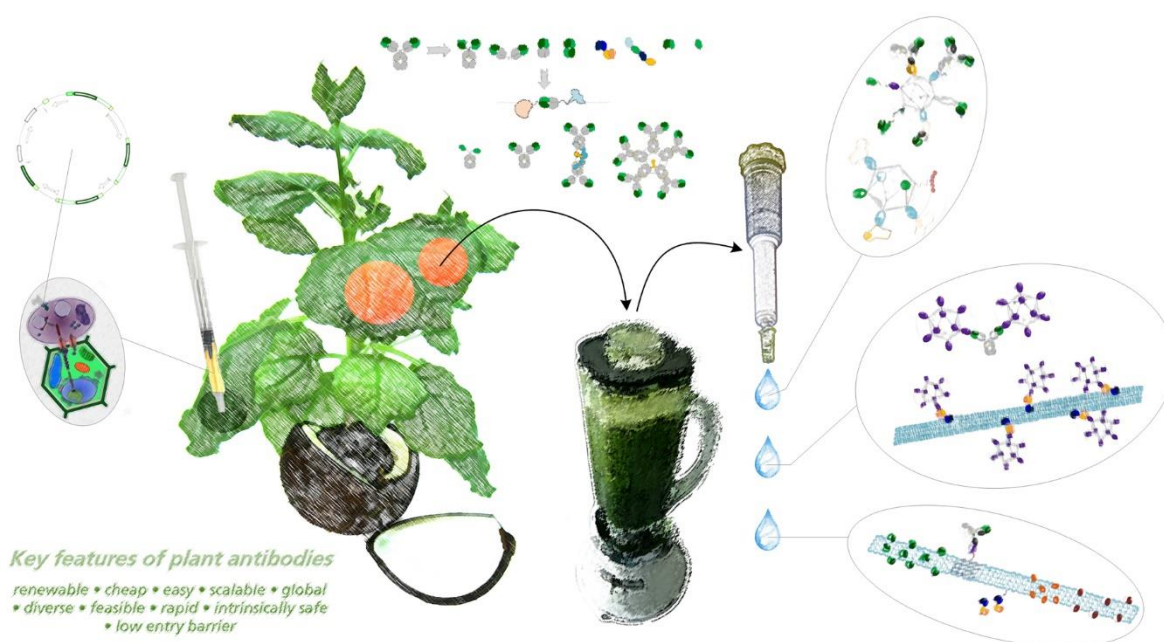
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## Graphical Abstract



## Abstract

Antibodies are produced as part of the vertebrate adaptive immune response and are not naturally made by plants. However antibody DNA sequences can be introduced into plants, and together with laboratory technologies that allow the design of antibodies that recognize any conceivable molecular structure, plants can be used as ‘green factories’ to produce any antibody at all. The advent of plant-based transient expression systems in particular allows the rapid, convenient and safe production of antibodies, ranging from laboratory-scale expression to industrial-scale manufacturing. The key features of plant-based production include safety, speed, low cost and convenience, allowing newcomers to rapidly master the technology and use it to its full advantage. Manufacturing in plants has recently achieved significant milestones and offers more than just an alternative to established microbial and mammalian cell platforms. The use of plants for product development in particular offers the power and flexibility to easily co-express many different genes, allowing the plug-and-play construction of novel bionanomaterials, perfectly complementing existing approaches based on plant virus-like particles. As well as producing single antibodies for applications in medicine, agriculture and industry, plants can be used to produce antibody-based supramolecular structures and scaffolds as a new generation of green bionanomaterials that promise a bright future based on clean and renewable nanotechnology applications.

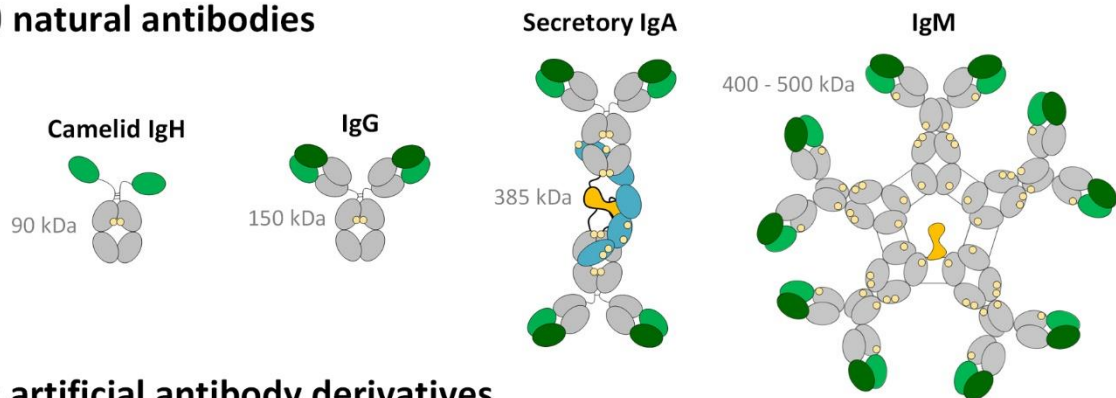
## Introduction

Antibodies are glycoproteins produced by the adaptive immune system in humans and other vertebrates. They recognize and bind particular target antigens with great affinity and specificity, allowing them to clear pathogens and other unwanted material from the body. Although plants do not naturally produce antibodies, they can be engineered to do so by introducing the corresponding immunoglobulin genes.<sup>1,2</sup> In this manner, plants can be instructed to make antibodies that target any antigen of choice, and they can make them efficiently and in all kinds of different formats.<sup>3,4</sup>

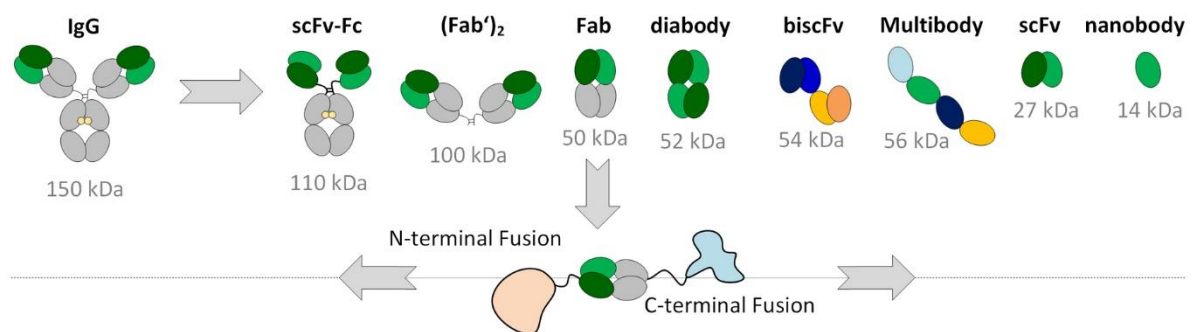
Most people picture antibodies as the typical mammalian serum-type immunoglobulin G (IgG), which comprises two identical heavy chains and two identical light chains joined by disulfide bonds (**Fig. 1**). The chains fold and assemble into a multimeric Y-shaped structure. Below the hinge, in what is known as the Fc region (fragment crystallizable), all IgG molecules are largely the same, and this region is responsible for the general effector functions of antibodies, such as immune cell recognition and complement fixation. This part of the antibody is also glycosylated. The hinge and the region above, where the antibody branches into its characteristic Y-shape, is known as the F(ab')<sub>2</sub> or Fab (fragment for antigen binding) and the distal variable region of the Fab gives antibodies their antigen-specific binding properties. Mammals produce five classes of immunoglobulins with different Fc regions and thus different effector functions (IgG, IgM, IgA, IgD and IgE) and some of these assemble into structures more complex than IgG. For example, the secretory-type IgA (sIgA) comprises four heavy chains and four light chains which assemble as two IgG-like tetramers along with two further components – a secretory component and a joining chain (**Fig. 1**). The expression of a full-size IgG in plants therefore requires two genes, for the heavy and light chains respectively, whereas the expression of a sIgA requires four genes, one for each component. However, the Fc portion of the antibody is not required for antigen binding and engineered antibodies often work better when the effector functions are removed. Smaller antibody derivatives which still require two chains include Fab and F(ab')<sub>2</sub> structures and minibodies (**Fig. 1**), whereas others, including large single chains, single chain variable fragments (scFvs), diabodies and camelid antibodies, require only a single polypeptide and therefore only one gene (**Fig. 1**). The variable domain of the camelid antibody, also known as a single-domain antibody (VHH) or nanobody, is the smallest antibody derivative that remains functional in terms of antigen binding.<sup>5</sup> More specialized derivatives include bispecific scFvs, which bring together the antigen-binding domains of two different antibodies and can therefore bind two different antigens simultaneously, and antibody fusion proteins, which pair the antigen-binding domain of an antibody with a separate domain that has a different function (e.g. a toxin to kill cancer cells).

The ability of antibodies to bind diverse antigens despite their largely conserved structure makes them tremendously useful as research reagents, diagnostics and therapeutics. The molecular mechanisms underlying the enormous diversity of natural antibodies can be mimicked in the laboratory,<sup>6,7</sup> and this together with the well-characterized modular structure of antibodies<sup>8</sup> and the plethora of available structures and structure-function relationships<sup>9,10</sup> have facilitated the development of antibody engineering and tailoring technologies that allow recombinant antibodies to be produced for diverse applications in research, medicine, agriculture and industry.<sup>11,12</sup> The modular structure and functional organization of antibodies is ideal for synthetic biology.<sup>13</sup> This allows the antigen-binding variable regions to be isolated by PCR or synthesized from first principles and placed on standard frameworks.<sup>14</sup> When these technologies are combined with the rapid and scalable production of antibodies in plants, the ingenuity of nature is placed at the disposal of humans to address modern-day challenges. In this sense, plants provide a unique platform for the production of recombinant antibody products.

## A) natural antibodies



## B) artificial antibody derivatives



**Figure 1.** Domain architecture of natural antibodies and some engineered recombinant variants. Domains representing the antigen binding site are indicated by the loops and constant domains by the straight line. The functional and structural separation of the variable domains (antigen binding site) and constant domains (structural arrangement, effector functions) and the free N- and C-terminal ends of the individual domains has given rise to a vast number of variants, derivatives and combinations.

In this review we consider the rationale for producing antibodies in plants, the history of the technology, the major breakthroughs and current applications, and also how antibodies from plants could be developed further into the next generation of nanomaterials. In particular we focus on transient gene expression mediated by *Agrobacterium tumefaciens*, which is by far the most flexible and versatile platform for research and, increasingly, full-scale antibody manufacturing. Key concepts, procedures and methods are outlined to facilitate the adoption of the technology by researchers outside the field. We also consider what is required to translate plant-derived antibodies from the research and technical environment to the next-generation of nanomedicines, which will require novel approaches during development, production according to good manufacturing practice (GMP) and when negotiating the regulatory system for pharmaceuticals and diagnostics.

## Why make antibodies in plants?

Recombinant antibodies have been manufactured very successfully using mammalian cells for more than three decades. So why should we want to make antibodies in plants? To fully grasp the importance of plant-derived antibodies we need to consider what drove the pioneers of this technology. To do this we must consider two different communities of researchers, one working on the production of valuable recombinant proteins in different host species and another working on the protection of plants against pests and diseases. The goal of the first community was to express antibodies as *products* that are isolated from the host, an approach sometimes described as *molecular farming*. Molecular farming in plants was considered by the pioneers as a way to address three major challenges associated with traditional platforms for antibody production based on

microbes and animal cells: safety, scalability and cost.<sup>15–23</sup> At around the same time, the second community realised that although plants do not naturally produce antibodies against their viral, bacterial and fungal pathogens, they could be engineered to express such antibodies if the correct antibody sequences could be developed in the laboratory. Or in other words, plants could be artificially endowed with certain aspects of the vertebrate immune system, and this would help to reduce the use of chemical pesticides and fungicides. The goal of this second community was therefore not to make antibodies as products, but to make pathogen-resistant plants as the products. The antibodies would not be purified from the plants, but would perform their intended function inside the plant.

The concept of expressing antibodies to function inside plants led to the realisation that such antibodies could not only target pathogens, but could potentially be used to target endogenous plant proteins and small molecules, thus allowing a wide range of *in planta* applications such as metabolic engineering and the modulation of signalling pathways. Recombinant antibodies expressed intracellularly to interfere with and modulate cellular processes are sometimes described as intrabodies<sup>24–26</sup> and they can be used as a research tool to inhibit small molecules, block protein-protein interactions, retarget, stabilize or destabilize endogenous proteins, and even to destroy particular cell types. Immunomodulation and protein interference by heterologous antibody expression in plant cells was somewhat overshadowed by the advent and rapid adoption of RNA-based methods such as antisense silencing, virus-induced gene silencing (VIGS) and especially RNA interference (RNAi). However, intrabodies are currently enjoying something of a revival now that many of the technical barriers affecting antibody expression in plants have been overcome, particularly because intrabodies can circumvent some of the weaknesses of RNA-based methods (e.g. the inability to target some genes specifically) and can exert more specific or subtle effects, such as abolishing the activity of proteins in a compartment-specific manner, abolishing the activity of individual protein domains, or the quantitative knock-down of protein activity. The diverse applications of plant-derived antibodies are summarized in **Table 1**.

The versatility of molecular farming in plants has also increased since the first pioneering experiments. The early practitioners faced an environment in which access to antibody genes was rather limited and the cloning and expression of recombinant antibody sequences was slow and tedious. Even in those days, several features of plant-based expression systems were already appealing, particularly the low cost of upstream production coupled with the unlimited scale-up potential of open-field cultivation (**Fig. 2**). These advantages promised to dramatically reduce the cost of biologicals once appropriate downstream processing methods and regulatory pathways were in place. In reality, the reluctance of big industry players to invest in a relatively untested (and unsupported) new technology, and public hostility to the open-field cultivation of GM crops in Europe, has slowed down the fulfilment of these promises. But the development of transient gene expression systems<sup>27–30</sup> has added speed to the panel of advantages offered by molecular farming in plants and the promises seem to be within reach once again.

**Table 1.** Application fields for plant-derived antibodies with examples from the literature.

Application field		Target or structure
Pathogen resistance	Virus	Artichoke mottled crinkle virus <sup>18</sup> , Tobacco mosaic virus <sup>22,69</sup> , Citrus tristeza virus <sup>135</sup> , Tomato spotted wilt tospovirus <sup>53</sup> , Beet necrotic yellow vein virus <sup>136,137</sup> , Grapevine leafroll-associated virus 3 <sup>138</sup> , Tombusviridae <sup>139</sup> , Potato virus Y/D and Clover yellow vein virus <sup>140</sup>
	Bacteria	Stolbur phytoplasma <sup>46</sup>
	Fungi	Fusarium spp. <sup>48,49</sup> , mycotoxin detoxification <sup>141</sup> , Sclerotinia <sup>142</sup> , <i>Botrytis cinerea</i> cutinase <sup>44</sup>
	Herbicide	Chlorpropham <sup>55</sup> , picloram <sup>143</sup> , carbamate <sup>144</sup>
	Nematodes	Nematode stylet secretions <sup>145,146</sup>
Immune modulation	Plant hormones	Absciscic acid <sup>19,147</sup> , phytochrome <sup>17</sup> , gibberellins <sup>148–150</sup> , jasmonic acid <sup>151</sup>
	Secondary metabolites	Solasodine glycosides <sup>152</sup> , plumbagin <sup>153</sup>
	Endogenous proteins	Arabidopsis histidine phosphotransfer protein <sup>154</sup> , heat shock protein <sup>155</sup>
Molecular farming	Therapy and passive immunization	CEA <sup>28,79</sup> , HIV <sup>87,88,156</sup> , Rabies virus <sup>157–159</sup> , <i>Streptococcus mutans</i> <sup>83,160</sup> , Ebola <sup>33</sup> , HSV <sup>70</sup> , ferritin <sup>161</sup> , interleukins <sup>162</sup> , EGFR <sup>163</sup> , CD40 <sup>43</sup> , anthrax toxin <sup>164</sup> , <i>Salmonella enterica</i> lipopolysaccharide <sup>165</sup> , EpCAM <sup>166,167</sup>
	Active immunization	B-cell lymphoma <sup>37,86</sup> , tetanus toxin <sup>168</sup>
	Diagnosis and research	Rhesus D antigen <sup>169</sup> , MAK33 <sup>40,41</sup> , hCG <sup>57</sup>
	Bionanomaterials	Antibody-ELP fusions <sup>170–172</sup> , tandibody <sup>112</sup>
	Affinity sorbent	Hepatitis B virus surface antigen <sup>173–174</sup>

Speed has become a major driving force behind molecular farming for two major reasons. First, transient expression radically shortens the development times for a product candidate, so both the product and process become available for testing, refinement and optimization more rapidly.<sup>31,32</sup> The breeding of transgenic plant lines can take years, whereas transient expression can reduce the gap between gene transfer and protein recovery to a few days. Second, the enormous potential of rapid and scalable transient expression systems has been recognized in the context of emergency medical responses and other time-critical applications, such as antibodies for active and passive immunotherapy. Notable examples include ZMapp, a cocktail of three monoclonal antibodies for the treatment of Ebola virus disease,<sup>33</sup> the use of plants to produce seasonal and emergency influenza vaccines,<sup>34,35</sup> which normally takes more than 6 months to stockpile using the traditional system based on chicken eggs,<sup>36</sup> and the individualized autologous idiotypic cancer vaccines produced for non-Hodgkin's lymphoma patients.<sup>37,38</sup> In addition to the speed, scale and economy, plants also benefit from their intrinsic safety compared to other systems – they do not produce endotoxins like bacteria and they do not support the replication of animal viruses and prions like animal cells. The key features of plants for the production of recombinant antibodies are summarized in **Fig. 2**.

## Key features of plant antibodies in a nutshell

**renewable** – light, water & soil

**cheap** – growing plants is cheap

**easy** – we know how to grow plants

**scalable** – 1 plant → billions of plants

**global** – we grow plants on the entire planet

**diverse** – Many different plants, tissues, and products

**feasible** – The knowledge and technology is available today

**rapid** – from gene to product in a few weeks with transient systems

**intrinsically safe** – animal and human pathogens do not infect plant cells

**low entry barrier** – less requirements for infrastructure, skills and supply logistics



**Figure 2.** Key features and selling points of plant-based systems for making antibodies and other recombinant proteins.

### Major milestones

Because plants do not naturally produce antibodies, it was initially unclear whether typical serum IgG molecules would assemble into functional multimers when expressed in plant cells. In the first experiment of this kind, tobacco plants were separately transformed with genes encoding the heavy and light chains of a catalytic IgG, and once the intact polypeptides had been detected individually the two lines were crossed so that both transgenes were expressed in progeny plants.<sup>15</sup> These plants produced the correctly assembled tetrameric antibody as well as various monomers and dimers. Importantly, the full-size recombinant antibody was shown to bind the correct antigen, demonstrating that the antibody was functional. This first report involved an antibody which bound to a low-molecular-weight hapten (P3 phosphonate ester) and its functionality was confirmed by demonstrating its ability to bind this hapten conjugated to bovine serum albumin. Subsequent reports dealt with antibodies that bound to medically-relevant antigens, and also investigated the expression of different antibody formats. Thus followed publications reporting the first plant-derived diabody, which recognized the neuropeptide substance P,<sup>39</sup> the first medically-relevant IgG and equivalent Fab and scFv derivatives recognizing human creatine kinase<sup>23,40,41</sup> and the remarkable demonstration that plants could also express sIgA antibodies, which as stated above require four separate polypeptide chains.<sup>21</sup> The latter was a significant breakthrough because although other antibody formats had been expressed in microbes and mammalian cell lines, sIgA synthesis requires two separate cell types even when expressed naturally in humans. As in the original description of IgG expression in tobacco,<sup>15</sup> the secretory antibodies were expressed by crossing transgenic tobacco plants expressing each of the individual components – although in this case the stacking of four different genes required two rounds of crossing. Later, both IgG and sIgA were also produced in plants via a single round of transformation with multiple genes.<sup>16,42</sup> Another key milestone was the first expression of an antibody fusion protein in plants, a scFv specific for the cell-surface receptor CD40 fused to the protein toxin bryodin, making this the first example of a plant-derived recombinant immunotoxin.<sup>43</sup>

The experiments described above are all examples of molecular farming, but parallel developments were taking place at the same time concerning antibodies that confer resistance against plant pathogens. Such antibodies are typically directed to accumulate in subcellular compartments that are occupied by the invading pathogen. Again, the field rapidly diversified to cover a range of antibody formats and targets, beginning with the expression of a scFv against *Artichoke mottled crinkle virus* coat protein in *Nicotiana benthamiana*<sup>18</sup> and a full-size IgG against *Botrytis cinerea* cutinase in transgenic tobacco.<sup>44</sup> Some of these early experiments were remarkably successful, with a 70% reduction in local lesion numbers achieved by expressing a full-size IgG against the *Tobacco mosaic virus* coat protein in tobacco<sup>22</sup> and a 90% reduction in local lesion numbers achieved by expressing a scFv targeting the same protein.<sup>45</sup> Early experiments also targeted phytoplasma, which are obligate intracellular bacterial parasites in plants.<sup>46,47</sup> One of the most significant breakthroughs was the first report describing a plant-derived antibody targeting a fungal pathogen rather than one of its virulence products, which was also the first report describing the use of an antibody fusion protein to confer antibody-mediated resistance in plants.<sup>48</sup> The authors mimicked the immunotoxin approach described above, in which the antibody sequence is fused to a toxic peptide, but in this case the peptides were derived from antifungal proteins tested *in vitro*, namely a wheat class I chitinase and antifungal peptides from *Raphanus sativus* and *Aspergillus giganteus*. After testing in *Arabidopsis thaliana*, this approach was shown to confer resistance against the major fungal pathogen *Fusarium asiaticum* in wheat.<sup>49</sup> Other interesting variants included the expression of a single variable domain to confer resistance against *Potato virus Y* in potato<sup>50</sup> and the expression of a scFv-GFP (green fluorescent protein) fusion to simultaneously confer resistance against *Tomato yellow leaf curl virus* and to visualize protein expression, which was also the first report of antibodies against a DNA virus in plants.<sup>51</sup> Although most attempts to confer pathogen resistance have involved the development of transgenic plants, *Potato virus X* has been used for the transient expression of several different scFvs conferring resistance against *Potato virus V*<sup>52</sup> and *Tomato spotted wilt virus*.<sup>53</sup> The versatility of expression strategies thus incorporates viruses as both friend and foe – as the vector used to achieve antibody expression and as the pathogen targeted by that antibody.

In a distinct application, *Potato virus X* has also been used to express antibodies against the enzyme granule-bound starch synthase I in order to modulate starch metabolism.<sup>54</sup> The expression of antibodies in plants can therefore be used not only to confer pathogen resistance but also to modulate the activity of endogenous proteins. In a similar manner, antibodies can also be used for the immunomodulation of plant hormones<sup>17,19</sup> and other small molecules, such as herbicides.<sup>55</sup>

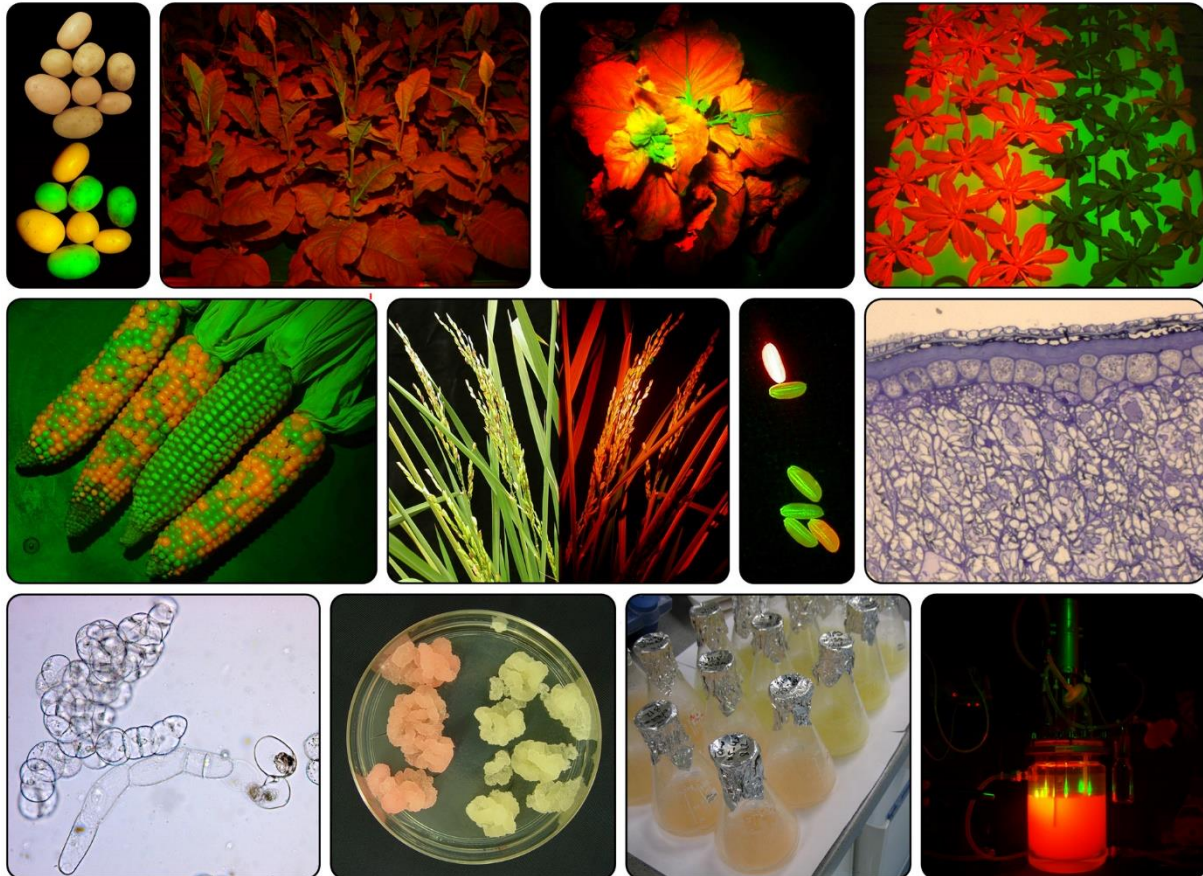
Whereas efforts to confer pathogen resistance usually involve the creation of transgenic plants, the use of plants for molecular farming can explore a wider range of approaches, including different expression methods, production hosts and protein targeting strategies. Around the turn of the millennium, the first medically-relevant antibodies were produced by transient expression in plants. Two different approaches were used, the first involving the infiltration of leaves with *Agrobacterium tumefaciens*.<sup>27</sup> A number of different antibodies and their derivatives were rapidly produced by agroinfiltration, including T84.66 which recognizes carcinoembryonic antigen<sup>28,56</sup> and a chimeric full-size IgG known as PIPP which recognizes human chorionic gonadotropin.<sup>57</sup> In both cases, plants were used to produce the full-size IgG as well as scFv and diabody derivatives. The second approach involves the use of plant viruses as vectors. McCormick et al.<sup>37</sup> were the first to use this system for the expression of antibodies – they used the well-characterized *Tobacco mosaic virus* as a vector to express therapeutic scFvs recognizing the mouse 38C13 B-cell lymphoma clone as a forerunner to the development of anti-idiotypic antibodies for the treatment of non-Hodgkin's lymphoma. Additionally, Verch et al.<sup>58</sup> produced a full-length IgG in tobacco plants by infecting them with two *Tobacco mosaic virus* vectors, one expressing the heavy chain and one the light chain. This study showed that

viral co-expression was compatible with the correct assembly and processing of multimeric recombinant proteins, although the mutual exclusion of competitive viruses (those with the same replication origin) resulted in low yields due to the small proportion of co-infected cells. The agroinfiltration and virus systems have been combined so that the bacterium delivers a DNA copy of the virus genome by transfection, which allows virus vectors to be used in a wider range of plants than the natural host range of the virus.<sup>59,60</sup> This magnification system has also been used to express antibodies at high levels in tobacco and other plants.<sup>61</sup>

Most of the early molecular farming experiments involved the expression of antibodies in leaves (usually tobacco or its close relative *N. benthamiana*) so the antibodies had to be recovered from leaf tissue. But there was soon a surge of interest in diverse expression hosts, providing the means to tailor the host for each application. Tweaking of the tobacco system allowed for variant approaches in which antibodies could be fully secreted and recovered from the root exudates or leaf guttation fluid, processes known respectively as rhizosecretion and phyllosecretion.<sup>62,63</sup> Antibodies have also been produced in tobacco hairy root and shooty teratoma cultures.<sup>64,65</sup> Many different antibody formats have also been expressed in tobacco cell suspension cultures, particularly the NT-1 and BY-2 cell lines. The first reports of the expression of different antibody variants came in rapid succession: scFv,<sup>66</sup> single heavy chain,<sup>67</sup> fusion protein,<sup>43</sup> full-size IgG<sup>68</sup> and a bispecific fragment.<sup>69</sup> Many other transgenic crops were soon used for antibody expression including soybean,<sup>70</sup> potato,<sup>71,72</sup> alfalfa,<sup>73</sup> rice and wheat,<sup>74</sup> barley,<sup>75</sup> pea,<sup>76</sup> rice cell suspension cultures,<sup>77</sup> maize,<sup>78</sup> and tomato.<sup>79,80</sup> Antibodies were also used as early model proteins for molecular farming in moss<sup>81</sup> and duckweed.<sup>82</sup>

Most of the antibodies produced in plants have been considered as proof-of-principle studies, but a small number have advanced into clinical development. The first candidates were Avicidin and CaroRx, and to date these remain the only antibodies derived from transgenic plants to have completed phase II clinical trials. Avicidin is a full-length IgG specific for EpCAM (a colorectal cancer marker) produced in maize and developed jointly by NeoRx and Monsanto. Avicidin demonstrated therapeutic efficacy in patients with advanced colon and prostate cancers but was withdrawn from development in 1998 due to side effects, which were not caused by the production system (the same issues were found with an equivalent mammalian antibody). CaroRx is a chimeric secretory IgA/G produced in transgenic tobacco plants, and binds to the major adhesin SA I/II of *Streptococcus mutans*, the bacterium responsible for dental caries (tooth decay) in humans.<sup>83</sup> The antibody completed phase II trials was registered as a medical device rather than a pharmaceutical in Europe because it can be administered topically, e.g. in toothpaste or oral rinse.<sup>84</sup> It is registered as an investigational new drug in the US. Several plant-derived antibodies have completed phase I clinical trials. One example is BLX-301, an IgG indicated for the treatment of non-Hodgkin's lymphoma, which was produced in duckweed by the former US Biotechnology company Biolex Inc. The company filed for bankruptcy in 2012 and recent developments suggest that the product and duckweed production system have been abandoned.<sup>85</sup> Similarly, the anti-idiotypic antibodies described above produced by transient expression using *Tobacco mosaic virus* vectors and have also been tested in phase I trials<sup>86</sup> and currently these products are being developed using the Icon Genetics magnification platform. One of the more remarkable clinical success stories is provided by the Pharma-Planta consortium, which brought a tobacco-derived HIV-neutralizing IgG (P2G12) all the way through development including a phase I clinical trial entirely via public funding.<sup>87,88</sup> The 2G12 antibody was taken through all development stages including gene cloning, platform selection (Fig. 3), transformation, expression analysis, plant breeding, process development, downstream processing, GMP accreditation, scale up, pre-clinical safety testing and clinical-grade manufacturing in concert with the appropriate regulatory bodies.<sup>89,90</sup> The project culminated in a first-in-human phase I safety study and represented a milestone in the commercial development of molecular

farming particularly notable for the absence of industry financial backing. The most recent development in this field, however, is the production of ZMapp, the experimental cocktail of three chimeric monoclonal antibodies indicated for the treatment for Ebola virus disease. The cocktail is being developed as a molecular farming product by Leaf Biopharmaceutical (a subsidiary of Mapp Biopharmaceutical) and is produced by transient expression in *N. benthamiana*.<sup>33</sup> ZMapp was approved by the FDA for compassionate use during the 2014 West Africa Ebola virus outbreak because the transient expression system allowed rapid production and earlier studies had shown positive results in nonhuman primates.<sup>91</sup> Following its deployment, ZMapp began formal clinical development and recently completed phase II trials in Liberia, Sierra Leone, Guinea and the US.<sup>92</sup>



**Figure 3.** The many flavors of plant-based production systems are illustrated here using the HIV-neutralizing antibody 2G12 as a case study. This antibody has been expressed in many different plant species, tissues and cells, including those shown here. The red fluorescent protein from *Discosoma* sp. has been introduced by placing the gene alongside those encoding the antibody chains to allow the rapid and convenient detection of antibody-expressing tissues (and to provide some idea of the yield) based on macroscopic fluorescence.

### A hitchhiker's guide to plant antibodies

The first leg of the journey towards the production of antibodies in plants is the selection and creation of the appropriate antibody genes. Once these are available the next stage is to transfer them into the plants that will be used as the expression host. Traditionally this was achieved by generating stable transgenic plants which carry the antibody genes permanently and transmit them from generation to generation. Transgenic plants are usually created by one of two methods – gene transfer mediated by the soil pathogen *Agrobacterium tumefaciens* or direct DNA transfer by particle bombardment. In both cases, the target tissue is usually undifferentiated callus which is regenerated

under selection to produce mature transgenic plants. There are also several alternative chemical and physical transfection methods which are applied to protoplasts but these are not widely used nowadays because the two primary methods are so efficient. Both methods may also be applied to plant cells to generate transgenic cell cultures, or the cells may be derived directly from transgenic plants.

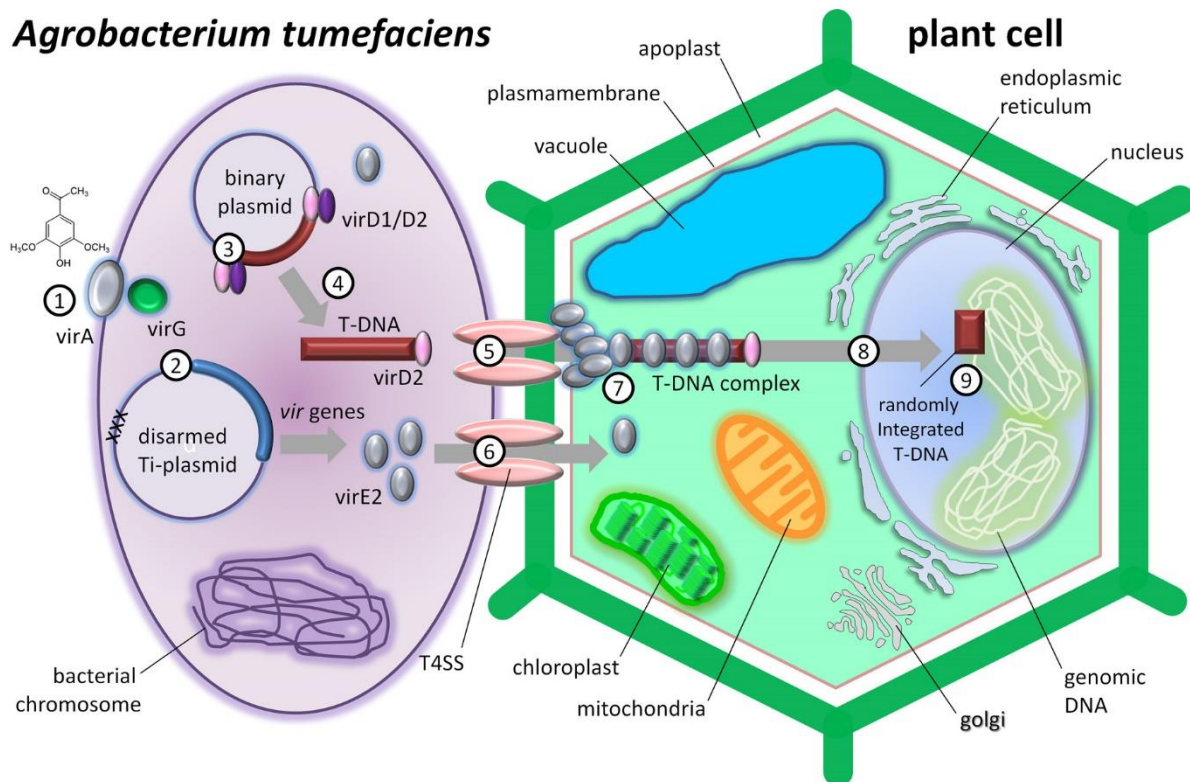
The *Agrobacterium*-dependent transformation method exploits the ability of these bacteria to transfer a segment of DNA carried in a resident plasmid to the plant genome.<sup>93–96</sup> This DNA is therefore termed ‘transferred DNA’ or T-DNA for short. Antibody genes placed within the borders of the T-DNA can be transferred to the plant cell, resulting in antibody expression. The T-DNA transfer process has two major stages: entry into the plant cell (transfection) and integration into the plant genome (transformation). The first stage is much more efficient than the second. Indeed, the second is so inefficient that the very rare transformed cells must be sorted from the background population of untransformed cells using powerful selectable marker genes. The differential efficiency of these stages means that for a short time many plant cells are transfected but not necessarily transformed, and this presents an opportunity for the short-term expression of any antibody genes on the T-DNA. This is the basis of transient expression processes that involve the infiltration of *A. tumefaciens* into the spaces between mesophyll cells in the leaf. This leads to the transfection of millions of cells, all of which can produce antibodies for a brief time. Similarly, the infection of plant cells with viruses carrying antibody genes can also allow the short-term expression of antibodies, before the plant counteracts the virus or is killed.

The transfection of plant cells by *A. tumefaciens* is probably the easiest, most versatile, robust and widespread method for the production of antibodies. It is best suited for rapid and scalable transient gene expression and thus probably most useful to those new to the field interested in making antibodies and green bionanomaterials in plants. Therefore, we discuss the biology of *A. tumefaciens* and the agroinfiltration method in more detail below, focusing on the binary vector system (pTRA) and associated protocols used in our laboratory. Other systems are described in the literature.<sup>97–101</sup>

*Agrobacterium tumefaciens* (recently renamed *Rhizobium radiobacter*) is a soil bacterium and plant pathogen that causes crown gall disease. Many crops including grapevine, nut trees, roses and stone fruits are affected. *A. tumefaciens* is a natural genetic engineer that reprograms plant cells by transferring genes that encode enzymes for the synthesis of plant hormones and opines.<sup>93–95</sup> The constitutive production of both auxins and cytokinins causes the plant cells to dedifferentiate and proliferate, thus causing the formation of the crown gall, which is a kind of tumor. Opines are amino acid derivatives that are used as a nitrogen and energy source by the bacteria. The virulence genes are located on the tumor-inducing (Ti) plasmid within the T-DNA, and are induced by phenolic compounds such as acetosyringone via a two-component signaling system comprising the membrane receptor histidine kinase VirA and the response regulator VirG (**Fig. 4** step 1). Following the phosphorylation of VirG (**Fig. 4** step 2), the virulence gene operons are induced (**Fig. 4** step 3) and the VirD1/VirD2 complex binds to short 25-bp imperfect repeats that form the left and right borders of the T-DNA, and introduce a nick on the bottom strand (**Fig. 4** step 4). VirD2 remains covalently coupled to the single stranded DNA (ssDNA) known as the T-strand, which is exported via a type IV secretion system (**Fig. 4** step 5). VirE2 is secreted separately (**Fig. 4** step 6). It binds to the plasma membrane of the plant cell and forms pores through which the T-DNA is translocated into the cytosol (**Fig. 4** step 7). VirE2 remains bound to the T-DNA and the nucleoprotein complex is imported into the nucleus via the nuclear localization signals of both VirD2 and VirE2 (**Fig. 4** step 8). The ssDNA is converted to double-stranded DNA (dsDNA) which integrates into the plant genome (**Fig. 4** step 9). The primary transcripts are processed and exported into the cytosol where depending on the presence of a signal peptide translation occurs on free or membrane-associated ribosomes. By using

additional targeting signals, the heterologous proteins can be targeted to different cellular compartments.

Because the only *cis*-acting elements required for T-DNA transfer are the left and right border sequences, the T-DNA can be deconstructed and used as a natural genetic engineering system by disarming the Ti-plasmid (removing the hormone/opine genes that generate tumors) and moving the T-DNA region to a second plasmid. To facilitate cloning, the T-DNA is moved to a shuttle vector that replicates efficiently in *Escherichia coli* but also contains a low-copy-number origin of replication for maintenance in *A. tumefaciens*.



**Figure 4:** Simplified schematic representation of the transfection and subsequent transformation of a plant cell by *Agrobacterium tumefaciens*. The individual steps are indicated by circled numbers and are explained in the text.

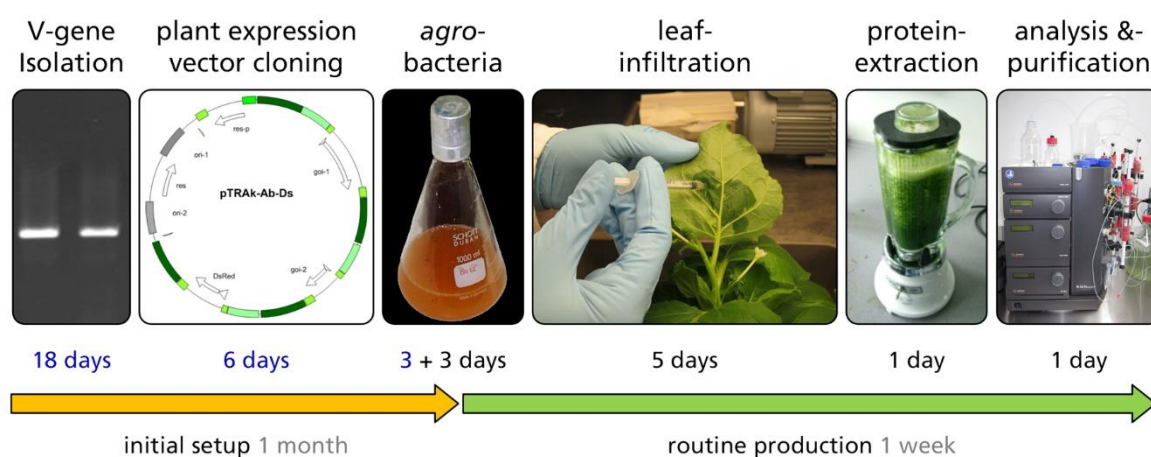
### Making an antibody in plants from first principles

The agroinfiltration method is very simple and requires only basic skills and standard laboratory equipment (**Fig. 5**). However, those new to the method are reminded of several important issues (**Box 1**). The only infrastructure needed in addition to a standard molecular biology laboratory is a place to grow the wild-type plants and a place to incubate the infiltrated leaves. The workflow is straightforward (**Fig. 6**). After the antibody transgene (or transgenes) has been cloned in a binary T-DNA vector (pTRA in our laboratory, but others include pEAQ, pRIC, magnICON, pORE and pGREEN), the plasmid is amplified in *E. coli*, isolated and used to transform *A. tumefaciens* by electroporation (common laboratory strains include GV3101, LBA4404 and EHA105). Recombinant bacteria carrying the expression constructs are selected on agar plates containing appropriate antibiotics (carbenicillin for pTRA plasmids). The bacteria are then grown in liquid culture at 28°C on an orbital shaker at 180 rpm (it is important to ensure that the temperature does not exceed 30°C because the plasmids are lost when the cells grow too quickly). *A. tumefaciens* grows more slowly than *E. coli* and when

inoculating a colony from an agar plate it typically takes 3 days before the liquid culture reaches an  $OD_{600} > 1$ . The culture is gently centrifuged and the cell pellet is resuspended in infiltration medium (4.3 g/L Murashige and Skoog standard salts, 30 g/L sucrose, 200  $\mu$ M acetosyringone, pH 5.6). The bacterial suspension is then injected directly into the intercellular spaces of a leaf using a 1-mL syringe or is infiltrated using a vacuum device, which allows the infiltration of entire plants. The infiltrated leaves are incubated for 3–6 days at 19–23°C <sup>102–104</sup> before the recombinant antibodies are extracted. This is achieved by homogenizing the leaf material in extraction buffer using an electropistil, by grinding using a mortar and pestle (optionally with liquid nitrogen) or using a standard kitchen blender. The resulting homogenate is effectively an ‘antibody smoothie’. This is centrifuged and the green liquid is collected for analysis and antibody purification. For antibody formats requiring two or more separate polypeptides it is not necessary to insert both transgenes into the same vector because the transfection process is highly efficient, and simply mixing different recombinant bacterial suspensions carrying the separate genes is sufficient to transfer both genes into large proportion of the transfected plant cells. This is an immense benefit when considering more complex expression strategies for novel green bionanomaterials, where multiple transgenes may be required. The agroinfiltration system is therefore ideal for such applications. It is also notable that the recombinant bacteria represent a common entry point that can lead to transient expression in, and the stable transformation of, diverse plant species and cell suspension cultures. Transient expression can therefore be used as a forerunner for the production of stable transgenic plants or suspension cell cultures without changing the vectors or the bacterial delivery vehicle.



**Figure 5:** Getting started: basic equipment and material required for transient gene expression in plants using the agroinfiltration method.



**Figure 6.** Make your own antibodies in plants using standard laboratory methods. Although the isolation of antibody variable genes can be challenging (e.g. if single-cell PCR is required), all the subsequent steps, including cloning, growing the bacteria and plants, the infiltration of leaves, and antibody extraction and purification, are straightforward laboratory methods that can be learned quickly. Notably, leaf infiltration and the subsequent incubation can be done in a non-sterile environment. A single plant can yield up to 100 mg of purified antibody.

**Box 1.** Important considerations for the agroinfiltration-based transient expression system

- Two plasmids are required: the helper plasmid and the binary T-DNA plasmid. Selection is necessary for both.
- The addition of 2 mM  $Mg^{2+}$  is required in many protocols.
- An incubation temperature greater than 28°C results in loss of the helper plasmid.
- Virulence genes are induced by phenolic compounds (e.g. acetosyringone) at a mildly acidic pH of 5.6.
- Infiltrated leaves must be allowed to get rid of the excess liquid to allow gas exchange within the intercellular space.
- The post-infiltration temperature should be 19–23°C.

### Antibodies from plants as next-generation bionanomaterials

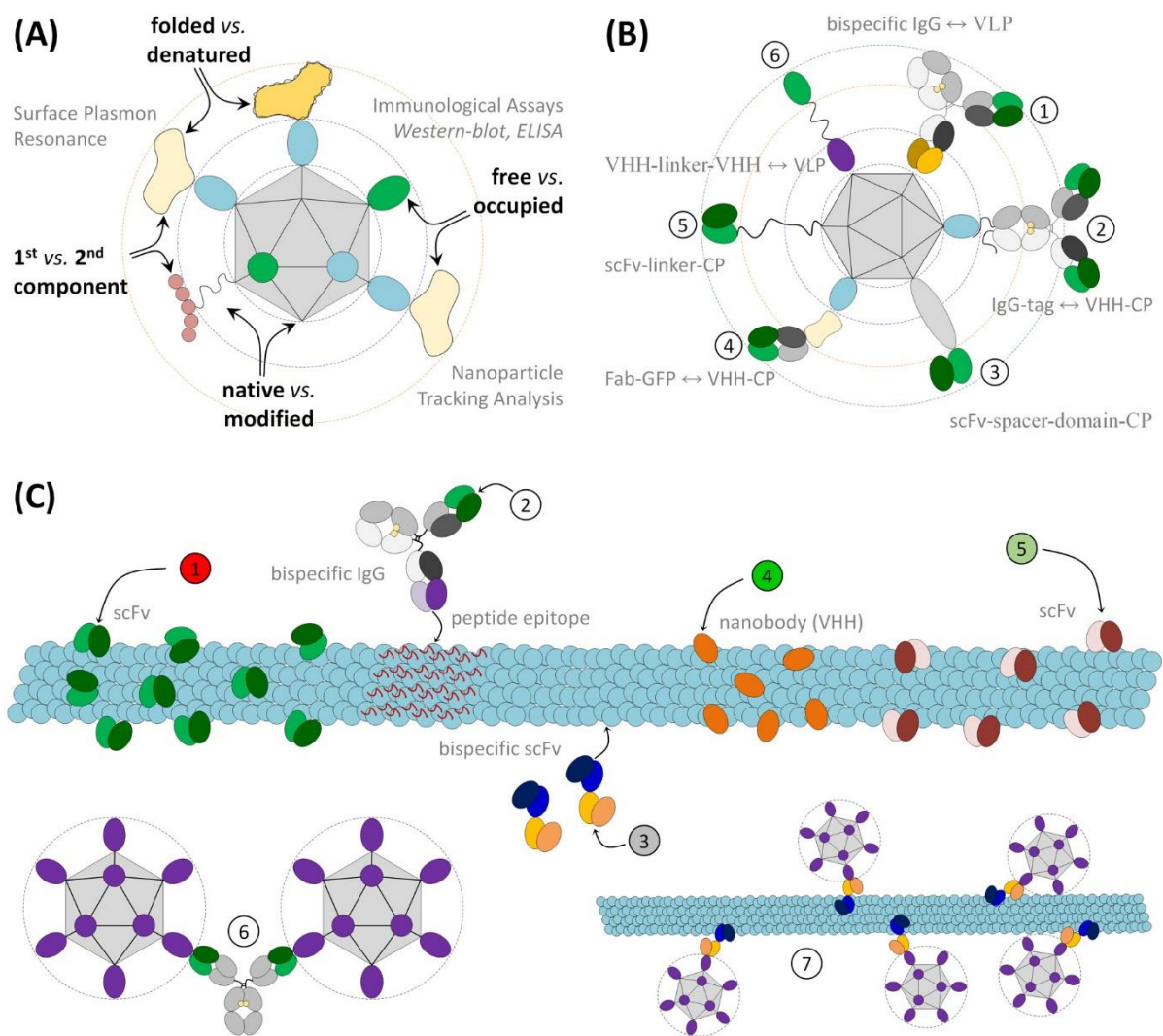
Plant-derived antibodies can be used in various ways to develop nanomaterials and nanomedicines, e.g. as reagents, building blocks and functional components. Some of the basic ideas are illustrated in **Figure 7**.

During research and development, plant-derived antibodies can be used like any other antibody available commercially or through academic collaborations, e.g. to analyze the expression, accessibility and stability of individual components and assembled nanostructures. However, making recombinant antibodies in plants offers several advantages that more than justify the extra investment needed to clone the variable domains. First, larger amounts the antibody can be produced easily in a do-it-yourself manner rather than restocking the reagent on multiple occasions. Second, the availability of the variable sequence corresponding to the antigen-binding domain instantly opens up all the possibilities of different antibody formats and fusion proteins. For example, monovalent derivatives are better suited for epitope titration, whereas fluorescent protein fusions can be used for particle characterization (e.g. nanoparticle tracking analysis)<sup>105</sup> and the directed coupling of SNAP-antibody fusion proteins facilitates the recruitment of nanomaterials to sensor surfaces.<sup>106,107</sup> Interestingly, the nanomaterial research community can easily share antibody expression clones to sustain and expedite research and development thus lowering entry barriers,

reducing running costs, making results more comparable and achieving a wider impact. The development of nanomaterials into nanomedicines requires powerful assays for the characterization of these materials from early product and process development through to GMP manufacturing and the quality control and release procedures required for the active pharmaceutical ingredient. High-quality antibody reagents are also likely to be required for clinical trials.

The use of antibodies as building blocks for nanomaterials exploits their high specificity and affinity, which can be employed to recruit other molecules. Importantly, antibodies can recognize not only peptides and proteins (their typical targets in the natural immune system) but almost any other structure, including small molecules (haptens), carbohydrates, nucleic acids, viruses and of course bionanomaterials. Antibodies can easily be engineered with different sizes, valencies, geometries and multiple specificities, allowing them to be used as building blocks and scaffolds. The expression of antibodies in plants is the ideal complement for the production of bionanomaterials in plants, such as plant virus-like particles.<sup>108,109</sup>

In one of the earliest examples of this approach, Smolenska et al.<sup>110</sup> described the generation of rod-shaped flexible PVX particles decorated with a scFv against the herbicide diuron. The authors used the short 2A peptide sequence from *Foot-and-mouth disease virus* to introduce a ribosomal skip in the coat protein gene, which provides a strategy to generate coat protein fusions (also known as the 'overcoat strategy'). Whereas this nanomaterial exploited the antibody as an affinity sorbent to bind small molecules, subsequent studies described nanomaterials comprising domains from protein A, a bacterial immunoglobulin-binding protein which is widely used in the bioprocessing industry for antibody purification.<sup>111</sup> Recently, Peyret et al.<sup>112</sup> described the development of a 'tandibody', a camelid nanobody fused to tandem copies of the *Hepatitis B virus* core protein. The antigen-binding specificity of this nanobody allows the decoration of the tandem core particle with the antigen or antigen-fusion proteins, providing a generic platform for highly immunogenic vaccine candidates. The approach is flexible because the outer antigen layer can be constructed during expression (by the co-expression of the antigen), during extraction or by mixing purified components. This study highlights an important step towards the next-generation of bionanomaterials involving the design of assembly strategies to combine different components quickly and efficiently. Antibodies can also be used as functional components to capture enzymatic activities, deliver the nanomaterial to specific target cells (e.g. tumor cells) and recruit effector cells. Their selective binding can also be exploited for targeting nanomaterials to higher-tier two and three dimensional structures, or to connect them to sensors. When all the components of these next-generation nanomaterials are produced or derived from plants they can be considered as 'green nanomaterials'.



**Figure 7.** The use of plant-derived antibodies for the analysis and construction of nanomaterials. **(A)** Plant-derived antibodies are not only useful for classical immunological assays such as western blots and ELISAs, but derivatives such as monovalent Fab and fluorescent SNAP-fusion proteins can facilitate surface plasmon resonance spectroscopy and nanoparticle tracking analysis. **(B)** Different derivatives that can integrate the functional properties of plant antibodies, particularly by targeting specific cell-surface molecules (indicated by the green domains): (1) bispecific IgG molecule bound to a virus-like particle (VLP); (2) full-size IgG with C-terminal peptide bound to a VHH fused to a virus coat protein (CP); (3) a scFv fused to a bridging domain fused to the virus CP; (4) a Fab fused to GFP, binding to  $\alpha$ -GFP-VHH-CP; (5) a scFv fused to a long linker peptide fused to a virus CP; (6) two VHH domains connected via a long linker peptide bound to a VLP. **(C)** Different plant antibody formats (scFv and nanobody) have already been used as fusions to virus CPs, whereas specific IgG and scFv formats have yet to be tested. Depending on the antibody specificity, a variety of molecules can be assembled into the nanomaterials (VLPs in this example): (1) small molecules such as herbicides or payloads; (2) large multimeric antigens; (3) enzymes; (4) fluorescent proteins; (5) or other nanomaterials. The latter may then be arranged into higher-order structures: (6) oligomers of spherical particles; or (7) combinations of spherical and rod-shaped particles.

## Translating green bionanomaterials into green bionanomedicines

The widespread adoption of plant-derived antibodies was initially discouraged by uncertainties in the regulatory framework,<sup>113</sup> differences in N-glycosylation between plants and humans<sup>114</sup> and the lack of large-scale manufacturing capacity.<sup>115,116</sup> Although antibodies are extremely versatile and useful at the laboratory scale, an important aspect for medical and industrial scientists as well as funding agencies is the need to advance plant-derived antibodies and green bionanomaterials into clinical trials and eventually into clinical practice. This has become increasingly likely as the major hurdles continue to fall and our knowledge base increases. Several plant-derived pharmaceuticals (including antibodies) have now progressed into clinical trials and the first products have reached the market, which means they are used in clinical practice. Plant-specific N-glycosylation, an important product-specific and application-specific issue for antibodies made in plants, has been addressed using classical genetic engineering approaches<sup>117–120</sup> as well as more recent genome editing technologies.<sup>121</sup> A number of auxiliary technologies have been used to address post-transcriptional gene silencing,<sup>121</sup> target protein folding and proteolysis,<sup>123–125</sup> and downstream processing strategies for extraction, clarification and purification.<sup>126</sup> Manufacturing capacity is being built at several international locations (**Table 2**) and models predicting the manufacturing costs are being refined.<sup>127–129</sup> All these advances clearly signify the maturation of plant-derived pharmaceuticals as a technology. Antibodies are among the pioneers in terms of clinical development because they are currently the most successful class of biological products.<sup>130</sup> With major bottlenecks and roadblocks resolved, future antibody products are expected to fully capitalize on the unique features of plant-based systems, and it will be interesting to watch green bionanomaterials following in their wake.

**Table 2.** Capacities of global GMP research and commercial production facilities.

Entity	Location	Capacity
Medicago / Mitsubishi Tanabe	Quebec, Canada and Research Triangle Park North Carolina, US	90,000 m <sup>2</sup> ; 10 million pandemic influenza vaccine doses per month <sup>175</sup>
iBio Inc. (Caliber Biotherapeutics LCC)	Bryan, Texas, US	> 4 million <i>N. benthamiana</i> plants up to 300 kg protein per year
Reynolds American Inc. (Kentucky BioProcessing LCC)	Owensboro, Kentucky, US	32,000 ft <sup>2</sup> (9753 m <sup>2</sup> ) / 30,240 plants
Fraunhofer Institute for Molecular Biology and Applied Ecology	Aachen, Germany	250 kg / 2000 tobacco plants per month (greenhouse); > 50 kg <i>N. benthamiana</i> per week (automated vertical farming unit, under construction)
Fraunhofer Center for Molecular Biotechnology	Newark, Delaware, US	13,000 ft <sup>2</sup> (400 m <sup>2</sup> ) greenhouse
Nomad (IconGenetics)	Halle, Germany	not disclosed;
Fiocruz - Bio-Manguinhos	Manguinhos, Rio de Janeiro, Brazil	not disclosed;

The translation of green bionanomaterials into green bionanomedicines has already taken advantage of the convenience, speed and versatility of scalable transient gene expression.<sup>131–133</sup> The next anticipated developments include the further scale-up and optimization of downstream processing strategies, the precise and accurate characterization of plant-based expression hosts, and improvements in GMP manufacturing. The intrinsic safety of both plant manufacturing hosts and

plant virus-derived green bionanomaterials offer significant risk assessment benefits that will encourage progress towards clinical trials. When combined with the speed and scalability of plant-based transient gene expression systems, we may well be looking forward to a Cambrian explosion in the development of highly creative and innovative biotherapeutics.

### A pledge for plant-derived antibodies

Plants can make antibodies! And they can make them efficiently and in all kinds of formats. Don't be afraid to use plants to make antibodies and bionanomaterials. It's easy, inexpensive and requires only basic laboratory skills. Plant-based production systems come in many flavors and scales, and are equally well suited to the high-tech environments of industrialized countries and the resource-poor settings of developing nations. Yes, there are still challenges and bottlenecks ahead, making research and development just as interesting as the diverse applications. But among the currently available expression systems, plants are probably the most versatile, globally accessible and possibly the most sustainable. Antibody diversity embraces not thousands but millions or even billions of different structures, providing an almost infinite source of structural and functional components for diverse applications, including the development of bionanomaterials and bionanomedicines. So let's explore! All it takes is basic recombinant DNA technology, the help of a little soil bacterium and a green thumb. The rest is just sun, soil and water.

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