



In planta production of plant-derived and non-plant-derived adjuvants

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Recombinant antigen production in plants is a safe and economically sound strategy for vaccine development, particularly for oral/mucosal vaccination, but subunit vaccines usually suffer from weak immunogenicity and require adjuvants that escort the antigens, target them to relevant sites and/or activate antigen-presenting cells for elicitation of protective immunity. Genetic fusions of antigens with bacterial adjuvants as the B subunit of the cholera toxin have been successful in inducing protective immunity of plant-made vaccines. In addition, several plant compounds, mainly plant defensive molecules as lectins and saponins, have shown strong adjuvant activities. The molecular diversity of the plant kingdom offers a vast source of non-bacterial compounds with adjuvant activity, which can be assayed in emerging plant manufacturing systems for the design of new plant vaccine formulations.

KEYWORDS: CTB • lectin • plant adjuvant • plant-made vaccine • recombinant protein • RTB • saponin

Vaccine adjuvants are substances coadministered simultaneously with an antigen, which potentiate the immune responses. At present, the only vaccine adjuvants licensed by the US FDA for human vaccines are aluminium salts, whose effect in enhancing diphtheria protection was first observed as early as 1926 by Glenny *et al.* [1]. In other countries, including members of the EU, other vaccine adjuvants have been approved for human use, such as a squalene-based oil-in-water emulsion (MF59) licensed for an influenza vaccine formulation (Fluad® [Novartis, Basel, Switzerland]) a decade ago; another oil-in-water, squalene-containing adjuvant (AS03 [GlaxoSmithKline, Brentford, UK]) was approved as a component of a prepandemic H5N1 vaccine (Prepandrix™ [GlaxoSmithKline]). AS04 adjuvant, a combination of monophosphoryl lipid A (MPL) and aluminium hydroxide, was approved for hepatitis B virus (HBV; Fendrix™ [GlaxoSmithKline]) and human papillomavirus (HPV; Cervarix™ [GlaxoSmithKline]) vaccines [2]. The mode of action of adjuvants is not always fully understood and several mechanisms have been proposed, including the formation of a depot of antigen in the site of inoculation, the protection of the antigen against degradation, the controlled release of

the antigen into the body, and/or the presentation of the antigen to the immunocompetent cells, leading to the production of different cytokines [2–4].

Adjuvants have significant effects on the nature of the immune responses, and can tilt the immune system in favor of Th1- or Th2-type response. A major challenge in the development of more effective vaccines is the development of potent adjuvants that can strongly, simply and safely enhance vaccine immunogenicity. Adjuvants that preferentially enhance Th1-type responses are particularly desirable, as these responses are believed to play the major role in immune resistance to diseases such as hepatitis, TB and cancer [5]. A major problem in the search for the ideal adjuvant is that adjuvants that promote cell-mediated (Th1) immunity (e.g., Freund's complete adjuvant) generally have unacceptable local or systemic toxicity that precludes their use in human vaccines.

Plants as vaccine factories: the quest for an effective immune response

Plants have been proposed as alternative platforms for the production of recombinant antigens for vaccination. The initial plant-made vaccine proposal aimed to match the

requirements of the ideal vaccine. This needed to be cheap, therefore affordable for developing countries; heat-stable, to facilitate distribution; and needle-free, therefore eliciting a response through the mucosal, preferably oral route [6]. In its most challenging proposition, a plant-made vaccine could be produced as an integral component of an edible plant organ (e.g., a tuber or a fruit), and dispensed through the oral route in a semiprocessed form, as a botanical complex mixture [7]. Expectations were high at the beginning, but have been conveniently leveled in recent years with the realization that issues such as downstream processing, *in planta* stability and dose control needed re-evaluation [8]. A particular issue of concern was the ability to mount an effective immune response through the mucosal/oral route, given that the delivery of antigens in a form equivalent to a food protein is likely to induce tolerance rather than protective immunity. To do so, it is important to escort the antigen with additional molecules/structures that stimulate the recruitment and activation of antigen-presenting cells (APCs), particularly dendritic cells (DCs) [9]. At this point, the attention of plant biologists moved partially from the antigen itself to the accompanying molecules that could contribute to enhance the immune response, particularly in the oral route [10,11]. Incidentally, it has been occasionally observed that certain plant-derived unpurified antigens produce an unexpectedly strong immune response, as if plant tissue 'contaminants' had conferred adjuvant activity to the mix. Tomato-made virus-like particles (VLPs) from Norwalk virus were highly immunogenic when administered orally in mice, whereas the same strategy using potato tubers was far less successful [12]. Certainly, these observations fit well with the historical origin of adjuvants in vaccine formulations, often referred to as 'vaccine's dirty little secret' [13,14]. Ultimately, this little secret has a molecular nature: adjuvancy is the result of the molecular action of one or more accompanying moieties conferring protection, targeting and/or costimulation to the antigen. In some sense, adjuvants bring context information to the antigen.

The interest of plants as vaccine/adjuvant production factories derives from two unique evolutionary features: first is the enormous productive capacity, probably derived from the evolutionary excess in production capacity arising from the conquest of firm land from aquatic environment [15]. Second, plants are masters in generating molecular variability, probably as a result of the diversification of lifestyles arising from the excess in production capacity. Whereas plant production capacity has driven the concept of the plant-made vaccine, plant genetic diversity is beginning to be exploited as a source for adjuvant capacity. Recently, some plant molecular entities from saponins to lectins, are being explored as candidates for adjuvant formulations. The discovery of adjuvant components in the plant kingdom often arises from a random experimental design, or even from serendipity, rather than a rational design. Only recently, the ability to rapidly test recombinant products in plants using transient expression methodology opens the way to rationally explore new adjuvant functions in plants and the ways to produce them. Here, we review the current trends in

the use of plant products as adjuvants, and discuss the role of plant genomics and plant biotechnology in the discovery and manufacturing of new adjuvants.

Plant-made protein fusions: bacterial-derived adjuvants & cytokines

A commonly used strategy for enhancing mucosal responses of plant-made antigens is the production of translational fusions with well-established adjuvants from a bacterial origin. One of the most potent natural adjuvants is the nontoxic B subunit of the cholera toxin (CTB), a homopentameric structure that binds the GM1 ganglioside receptor at the surface of intestinal cells [16]. CTB pentamers are properly assembled in the plant cell when targeted to the endoplasmic reticulum and maintain their ability to bind GM1 ganglioside, leading to a protective immune reaction against bacterial endotoxin in mice [17,18]. Recombinant CTB has been produced in different plant tissues and species, showing expression levels of up to 0.5% of the total soluble protein (TSP) in nuclear transformants when targeted to the plant endomembrane system [19,20] (TABLE 1). CTB is apparently not stable in the plant cytoplasm and, therefore, cytoplasm antigens fused to CTB need to be directed to the ER to achieve significant expression levels [21]. Plastids are also an attractive production system, as Daniell *et al.* demonstrated that CTB is properly assembled in this organelle [22]. The M-cell-targeting ability of CTB has brought cholera vaccines to the front-line of plant mucosal vaccine strategies: a rice-produced CTB vaccine (MucoRice) was shown to elicit a cholera toxin (CT)-specific immune responses with toxin-neutralizing activity in both systemic and mucosal compartments when administered orally to macaques, even in the presence of pre-existing anti-CT antibodies [23,24]. Interestingly, rice-produced CTB, localized in protein bodies, remained resistant to pepsin digestion. This finding highlights the importance of antigen protection in oral vaccination and suggests that CTB may be a good fusion partner in mucosal strategies. Used as a chimerical partner, CTB promotes the endocytosis of its fused antigen, enhancing both humoral and cellular responses. Several CTB fusion strategies have been assayed in plants (TABLE 1). Transplastomic plants expressing CTB fused to 2L21 peptide from virulent canine parvovirus (CPV) retained pentamerization and GM1-ganglioside binding characteristics of the native CTB, and induced antibodies able to recognize CPV [25]. Rabbits immunized with pea-produced VP60, the major structural protein of rabbit hemorrhagic disease virus (RHDV), fused to CTB, showed anti-VP60-specific antibodies and survived RHDV challenge, suggesting that CTB fusions can also be used to enhance the immunogenicity of veterinary antigens, as a way to make the vaccination costs accessible for veterinary approaches [26]. As a mucosal adjuvant, CTB fusions have also been effective in inducing oral tolerance against allergy reactions. T-cell epitopes of Cry j 1 and Cry j 2 cedar pollen allergens were expressed in rice seed as a fusion protein with either CTB or rice gluten as a control. CTB-fused T-cell epitopes suppressed allergen-specific IgE responses and pollen-induced clinical symptoms at 50-fold lower oral doses than controls [27].

Plant-made cytokines have also been produced in plants as immune modulators (TABLE 1). There are several examples in the literature showing recombinant cytokine expression and proper processing *in planta*. Interestingly, Ma *et al.* showed that the combination of plant-produced IL-4 and glutamic acid decarboxylase (GAD) self-antigen was effective in inducing oral tolerance to prevent diabetes in a mouse model [28].

Another commonly used fusion partner with adjuvant effects is the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB). In plants, different antigens such as a contraceptive epitope [29], ESAT-6 TB antigen [30], or porcine epidemic diarrhea virus [31] have been separately expressed as LTB fusion proteins, thus resulting in chimeric proteins displaying antigenic determinants of both components. In another recent example, LTB has been used to induce humoral and cellular T-helper (Th)1 and Th3 immune responses in mice orally challenged with a *Chlamydomonas psittaci* antigen [32]. An important breakthrough in enterotoxigenic *E. coli* (ETEC) plant vaccine production was recently reported by Rosales-Mendoza *et al.* They reported the production in tobacco chloroplasts of a translation fusion between the poorly immunogenic heat-stable toxin (ST) and LTB. Expression levels of LTB-ST in tobacco leaves reached up to 2.3% of the TSP. Oral uptake of freeze-dried leaf tissue expressing ST-LTB fusions led to the induction of both serum and mucosal LTB-ST-specific antibodies [31]. Most interestingly, it elicited protective immune responses against the CT [33].

In recent years, VLPs have arisen as systems for the development of new vaccines. VLPs are made of viral structural proteins that retain the ability to self-assemble without the presence of the viral genome. VLPs are highly immunogenic owing to a number of mechanistic reasons, such as the optimal size for uptake by DCs, the efficient activation of the APCs or their ability to cross-link B-cell receptors [34]. Vaccination strategies using plant-made antigen subunits structured as VLPs have been extremely successful but are not discussed here as they are beyond the scope of this article. Conversely, the use of immunogenic peptides genetically fused to viral structural proteins should be mentioned, which end up being exposed on the surfaces of the assembled VLPs. Several plant-made adjuvant VLPs have been successfully used for the presentation of murine and human epitopes. In most cases, the basic structural subunits of VLPs derive from plant viruses, as is the case for cowpea mosaic virus, tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus or papaya mosaic virus, among others [35–38]. Alternatively, non-plant viruses can be used as antigen carriers, as recently reviewed for Norwalk VLPs [39].

The list of immunomodulatory xenoproteins produced in plants is expected to increase as more adjuvants from nonplant origin are newly described. Plants, being a safe (bacterial endotoxin-free) and cost-effective production platform offer advantages for manufacturing the new wave of immune modulators. In a recent example, Farran and coworkers showed the successful production of the extradomain A (EDA) from fibronectin in plastids using an enhancer N-terminal extension to the protein [40]. Similarly to lipopolysaccharides, EDA is known to activate Toll-like receptor

(TLR)4, therefore inducing DC maturation. Plastid-made EDA retained its proinflammatory properties as an adjuvant, showing the feasibility of using transplastomic tobacco as a platform for nonplant adjuvants.

Plant lectins as immunomodulators

Plant lectins are a class of carbohydrate-binding proteins distributed in a variety of plant species. Lectins are usually glycoproteins themselves and specifically bind carbohydrate moieties without introducing biochemical modifications. The interest of lectins in vaccine development comes from their unique ability to bind carbohydrate moieties at cell surfaces, particularly M cells at the Peyer's patches of mucosal surfaces. The Peyer's patch is the main site of immune induction in the GI tract, whereby immune complexes and antigens in the gastrointestinal lumen are sampled and delivered to underlying mucosal immune cells. M cells display a unique ability to transcytose particles or antigens. DCs can then take up these transcytosed particles or antigens and migrate to the underlying lymphoid tissue, where they interact with T and B cells. This property has led to postulate some plant lectins as possible antigen-delivery agents (TABLE 2).

The term lectin derives from the Latin *legere*, literally, to choose [41]. Initially, plant lectins drew the attention of researchers for their ability to agglutinate blood cells. The first lectin to be isolated was concanavalin A from jack bean (*Canavalia ensiformis*) seeds [42]. Since then, plant extracts have been screened for agglutinating activities leading to a breakthrough in hematology owing to their ability to label and sort blood cells.

Plant lectins conform a group of biochemically and structurally diverse proteins. On the basis of their structure, plant lectins can be classified into different groups [41] (also see online data at [201]). The first group corresponds to legume lectins: legume seeds are rich in a structurally conserved group of lectins showing a wide range of sugar-binding specificities, and whose best-studied representatives are the *Phaseolus vulgaris* phytohemagglutinin (PHA), soybean (*Glycine max*) lectin (SBA), *Ulex europaeus* lectin (UEA-1) and jack bean Concanavalin A (ConA). Structurally unrelated to legume lectins are hevein lectins, the chitin-binding wheat germ agglutinin (WGA). The GlcNAc-binding solanaceous lectin group, including potato and tomato lectin (LEA), are also composed of hevein domains [43]. The widespread β -prism-I structure of jackfruit seed lectin, jacalin A, defines the group of jacalin-like lectins [44]. Some monocots accumulate bulb D-mannose-specific lectins, with snowdrop (*Galanthus nivalis*) as the class representative. Finally, the group of β -trefoil lectins deserves special attention for their potential application as a vaccine adjuvant, whose best characterized representative is the B subunit of ricin (ricin B) of the *Ricinus communis* ricin toxin.

The biological functions of plant lectins are not always fully understood. The structural diversity of this group of proteins probably also reflects a certain degree of functional diversity. The role of many lectins in plant defense is well established, acting as antinutrients and, therefore, protecting against animal predation through toxicity. This is probably on the basis of their unique binding capacity to digestive structures. PHA, for

Table 1. Plant-made adjuvants/adjuvant-antigen fusions: bacterial toxins and cytokines.

Adjuvant	Antigen	Expression host	Activity reported	Ref.
CTB	D2	<i>Chlamydomonas reinhardtii</i>	Oral administration protects mice against lethal doses of <i>Staphylococcus aureus</i>	[112]
CTB	RHDV-VP60	Pea	Enhanced VP60-specific antibody titer in rabbit after oral or intraperitoneal immunization	[26]
CTB	MPR ₆₄₉₋₆₈₄	<i>Nicotiana benthamiana</i>	Mucosal and serum anti-MPR ₆₄₉₋₆₈₄ (IgA and IgG) after mucosal prime-systemic boost immunization in mice	[113,114]
CTB	GFP	Tobacco	Internalization of CTB-GFP by the mouse intestinal mucosal cells as well as the antigen-presenting cells in the intestinal mucosa and submucosa following oral delivery in mice Detection of GFP in liver and spleen; CTB remains in the intestinal cell	[115]
CTB	3Ccp	Rice	Suppressed allergen-specific IgE responses and pollen-induced clinical symptoms in mice after oral administration	[27]
CTB	As16	Rice	Oral administration to mice elicited an As16-specific serum antibody response and protection against <i>Ascaris suum</i> challenge but required coadministration of CT	[116]
CTB	Pins	Tobacco/lettuce	Preservation of insulin-producing β -cells in the pancreatic islets, increased expression of immunosuppressive cytokines IL-4 and IL-10, and elevated serum levels of Ig ₁ in nonobese diabetic mice after oral administration	[117]
CTB	INS	Potato	Reduction in pancreatic islet inflammation and delay in the progression of clinical diabetes in nonobese diabetic mice following oral administration	[118]
CTB	2L21	Tobacco	Anti-2L21 sera antibodies (IgG ₁ > IgG _{2a}) in mice and rabbit after intraperitoneal or intradermal immunization, and weaker humoral response after oral delivery; efficient CPV neutralization by rabbit serum following intradermal but not oral administration	[25,119]
CTB	CFA/I, NSP4 ₂₂	Potato	Detection of serum (IgG) and intestinal (IgG and IgA) antibodies against CTB, CFA/I and NSP4 ₂₂ ; elevated levels of IL-2 and IFN- γ ; increased CD4 ⁺ lymphocyte numbers, and reduced severity of diarrhea symptoms following rotavirus challenge in orally immunized mice. Protection by immunized mice sera against toxic <i>Escherichia coli</i> binding to Caco-2 human colon carcinoma cells	[120,121]
CTB	HVR1	<i>N. benthamiana</i>	Anti-CTB and anti-HVR1 serum antibody in mice following intranasal immunization	[122]
LTB	MOMP	Rice	Detection of anti-MOMP IgG and IgA in sera and feces, splenocyte proliferation, increased levels of IL-2, IFN- γ and TGF- β , and stimulated CTL response in orally immunized mice	[32]
LTB	ST	Tobacco	Induction of anti-LTB-ST IgG in serum and IgA in intestine, and decrease of intestinal fluid accumulation observed in mice following oral immunization	[33]
LTB	ESAT-6	<i>Arabidopsis thaliana</i>	Induced antigen-specific responses from CD4 ⁺ cells, increased IFN- γ and induced Th2 response in the Peyer's patch following oral administration. No protection against challenge with <i>Mycobacterium tuberculosis</i>	[30,123]

2L21: Epitope of the VP2 protein from the canine parvovirus; 3Ccp: T-cell epitopes P1-277–290 of Cry j 1 and P2-246–259 and P2-70–83 of Cry j 2 Japanese cedar pollen allergens; As16: *Ascaris suum* antigen; CFA/I: Enterotoxigenic *E. coli* fimbrial colonization factor; COE: Core-neutralizing epitope of porcine epidemic diarrhea virus; CPV: Canine parvovirus; CT: Cholera toxin; CTB: Nontoxic B subunit of the cholera toxin; D2: *Staphylococcus aureus*-specific epitope D2; ESAT-6: Early secretory antigenic target 6 kDa of *Mycobacterium tuberculosis*; GAD: Glutamic acid decarboxylase; GFP: Green fluorescent protein; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HVR1: Hypervariable region 1 of hepatitis C virus; INS: Human insulin; LTB: B subunit of *Escherichia coli* heat-labile enterotoxin; MOMP: Major outer membrane protein of *Chlamydia psittaci*; MPR₆₄₉₋₆₈₄: Membrane proximal (ectodomain) region of gp41 envelope protein of HIV-1; NSP4₂₂: 22-amino acid immunodominant epitope of the murine rotavirus enterotoxin; PEDV: Porcine epidemic diarrhea virus; Pins: Human proinsulin; RHDV: Rabbit hemorrhagic disease virus; rHcv b 3: Allergen of the latex of *Hevea brasiliensis*; ST: Heat-stable toxin of *E. coli*; Th: T helper; VP60: Viral protein 60; ZP3: Epitope (amino acids 336–342) from the mouse zona pellucida 3 glycoprotein.

Table 1. Plant-made adjuvants/adjuvant-antigen fusions: bacterial toxins and cytokines (cont.).

Adjuvant	Antigen	Expression host	Activity reported	Ref.
LTB	rHev b 3	<i>N. benthamiana</i>	Anti-LTB and Hev b 3 IgG ₁ and IgG _{2a} and anti-Hev b 3 IgE in mice following intranasal administration. Increased IL-5:IFN- γ ratio	[124]
LTB	COE	Rice/tobacco/lettuce	Protein functionally active in binding to the GM1 receptor	[31,125,126]
LTB	ZP3	Tomato	Protein functionally active in binding to the GM1 receptor	[29]
IL-4 (mouse)	GAD65 (human)	Tobacco	Enhanced levels of IgG ₁ anti-GAD antibodies, increased Th2 immune response (splenocyte IL-4/IFN- γ cytokine responses), and production protective regulatory T cells in NOD mice following oral administration	[28]
IL-10 (mouse/viral)	-	Tobacco	Recombinant IL-10 was able to activate the IL-10 signaling pathway and to induce specific anti-inflammatory responses in mouse J774 macrophage cells	[127]
IL-10 (human)	-	Tobacco	Reduced severity of colitis, downregulation of TFN- α , increased expression of IL-2 and IL-1- β in mice following oral administration	[128]
IL-12 (human)	-	Tobacco	Recombinant protein is biologically active (stimulation of IFN- γ production by natural killer cells)	[129]
IL-12 (mouse)	-	Tomato	Increased resistance to infection by <i>M. tuberculosis</i> in Balb/C mice after oral administration	[130]
IL-12 (mouse)	-	Tobacco, tomato	Increased Th1 and reduced Th2 response	[131,132]
IL-12 (chicken)	-	Tobacco	Recombinant protein induced IFN- γ secretion from mouse splenocytes and stimulated splenocyte proliferation	[133]
IL-13 (human)	-	Tobacco	Recombinant protein is biologically active (stimulation of IFN- γ production in splenocytes)	[134]
GM-CSF (mouse)	-	<i>Nicotiana tabacum</i>	Recombinant protein is biologically active (stimulation of TF-1 cells proliferation)	[135]
<p>2L21: Epitope of the VP2 protein from the canine parvovirus; 3Ccp: T-cell epitopes P1-277-290 of Crv j 1 and P2-246-259 and P2-70-83 of Crv j 2 Japanese cedar pollen allergens; As16: <i>Ascaris suum</i> antigen; CFA/II: Enterotoxigenic <i>E. coli</i> fimbrial colonization factor; COE: Core-neutralizing epitope of porcine epidemic diarrhea virus; CPV: Canine parvovirus; CT: Cholera toxin; CTB: Nontoxic B subunit of the cholera toxin; D2: <i>Staphylococcus aureus</i>-specific epitope D2; ESAT-6: Early secretory antigenic target 6 kDa of <i>Mycobacterium tuberculosis</i>; GAD: Glutamic acid decarboxylase; GFP: Green fluorescent protein; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HVR1: Hypervariable region 1 of hepatitis C virus; INS: Human insulin; LTB: B subunit of <i>Escherichia coli</i> heat-labile enterotoxin; MOMP: Major outer membrane protein of <i>Chlamydomonas psittaci</i>; MPP₆₉₄₋₆₈₄: Membrane proximal (ectodomain) region of gp41 envelope protein of HIV-1; NSP₂₂: 22-amino acid immunodominant epitope of the murine rotavirus enterotoxin; PEDV: Porcine epidemic diarrhea virus; Pns: Human proinsulin; RHDV: Rabbit hemorrhagic disease virus; rHev b 3: Allergen of the latex of <i>Hevea brasiliensis</i>; ST: Heat-stable toxin of <i>E. coli</i>; Th: T helper; VP60: Viral protein 60; ZP3: Epitope (amino acids 336-342) from the mouse zona pellucida 3 glycoprotein.</p>				

Table 2. Plant lectins as vaccine adjuvants.

Adjuvant	Antigen	Activity reported	Ref.
RTB	GFP	Production of RTB–GFP fusion protein in tobacco. Intranasal immunization of mice with purified ricinB–GFP tobacco root cultures induced GFP-specific serum IgGs (IgG ₁ > IgG _{2a}) and serum and fecal anti-GFP IgA	[50]
RTB	VP7	Production of RTB–VP7 fusion protein in potato	[52]
RTB	INS	Production of RTB–INS fusion protein in potato	[53]
KML-C	H1N1	Influenza-specific antibodies (with dominant IgG ₁ in serum, IgG in genital secretions and IgA in saliva), and influenza-specific lymphocyte proliferation and cytotoxic activity in spleens and in mediastinal lymph nodes enhanced by KML-C in mice via intranasal immunization. Protection against challenge with homologous (H1N1) and heterologous (H3N2) influenza virus	[136]
KM ⁺	SLA	Enhanced IFN- γ production, reduction of parasite load, and induced dendritic cell maturation (enhanced expression of MHC II, CD80 and CD86) in mice after subcutaneous immunization	[137]
ML-I, II, III	OVA, gD2	Enhanced serum (high ratio IgG ₁ :IgG ₂) and mucosal antibody responses to the codelivered antigen	[138,139]
UEA-1	HIV peptides	Enhanced serum (IgG ₁ and IgG _{2a} /IgG _{2b}) antibody responses in mice after intranasal immunization. High percentage of HIV-IIIIB strain virus inhibition	[140]
UEA-1	HIV-1	Elevated systemic and mucosal antibody responses in mice immunized intranasally	[141]
UEA-1	<i>Helicobacter pylori</i> , <i>Campylobacter jejuni</i>	Increased serum and intestinal antibody levels and protection against live pathogen in mice following orogastrical delivery	[57]
crMCL	β -gal	Antigen-specific IgG antibody significantly enhanced by crMCL via intramuscular administration with the prime–boost regimen	[142]

β -gal: β -galactosidase; crMCL: Crude *Momordica charantia* lectin; gD2: Herpes simplex virus glycoprotein D2; GFP: Green fluorescent protein; H1N1: Inactivated influenza virus; INS: Proinsuline; KM⁺: *Artocarpus integrifolia* lectin; KML-C: Korean mistletoe lectin C; ML: Mistletoe lectin; OVA: Ovalbumin; RTB: Ricin toxin B subunit; SLA: *Leishmania amazonensis* antigen; UEA-1: *Ulex europaeus* I lectin; VP7: Outer capsid glycoprotein of simian rotavirus SA11.

instance, is known to be toxic for mammals in general, and to the potato leafhopper (*Empoasca fabae*). Here, PHA binds to the midgut epithelial cells and leads to severe disorganization and finally to occlusion of the lumen [45,46]. Probably the best-studied antinutrient lectin is the ricin B. The lectin subunit binds to the cell surface and directs the entry of A subunit into the cell membrane. The imported protein then moves by retrograde migration along the secretory pathway of animal cells, arriving finally to the ribosomes. Here, the toxic A subunit inactivates ribosome activity by cleaving 28S rRNA at an essential position for binding of elongation factors, therefore blocking protein synthesis and causing cell death [47].

A comprehensive investigation of the possible use of plant lectins as mucosal adjuvants was carried out by Lavelle and coworkers by comparing several plant lectins ML-I, LEA, PHA, WGA and UEA-I. Plant lectins were compared with CT as adjuvants for a bystander antigen, ovalbumin (OVA) [48]. It was found that, although most lectins had certain antigenic capacity, antigenicity and adjuvant capacity does not necessarily correlate. Whereas CT and ML-1 stimulated an immunological reaction against both lectin and OVA, WGA and UEA-I stimulated high anti-OVA IgG production and low antilectin reactions, as expected for a low toxicity adjuvant. In spite of this, a recent study re-evaluates the toxic effect of WGA on an *in vitro* model and shows unexpected bioactive effects of WGA on immune cells at nanomolar concentrations [49].

Ricin B has been experimentally tested to serve as a carrier for delivery of antigens to the mucosal immune system. Fused to a model antigen, green fluorescent protein (GFP), it was expressed in tobacco plants and hairy root cultures to test for utility in mucosal vaccine delivery/adjuvancy [50]. Intranasal immunization of mice with galactosamine-affinity purified ricin B–GFP triggered significant increases in GFP-specific serum IgGs, comparable with that observed following GFP immunization with CT adjuvant. Also as a viral antigen carrier, a ricin B N-terminus fusion with a 90-amino acid peptide from simian rotavirus SA-11 nonstructural protein NSP490, produced in *E. coli*, stimulated a strong Th1 cell-mediated immune response [51]. The same fusion produced in potato tubers was biologically active and made up approximately 0.03% of the transformed tuber TSP [52]. More recently, RTB–insulin fusions have also been reported in plants as a proposed adjuvant strategy for inducing oral tolerance against Type 1 diabetes [53].

Plant lectins have also been used to target antigens to M cells of the Peyer's patches in microencapsulation strategies. Microencapsulation of vaccine antigens and adjuvants has been studied for years as a strategy for mucosal immunization. A biodegradable copolymer of lactic and glycolic acids, the polylactide-coglycolic acid (PLGA), is most commonly used for microparticle manufacturing due to its excellent toxicological profile [54]. By including M-cell-targeting lectins in the microcapsules composition, the number of microcapsules that

effectively reach M cells is expected to increase. The targeting potential of the lectinized nanoparticles has been assayed using UEA-1, which appears to owe its M-cell specificity to a specific receptor on the apical surface of M cells. UEA-1-lectinized nanoparticles have demonstrated an approximately fourfold increase in the degree of interaction with the M cells at the bovine submaxillary mucin compared with plain nanoparticles. Sugar specificity of the lectinized nanoparticles was also maintained. These stabilized, lectinized nanoparticles could be a promising carrier–adjuvant for the targeted oral–mucosal immunization [55]. Similar behavior was also observed with *Arachis hypogaea* (peanut agglutinin) lectinized nanoparticles [56]. Recently, a new strategy for vaccination against *Helicobacter pylori* and *Campylobacter jejuni* was assayed involving the lectinization of whole-bacteria preparations. Killed bacteria preparations were agglutinated with UEA-1 lectin. Oral delivery of lectin-agglutinated bacteria in mice induced a significant increase in both serum and intestinal antibody levels. This response was specifically dependent on lectin-directed M-cell targeting, since unagglutinated bacteria, or bacteria agglutinated with non-M-cell-specific *Bandeiraea simplicifolia* I (BS-I) lectin did not trigger the same response [57]. The glycospecificity conferred by plant lectins has potential applications in medicine that go beyond the concept vaccine adjuvants in a strict sense. These include the potential use as mucosal biocides against viral infection and particularly HIV. Several anti-HIV lectins have been described such as the cyanobacterial cyanovirin-N [58], snowdrop lectin GNA and *Hippeastrum* hybrid (*Amaryllis*; HHA) [59] and, most recently, a jacalin-related lectin (BanLec) isolated from banana fruit (*Musa acuminata*) [60]. Finally, there is an increasing interest in plant lectins as antitumor antigens as a result of their ability to induce programmed cell death and/or autophagocytosis in cancer cells (recently reviewed in [61]).

Plant saponins are essential components of immunostimulatory complexes

Saponins are secondary metabolites found in particular abundance in various plant species. Their basic structure comprises of a lipophylic part (sapogenin), formed by a steroid or other triterpene aglycon, decorated with one or more hydrophilic glycoside moieties. Aglycone derivatives can also incorporate nitrogen, so that some saponins also present with chemical and pharmacologic characteristics of natural alkaloid products. The amphipatic nature of saponins determines their name, by the soap-like foaming they produce when shaken in aqueous solutions. Plants produce a wide variety of saponins that exhibit many different biological activities, from antimicrobial to cytotoxic and antitumoral, which are on the basis of many uses of plants in traditional medicine [62]. Certain saponins have been found to activate the mammalian immune system, leading to significant interest in their potential as vaccine adjuvants. On the basis of their common use within the food and beverage industries, with no documented toxicity in humans at the present levels of consumption, food-grade saponins have also been proposed as adjuvants for use with plant-made vaccines [63].

The lead adjuvant candidate saponin is QuilA, a saponin extract from the bark of the tree *Quillaja saponaria*. QuilA is actually a complex mix of saponin compounds. A detailed analysis of QuilA HPLC fractions led to the isolation of peak QS-21, which retains most of its adjuvant capacity and low toxicity. A QS-21 fraction has been included in several experimental human vaccine formulations, including some against HIV, cancer and malaria in primates and humans [64–68]. However, serious drawbacks have limited its use as an adjuvant in human vaccination, such as high toxicity, hemolytic effect and instability [69,70], although it has not precluded its use in experimental and commercial veterinary vaccines such as foot-and-mouth disease [71,72].

QuilA is also a fundamental component in the formulation of immunostimulatory complexes (ISCOMs), particulate delivery systems composed of antigen, cholesterol, phospholipid and saponin. ISCOMs and ISCOMATRIX particulate adjuvants (both made up of the same components with and without antigen respectively) combine the advantages of a particulate carrier system with the presence of the adjuvant QuilA. Consequently, ISCOMs are more immunogenic than other colloidal systems such as liposomes and protein micelles, while having reduced toxicity associated with the presence of QuilA [73].

The relative success of QuilA in vaccine formulation has triggered an intense search for new plant saponins with immunomodulatory and/or other pharmacological effects, preferably with lower toxicity effect (recently reviewed in [62] and [74]). For instance, ginseng saponins (ginsenosides) improved the antibody response of pigs to porcine parvovirus and *Erysipelothrix rhusiopathiae* [75].

Also relevant for the field of plant mucosal vaccines is the presence of possible saponin-based immunomodulators in edible crops, which could eventually act as endogenous adjuvants of plant-based vaccines. In this regard, solanaceous crops are rich in steroidal saponins often classified as glycoalkaloids. Potato contains mainly α -solanine and α -chaconine, whereas green tomatoes are rich in α -tomatine. In a study aimed at evaluating novel aggregate structures as adjuvants, Rajanathan and coworkers formulated 'tomatine adjuvant', a α -tomatine-based mixture also containing *n*-octyl- β -D-glucopyranoside, phosphatidylethanolamine and cholesterol. Tomatine adjuvant was a highly effective immunostimulator, being capable of generating anti-ova humoral responses in mice after a single immunization. Immunizations using ova formulated with Tomatine adjuvant resulted in antibody titers that were greatly superior to those achieved by immunization with conventional reference adjuvants, alum and incomplete Freund's adjuvant (IFA). Interestingly, these responses were achieved in the absence of toxicity (i.e., inflammation) [76]. Subsequently, the potential utility of tomatine as a vaccine adjuvant has been assayed in experimental disease models. Tomatine adjuvant was shown to enhance a cellular immune response to malaria *Plasmodium berghei* CS 9-mer antigen, yielding antimalaria protective capacity upon *in vivo* immunization [77]. The adjuvant effects of the tomatine mix were also confirmed in combination with irradiated *Francisella tularensis* immunization against modelled tularemia and in T-cell-mediated regression of EG7-OVA murine lymphoid tumors [78].

Plant polysaccharides as low-toxicity adjuvants

Immunologically active plant-based polysaccharide particles have emerged as strong candidates for use as human vaccine adjuvants, combining potent adjuvant efficacy with good tolerability and safety. Among them, the fructan inulin, on which the ADVAX family of adjuvants is based, is one of the best studied [79–81]. Inulin is a fructan, that is, a polymer comprising mainly of fructose. Together with starch and sucrose, fructans are used as reserve carbohydrates in approximately 15% of flowering plants. Apart from the obvious function as an energy store, fructans are also involved in adaptation to drought and cold stress [82]. Inulin is made exclusively of linear $\beta(2\rightarrow1)$ fructosyl \rightarrow fructose-linked fructose polymers with a terminal glucose unit. Inulin is generally regarded as safe (GRAS) by the FDA. It can be prepared free of endotoxin or other contaminants and it is heat stable with a long shelf-life. Inulin is widely used in human nutrition, for example, as a low-calorie sweetener, as dietary fiber or as a fat substitute [83]. On an industrial scale, chicory (*Cichorium intybus*) roots are the main source for inulin extraction, although adjuvant inulin is obtained from dalia tubers. There has been a certain biotechnological interest in transferring inulin biosynthesis route to nonfructan accumulating species as a dietary fiber fortification strategy. Potato tubers were engineered by transferring the sucrose:sucrose 1-fructosyltransferase (*I-SST*) and the fructan: fructan 1-fructosyltransferase (*I-FFT*) genes of globe artichoke (*Cynara scolymus*) to transgenic potato plants [84]. Inulin made up 5% of the dry weight of transgenic tubers, and a low level of fructan production was also observed in fully expanded leaves.

Inulin can be isolated in different forms, but particulated γ -inulin isoform shows superior immunomodulatory activities. Inulin activates and exhausts the complement system when incubated with human serum, and was one of the first substances used for this purpose. It can boost both cell-mediated and humoral immunity efficiently, promoting both Th1 and Th2 immune responses [80,85]. In addition, γ -inulin adjuvant has been shown to improve vaccine responses in animal tests against hepatitis B [86], malaria [87] and influenza in a spray freeze-dried preparation [88], and effectiveness in stimulating contraceptive and antitumor strategies has also been proposed [85].

A number of additional plant-derived polysaccharides have been assayed in recent years, such as *Lemna minor* apiogalacturonic peptin, *Actinidia eriantha* polysaccharide or, more recently, *Lycium barbarum* polysaccharide–protein complex (LBP) (TABLE 3). The latter complex has recently been shown to induce phenotypic and functional maturation of DCs with strong immunogenicity, which makes LBP a potent candidate adjuvant for the design of DC-based vaccines [89].

Expert commentary

For centuries, plants have been man's main source of chemically complex commodities, particularly those needed at large scale. In the last two decades, the ability to manipulate crop genomes has opened the door for manufacturing subunit vaccines from plants. However, in the race for high levels of antigen subunits, the intrinsic capacity of the plant cell to generate a vast

Table 3. Plant polysaccharides as vaccine adjuvants.

Adjuvant	Antigen	Activity reported	Ref.
Advax	JEV	Balanced Th1/Th2 immune response against JEV (IgG ₁ and IgG _{2b}) in mice after subcutaneous immunization. Protection against live JEV challenge	[143]
γ -inulin, liposomes, vitamin E	HE2	Elicitation of specific HE2 antibodies	[144]
Starch microparticles	HSA	IgA response elicited in mice after oral, but not subcutaneous or intramuscular, immunization. Stronger Th2 response after oral primary immunization than after intramuscular primary administration, but the relative Th1 response was stronger after an oral booster than after an intramuscular booster	[145]
Starch microparticles	DF, CRM-197	IgG ₁ , IgG _{2a} and IgG _{2b} response in mice after oral or parenteral immunization. Oral administration elicited mucosal IgA response. Strong diphtheria toxin-neutralizing antibody response following oral or parenteral administration	[146,147]
LM	OVA	Increased levels of serum IgG ₁ and IgG _{2a} and intestinal IgA, and increased adhesion of macrophages in orally immunized mice	[148]
LBP	-	LBP upregulated DC expression of CD40, CD80, CD86 and MHC class II molecules; enhanced DC allostimulatory activity; induced IL-12p40 and p70 production. LBP-treated DCs enhanced Th1 and Th2 responses <i>in vitro</i> and <i>in vivo</i>	[90]
AEPS	OVA	Enhanced OVA-specific IgG, IgG ₁ , IgG _{2a} and IgG _{2b} antibody titers, IL-2, IFN- γ and IL-10 levels, and killing activities of NK cells in mice immunized subcutaneously	[149]
CPP	OVA	Enhanced OVA-induced splenocyte proliferation and IgG, IgG ₁ and IgG _{2b} antibody levels in serum in mice immunized subcutaneously	[150]

AEPS: *Actinidia eriantha* polysaccharide; CPP: *Codonopsis pilosula* polysaccharide; CRM-197: Nontoxic derivative of diphtheria toxin; DC: Dendritic cell; DF: Diphtheria toxin; HE2: Human epididymis gene product residues 15–28; HSA: Human serum albumin; JEV: Japanese encephalitis virus; LBP: *Lycium barbarum* polysaccharide–protein complex; LM: *Lemna minor* apiogalacturonic peptin; NK: Natural killer; OVA: Ovalbumin.

amount of partner molecules for cell interaction has somehow been neglected. The only exception to this rule, to our knowledge, is the use of ricin B as a fusion partner in subunit vaccine candidates. This in spite of the obvious realization that many of the most promising new adjuvant formulations, as previously reviewed, contain plant-made products among the key active components. Conversely, traditional botanical mixes as *Echinacea* and ginseng exhibit striking adjuvant activities whose molecular mechanism are yet to be identified. Most recently, orally administered Rehmannia Six Formula, a traditional Chinese herbal medicine, has been shown to stimulate immunity against tetanus toxoid and diphtheria toxin [90,91]. Therefore, plant-based manufacturing platforms are ideal biofactories for the synthesis of the immunomodulators of the future, at least those of plant origin. The future plant-made vaccines may well go hand-in-hand with the discovery and recombinant biosynthesis of new adjuvant molecules.

Plant defense molecules & the human immune system: an unexploited gold mine?

It is interesting to observe how most of the plant molecules with proven immunomodulator activity, from saponins to lectins, have been proposed to have endogenous defensive roles, particularly against predators. It is tempting to speculate whether or not this responds to a sort of arms race between predator and prey at the molecular level in the context of the digestive mucosa. Most legume lectins accumulate at high levels in endoplasmic reticulum-derived protein bodies of seeds, at the same or similar locations as to where seed storage proteins such as gluteins, zeins or legumins are also accumulated to serve as a nitrogen source for the developing seed. M-cell-specific binding of seed lectins might well be a self-defense mechanism to discourage seed predation. Binding to insect gut structures and resistance to proteolytic degradation by insect digestive enzymes are the two main prerequisites for lectins to have toxic effects on insects [92]. Reversely, recognition and internalization of plant lectins in Peyer's patches might involve an adaptation of the predator to favor, for example, induction of mucosal tolerance towards seed proteins and metabolites. An interesting observation in this regard is the ability of certain jacalin-like molecules to agglutinate (i.e., to particulate) seed proteins such as β -glucosidases. For several years, some maize genotypes were regarded as homozygous for a 'null' allele at the maize β -glucosidase isozyme Glu1 (*glu1*) gene, on the basis of the absence of a glucosidase activity band in the zymograms from seed extracts. Only recently it was found that β -glucosidases were indeed present in *glu1* genotypes, but in the form of large insoluble complexes, which were unable to enter zymogram gels. The factor responsible for the *glu1* null phenotype was shown to be a β -glucosidase-aggregating factor (BGAF) containing a jacalin-related lectin (JRL) domain with β -glucosidase agglutinating activity [93–95]. The biological significance of the interactions between β -glucosidases and their partners is not known. In the most widely accepted hypothesis, insect/herbivore attack induces jacalin-mediated agglutination of β -glucosidase, which somehow enhances insect toxicity. The

proposed function of maize β -glucosidases is the release of toxic aglycones, such as hydroxamic acids, from their glucosides. Agglutinated (i.e., particulated) β -glucosidase complexes have shown resistance to proteolytic activity [96], but the interplay between these complexes and the mucosal immune system has been poorly investigated.

It might also be just a coincidence that jacalin A from jackfruit seeds binds human secretory (s)IgA. Jacalin A binds *O*-glycosylated groups at the hinge region of human IgA₁, and it is, together with *Staphylococcus aureus* Ssl7 protein, the only commercially available product for IgA affinity purification [97–99]. Ssl7 interacts with circulating monomeric IgA at the Fc α receptor binding site, blocking the immune response and facilitating infection. Similarly, various microbial adhesins and toxins bind sIgA through their carbohydrate moieties. Given that sIgA is retro-transported across M cells, and triggers mucosal and systemic immune responses, it is conceivable that jacalin–sIgA complexes contribute to the regulation of immune responses against dietary proteins. Whereas the role of microbial lectins in IgA binding is well established, a biological function for seed lectin binding to sIgA remains speculative. However, the ability of defense molecules from plant edible organs to perform relevant activities for the host immune system, such as binding sIgA, agglutinating toxins, targeting them to antigen-sampling areas and/or enhancing membrane permeability, needs to be further investigated following a multidisciplinary approach. Regardless of the existence of a biological rationale underlying the responsiveness of the human mucosal immune system to the defensive molecules produced by plants, this fact can be biotechnologically exploited in the context of plant-made vaccines. Plant-made immunomodulators can either be produced as built-in adjuvants in crude or partially purified plant-derived formulation or, perhaps more realistically in the short term, they can be manufactured separately or as genetic fusion partners in plant-based subunit vaccine strategies.

Plant-based adjuvants at the post genomic era

Up until now, only a minute fraction of the variability of the plant kingdom has been exploited for its potential to provide immunostimulatory molecules. Until now, chromatography separation of plant extracts has constituted the main tool for plant adjuvant discovery. This might well change as the ever-increasing list of plant genomes being sequenced opens the way for surveying genetic variability at the basis of DNA sequence homology rather than the biochemical profile. Simply in *Arabidopsis thaliana*, Raval *et al.* found 118 entries with a jacalin-like domain [44]. A plant-lectin database created in 2006 resulted in a total of 947 entries of unique, non-redundant entries spread across 241 different plant source entries [100]. The huge pool of glycointeractors offered by the genetic diversity in the plant kingdom enters a new dimension in the post-genomics era. The biochemical/immunological properties of the genetic pool of plant lectins, for instance, can be screened using increasingly efficient and versatile expression systems (see later), leading to a more rational design of new adjuvant molecules. The situation is a bit more complicated in the case of nonprotein adjuvants such as saponins. However, the recent efforts in plant metabolomic profiling,

and the increasingly efficient bioinformatic tools for cross-examination between metabolomic and transcriptomic data sets and plant genomic markers, are contributing to unveiling the genetic basis of many complex routes in secondary plant metabolism. This has not only biotechnological but also ecological implications: as an example, the overexploitation of the *Q. saponaria* bark has caused important ecological damage and a considerable shortage of the available supplies [101]. A better understanding of the genetic basis of plant secondary metabolism would enable the genetic transfer of whole biosynthesis routes to plant heterologous systems. This would enable the production, at medium/large scale and under controlled and sustainable conditions, of large quantities of plant metabolites with adjuvant activities.

From discovery to synthesis: new opportunities from plant synthetic biology

Massive sequencing tools are pouring out tonnes of information on the genetic diversity of the plant kingdom. The next challenge is to move from the analytical to the synthetic side of the equation in order to take full advantage of its pharmacological potential. In this sense, the new advances in the field of recombinant protein production in plants are encouraging. A major paradigm shift in recent years has come from the sudden burst in plant-based transient expression systems. Initially, expression vectors based on plant viruses, such as those developed initially by the company Large Scale Biology based on TMV, combined speed and scalability in the production of recombinant plant products [102]. More recently, the group led by Yuri Gleba at ICON Genetics engineered a TMV vector optimized for *Agrobacterium tumefaciens* delivery into the plant cell [103–105]. This was a major breakthrough in the use of plants as biofactories, as it combined the versatility of agrodelivery with the expression power of the viral systems. With these technologies in place, the use of tobacco-related species and, in particular, *Nicotiana benthamiana* for transient expression of recombinant proteins, has become routine practice in many molecular farming laboratories. Yet the main limitation of the viral technology kept being the inability to achieve combined expression of multigenic constructs within a single cell. This restriction arises from two factors inherent to the self-replicating nature of the virus: one is that the size of recombinant DNA that can be added to the viral genome without affecting its fitness is limited. The second constrain comes from the phenomenon known as viral exclusion, whereby two or more sequence variants from the same virus accumulating simultaneously within a single cell may exclude one another. The problem was partially solved with the use of two noncompeting viruses, which led to the successful transient coexpression of dimeric proteins as antibodies [106], but the limitations for multigene approaches are still in place. Nevertheless, the use of viral vectors has been successfully exploited for the biosynthesis of nanostructures [107], including those with improved antigenic potential such as VLPs, which will be reviewed elsewhere in this special focus issue.

Regarding the ability to produce multigenic structures in transient plants systems, it is interesting to pay attention to the new pEAQ group of vectors based in cowpea mosaic virus (CMV)

produced by the group led by George Lomonosoff. Using mutagenized CMV sequences and *Agrobacterium*-mediated gene delivery, this set of vectors is able to produce high levels of transgene expression in the absence of viral replication [108,109]. This technology eliminates the viral exclusion and transgene size constrains of replication-based viral systems, allowing the engineering of multigene structures and complex metabolic pathways via transient expression. This and similar technologies may open the way for the *in planta* engineering of complex antigen–adjuvant structures based on, for example, agglutinating lectins and/or immunocomplexes that go beyond linear genetic fusions. Moreover, the speed and versatility of transient expression systems should facilitate the *in vitro* testing of the modulatory activities of different structural variants and perhaps the introduction of high-throughput screening methods that link analytical and synthetic branches of discovery.

To facilitate these goals, there is an urgent need for versatile biotech tools that facilitate the engineering of multigene constructs at the DNA level, preferably in a modular and exchangeable fashion, which could be subsequently transferred to the plant genome for the biosynthesis of increasingly complex combined structures and/or pathways. In this sense, the initiatives taken in the field of synthetic biology for the engineering of new biological devices based on microbial genomes are inspiring for plant biotechnologists [110].

One of the most promising proposals in the field of molecular farming is the manufacture of personalized, anti-idiotypic vaccines against non-Hogkin's lymphoma using TMV-based transient expression vectors [111]. The ability to provide a personalized response to patient needs is one of the emerging paradigms of 21st Century medicine. The protective and possible adverse effects of vaccines are likely to be genetically determined and, therefore, are predictable. The ability to put fast and versatile tools in place for the production of vaccine components, including adjuvants, and to provide tailor-made vaccine formulations based on plant transient technology is anticipated for the future.

All of the above does not preclude the potential use of stable transgenic crops as large-scale production systems for adjuvants or antigen–adjuvant combinations. Vaccine production at the agricultural scale remains an inspiring and perhaps indispensable goal for meeting the needs of an ever-increasing world population. Transient expression systems can be considered in this regard as a convenient technology bridge for reaching heat-stable and endogenously adjuvanted oral plant-made vaccines.

Five-year view

The plant kingdom will continue being one of the main sources of diversity in the search for new adjuvant properties in the next 5 years and beyond, and it is likely that in the coming wave of vaccines to be approved by the FDA, EMA or equivalent agencies, plant-derived molecules with immunomodulatory activity will be part of their formulation. A different matter to consider is if plant recombinant technologies will be put in place for the production of plant and nonplant adjuvant molecules. The future of plant recombinant adjuvants is likely to run in parallel with other plant-made recombinant products such as vaccines and antibodies.

This will depend on the success of the leading molecular farming products such as Bayer's (Leverkusen, Germany) non-Hodgkin's lymphoma vaccines, Protalix (Carmiel, Israel) Cerebridase, Sembyosis' (Calgary, Canada) insulin or MucoRice vaccine. Whereas no adjuvant innovations are expected in the first wave of plant-based products to reach the market, the influence of a success story can cause a burst of interest and lead to the development of new plant-produced adjuvants, either as independent recombinant products or as new components of previously successful plant-made vaccines. In the light of new advances in recombinant expression systems, the plant manufacturing of, for example, lectinized nanostructures with improved presentation potential can become an emerging field in adjuvant technology in the coming years.

Veterinary vaccines may become an advanced experimental field. Here, ethical constraints are more relaxed and low costs are an increasing driving force. The production of highly structured

antigens based on multigene expression of viral subunits, such as the empty capsid-like particles of the foot-and-mouth disease veterinary vaccines, is an example of the power of these new technologies. In the short-to-medium term, the *in planta* production of adjuvant–antigen nanostructures may become an efficient and economic technology for veterinary vaccines.

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Key issues

- The plant-based production of antigen–adjuvant genetic fusions has emerged as a simple and effective method for enhancing protective immunity in plant-made vaccines. The B subunits of cholera toxin, *Escherichia coli* heat-labile toxin, plant ricin toxins or coat proteins forming virus-like particles have been used as fusion partners with proven immune-stimulating activity.
- Natural compounds of plant origin as lectins and saponins are key components in front-line vaccine formulations owing to their adjuvant activity.
- Many plant adjuvants play endogenous defensive roles as herbivore deterrence factors. This suggests a functional link between the plant defense system and the immune-stimulating activity of plant antinutrients, a link that is not devoid of biotechnological implications.
- The analysis of sequenced plant genomes, proteomes and metabolomes is yielding an increasing number of candidate adjuvants.
- New manufacturing platforms based on transient plant transformation allow for fast plant-based synthesis and screening of candidate adjuvants.

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