Plant Produced Biopharmaceuticals against SARS-CoV-2

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Contributor: Corbin England, Jonathan TrejoMartinez, Paula PerezSanchez, Uddhab Karki, Jianfeng Xu

SARS-CoV-2 is an enveloped RNA virus with a single-stranded, positive-sense genome of ~29.9 kB in size. The virus consists of four major structural proteins, named spike (S), nucleocapsid (N), envelope (E), and membrane proteins (M). The S protein which is present as a crown-like spike on the outer surface of the virus plays a major role in viral entry into mammalian cells. Specifically, the virus uses the receptor binding domain (RBD) on the S protein to interact with human angiotensin-converting enzyme 2 (ACE2) receptor as a critical initial step to enter target cells. Plants have provided a promising production platform for both bioactive chemical compounds (small molecules) and recombinant therapeutics (big molecules). Plants naturally produce a diverse range of bioactive compounds as secondary metabolites, such as alkaloids, terpenoids/terpenes and polyphenols, which are a rich source of countless antiviral compounds. Plants can also be genetically engineered to produce valuable recombinant therapeutics. This molecular farming in plants has an unprecedented opportunity for developing vaccines, antibodies, and other biologics for pandemic diseases because of its potential advantages, such as low cost, safety, and high production volume.

Keywords: coronavirus ; COVID-19 ; vaccines ; plant

1. Introduction

Plant-based expression systems, or plant molecular farming, have emerged as a promising alternative for the production of biologics. As eukaryotic organisms, plant hosts are able to perform correct post-translational modifications, such as glycosylation, allowing the development of *authentic* biologics with their efficacy being similar to those produced using other expression systems, such as mammalian or yeast-based cell cultures [1][2]. Plant-produced biologics are also regarded as safe because they do not pose the risk of introducing human and animal pathogens into biopharmaceuticals [3]. In addition, plant expression systems, particularly transient expression systems, could prompt rapid (4–8 weeks) manufacturing of target biologics on a large scale [4][5], which meet emergency demands, such as in the case of the COVID-19 pandemic. Given the aforementioned factors, plant-based expression systems have been actively adopted by pharmaceutical manufacturers. A wide range of recombinant proteins, such as vaccines, antibodies, hormones, cytokines, therapeutic enzymes, and nutritional proteins have been produced via stable and transient expression in entire plants or plant cell cultures [3][6]. The first plant-produced biologic for human use, taliglucerase alfa (Elelyso®), was approved by the FDA in 2012 for the treatment of Gaucher disease [7]. In 2019, a plant-produced influenza virus vaccine completed Phase III clinical trials with encouraging results [8]. In early 2022, the plant-made COVID-19 vaccine, Covifenz®, won first approval in Canada [9]. These successes have revived people's interest in plant-based production of biologics for human use. To combat COVID-19, plants have been used to produce vaccines [10], monoclonal antibodies [11], and other biologics that block the interactions between angiotensin-converting enzyme 2 (ACE2) and the S proteins, such as soluble ACE2 [12] and its fusion with the Fc region of human IgG1 (ACE2-Fc) [13]. In addition, plant-produced antiviral lectin has also been tested for inhibition of SARS-CoV-2 infection [14] (**Figure 1**).

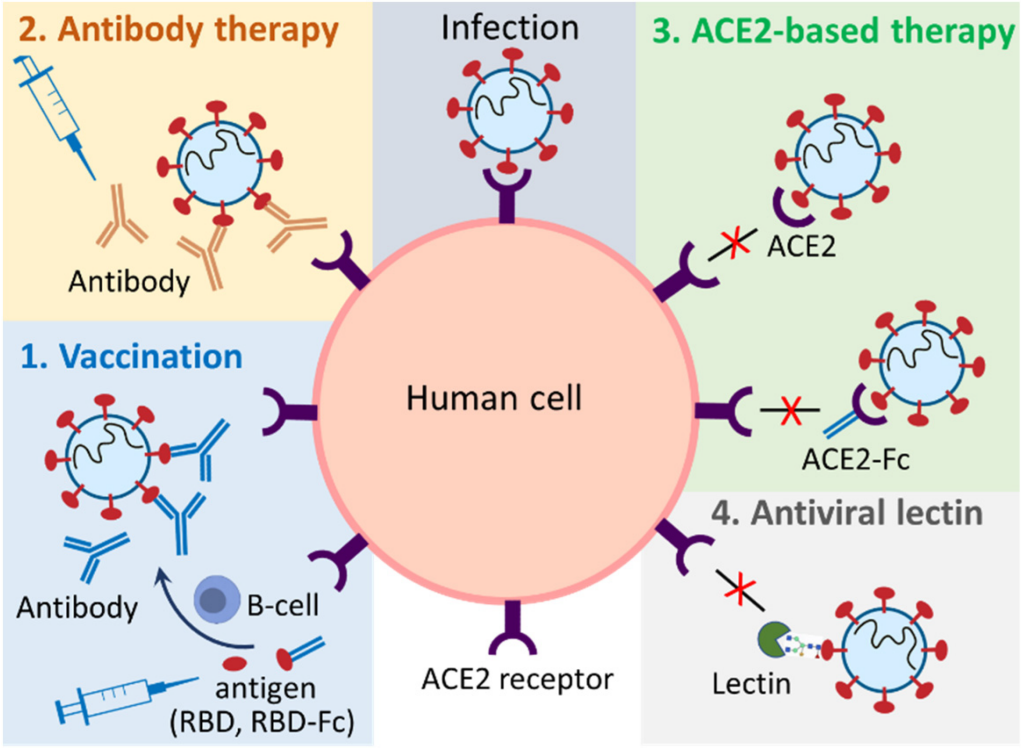

Figure 1. Schematic diagram of the plant-produced biologics functioning in preventing and treating SARS-CoV-2 infection. Plant-produced vaccines (1), antibodies (2), ACE2-based biologics (ACE2-immunoadhesin, ACE2-chewing gum) (3), and antiviral lectins (4) can be used to combat COVID-19.

2. Plant-Produced Vaccines

Although traditional inactivated viral vaccines and the new adenovirus vector- and mRNA-based vaccines haven been approved and widely used in the world to combat the pandemic, other types of modern vaccines, such as the protein subunit [15] or virus-like particle (VLP) varieties [16], have multiple advantages over currently used vaccines [17]. The minimum requirement for either type of vaccine is the genetic sequence of a single viral antigen rather than the genetic sequence of either virus [17]. This is safer for recipients of the vaccine, because lone antigens cannot cause or spread disease, and safer for scientists researching and manufacturing the vaccine, since no handling of live virus is required once the antigen has been sequenced [18]. SARS-CoV-2 replicates by infecting human cells via the interaction of the receptor binding domain (RBD) on the viral S protein with ACE2 receptors on human cells [19], therefore, the S protein, particularly RBD, has become the focus of vaccine development efforts in the pandemic [17]. A full list of subunit vaccines and VLP vaccines that have reached or passed Phase I human trials according to the COVID-19 vaccine tracker website. Plants, either whole plants or cell suspension cultures, are suitable for producing either type of vaccine [20]. A recent comprehensive review of the use of plant-based vaccines for the prevention and cure of human viral diseases can be found in the literature [5][21][22][23]. So far, a few plant-based subunit or VLP vaccines have been developed and some of them have moved to clinical trials (**Table 1**).

Table 1. Plant-produced SARS-CoV-2 vaccines. Four of them have progressed to clinical trials.

Table	Trade Name	Antigen	Plant	Manufacturer	Efficacy	Commercialization Progress	Source
Virus-like particles	Covifenz	S protein	*N. benthamiana*	Medicago	69.5% to 78.8% (Phase III)	Approved: Canada Phase III Trials: Argentina, Brazil, United Kingdom, USA	[9][24][25]
	KBP-201	RBD	*N. benthamiana*	Kentucky Bioprocessing	100% (K18-hACE2 mice)	Phase I/II Trials: USA	[25][26] [27][28]
	IBIO-200, IBIO-201, and IBIO-202	S protein	*N. benthamiana*	iBio, Inc.	n.d.	Pre-clinical trials	[21][29] [30]
Subunit	n/a	S protein	*N. benthamiana*	n/a	n.d.	no	[31]
	Baiya SARS-CoV-2 Vax 1	RBD-Fc	*N. benthamiana*	Baiya Phytopharm	100% (K18-hACE2 mice)	Phase I Trials: Thailand	[25][32]
	Baiya SARS-CoV-2 Vax 2	RBD-Fc	*N. benthamiana*	Baiya Phytopharm	Unknown	Phase I Trials: Thailand	[25][32]
	n/a	RBD-Fc	*N. benthamiana*	n/a	n.d.	no	[10]
	n/a	RBD	*N. benthamiana*	n/a	n.d.	no	[33][34] [35]
	n/a	S protein, RBD	Tobacco BY-2 and Medicago truncatula A17 cell	n/a	n.d.	no	[36]

n/a: not applicable; n.d.: no data.

2.1. Plant-Produced Subunit Vaccines

In their simplest form, subunit vaccines require only a viral protein capable of eliciting an immune response and an adjuvant. These proteins are capable of eliciting a response from B cells, helper T cells, and cytotoxic T cells, but this response is weak relative to traditional inactivated viral vaccines and necessitates the addition of an adjuvant [18]. A subunit vaccine developed by Novavax (USA) has already been granted EUA by the FDA in 2022 [37]. Studies have showed that this subunit vaccine was about 90% effective in preventing SARS-CoV-2 infections [38](Centers for Disease Control and Prevention, CDC, USA), which is similar to the efficacy of Moderna (94%) and Pfizer (95%) and better than Johnson & Johnson (66%) [39].

Plant expression platforms, mainly transient expression with *Nicotiana benthamiana*, have been used to produce subunit vaccines against SARS-CoV-2 (**Table 1**). The RBD alone and its fusion with the Fc region of human IgG1 (RBD-Fc) are utilized as an antigen to develop subunit vaccines. The recombinant RBD and RBD-Fc showed specific binding to human ACE2 receptor [10][33][34][35]. In animal tests, the plant-produced RBD and RBD-Fc antigens elicited potent neutralizing responses in mice and non-human primates [10][33]. In order to increase the immunogenicity of the antigen, RBD fused to flagellin of *Salmonella typhimurium* (Flg), known as mucosal adjuvant, was also transiently expressed with *N. benthamiana* using a self-replicating viral vector [40]. As an alternative to the transient expression platform, tobacco BY-2 and *Medicago truncatula* A17 cell suspension cultures were also used to stably express both full-length S protein and RBD [36]. The results showed that recombinant S protein and RBD could be secreted into the culture medium, which facilitated the subsequent purification and reduced the downstream processing costs. This represents the first report on the stable expression of SARS-CoV-2 antigen protein with plant cell culture system, though the bioactivity of the expressed proteins was not assessed.

Plant-produced subunit vaccines have been moved to commercial development. Of particular interest is the subunit vaccines developed by Baiya Phytopharm Co., Ltd. (Bangkok, Thailand), trade names Baiya SARS-CoV-2 Vax 1 and Baiya SARS-CoV-2 Vax 2, that utilizes a *N. benthamiana*-produced RBD as its antigen. A publication on preclinical results states that the RBD protein has been modified by fusing it with the Fc region [10]. When used with alum as adjuvant, Vax 1 induced potent immunological responses in both mice and cynomolgus monkeys [32][41][42]. Vax 1 was also reported to

show 100% efficacy against infection in K18-hACE2 mice [43]. The efficacy of, and adjuvant for, the Vax 2 variant has not been revealed so far.

2.2. Plant-Produced Virus-Like Particles Vaccines

VLP vaccines make use of one or more viral structural proteins that are capable of self-assembling into nanostructures that mimic the size and shape of a virus [44]. The building blocks of the particle may serve as viral antigens capable of eliciting an immune response and the shape of the overall VLP may conform to a pathogen-associated molecular pattern recognized by the immune system [45]. Where a true virus has a cavity that contains its genetic material, VLPs have a hollow cavity that may be used to deliver small molecules to further enhance the immune response triggered by the vaccine [46].

According to the COVID-19 Vaccine Tracker website (https://covid19.trackvaccines.org/, accessed on 15 January 2023), maintained by scientists at McGill University, at the time of writing only one plant-produced SARS-CoV-2 vaccine has been approved for use. As detailed in **Table 1**, this VLP vaccine, with the trade name of Covifenz® (Medicago, Canada), has only been approved for use in Canada and it is in Phase III trials in several others. Three other plant-produced vaccines have reached the point of clinical trials, but none of these have yet passed the Phase III trials. Medicago's VLP vaccine utilizes the recombinant, full-length S protein from an original SARS-CoV-2 strain. These proteins associate into trimers within a lipid membrane from the cell membranes of the host *N. benthamiana* cells. The protein contains modifications made to improve the stability of the protein as well as increase the formation of VLPs [47]. According to Phase III human trials, the vaccine efficacy was 69.5% against symptomatic infection and 78.8% against infection with symptoms ranging from moderate to severe [9]. According to the Canadian government, Covifenz® is administered in two doses 21 days apart and alongside the adjuvant AS03 [24].

Kentucky Bioprocessing's VLP vaccine, trade name KBP-201, utilizes a recombinant S protein's RBD and inactivated tobacco mosaic virus. The S protein's RBD, serving as the antigen, and tobacco mosaic virus, serving as the VLP's structural component, are each expressed in *N. benthamiana*, and chemically conjugated together following purification [28]. KBP-201's RBD is fused with the Fc domain of human IgG1 to improve protein stability and an *N. benthamiana* extensin peptide to allow protein secretion and folding in the host species [28]. Preclinical trials with K18-hACE2 mice showed efficacies of 71.4% and 100%, for one and two doses effectively, against lethal infection [27]. The combined Phase I/II trial listed on ClinicalTrials.gov details a two dose regimen 21 days apart using cytosine phosphoguanine as an adjuvant [26].

In addition to Covifenz® and KBP-201, more potential SARS-CoV-2 vaccines (VLP) from iBio, Inc. (Bryan, TX, USA): IBIO-200, IBIO-201 and IBIO-202 have been reported as in pre-clinical trials in separate review publications [21][30]. However, tracking the progress of potential SARS-CoV-2 vaccines that are still in pre-clinical stages poses certain challenges that prevent us from providing accurate, current information on their status. While success in benchtop and animal models may be publicized by academic labs, corporate labs may restrict publications until entry into clinical trials. This is the case with iBio's VLP vaccines. The citations provided for the status were from iBio's website (ibioinc.com), a news media website, or another review publication over the same topic rather than a direct, peer reviewed article from scientists responsible for the research. A press release by iBio, dated after the publication of these reviews, has stated that the company will no longer continue development of IBIO-202 and none of the three vaccines appear on the company's public pipeline [48][49].

3. Plant-Produced Antibodies

Rather than providing the long-term protection of a vaccine, therapeutic antibodies can be used in the moment to treat people infected by a disease. mAbs targeting epitopes on the virus or infected cells may, alone or as a cocktail, reduce the viral load and thereby reduce the severity of symptoms experienced by the patient [50]. mAb-based therapeutics against the S protein have been shown to be effective treatments for SARS-CoV-2 infection, especially the original viral strain. Up to now, five kinds of FDA approved (EUA) antibodies, either alone or as a mAb cocktail, have been developed to treat COVID-19. However, the current mAbs produced in mammalian cells are expensive and might be unaffordable for many [51]. Plants may provide a low-cost and safety-friendly alternative platform to produce efficacious and affordable antibodies against SARS-CoV-2. As a new production platform, plants have already been demonstrated to have the capability of producing mAbs with quality and characteristics matching those produced in mammalian cells [52]. For example, a plant-made anti-HIV mAb has been found to meet all regulatory specifications for human application in a clinical study [53]. However, to the best of our knowledge, at the time of writing no plant-based antibodies for the treatment of SARS-CoV-2 were in clinical trials. The scope of this portion of the review has been restricted to antibodies expressed

within plant cells that have been demonstrated, either in vitro or in vivo, to have a neutralizing effect on at least one variant of a SARS-CoV-2 lineage (**Table 2**).

Table 2. Plant-produced antibodies against the SARS-CoV-2 virus. Neutralizing capability is indicated by neutralizing titer *, meaning the dilution factor needed to reduce antibody levels below detectable limits; IC_{50} †, meaning half maximal inhibitory concentration; or NT_{100} ‡, meaning complete protection from cytotoxic effects of infection.

Antibody Name	Plant	Affected Lineages	Neutralizing Capability (Neutralizing Titer *, IC_{50} † or NT_{100} ‡)	Source
CR3022	*N. benthamiana*	Original strain	Fail to neutralize *	[34]
B38	*N. benthamiana*	Unidentified	640 at 0.492 µg/mL *	[54]
H4	*N. benthamiana*	Unidentified	40 at 5.45 µg/mL *	[54]
H4-IgG1-4	*N. benthamiana*	Unidentified	591 nM for H4-IgG3 ‡	[55]
CA1	*N. benthamiana*	Original strain, Delta	9.29 nM: Original † 89.87 nM: Delta †	[51]
CB6	*N. benthamiana*	Original strain, Delta	0.93 nM: Original † 0.75 nM: Delta †	[51]
11D7	*N. benthamiana*	Original strain, Delta, Omicron	25.37 µg/mL: Original † 59.52 µg/mL: Delta † 948.7 µg/mL: Omicron †	[4]

The first reported plant-made functional mAbs against SARS-CoV-2 were B38 and H4, which were collected from blood sera of a convalescent patient [11]. These antibodies could block binding between the RBD of the virus and the cellular receptor ACE2. Transient co-expression of heavy- and light-chain sequences of both the antibodies in *N. benthamiana* by using a geminiviral vector resulted in rapid accumulation of correctly assembled mAbs in plant leaves. Both mAbs purified from plant leaves demonstrated specific binding to RBD of SARS-CoV-2 and exhibited efficient virus neutralization activity in vitro [54]. Before this, the same research group tried to express another mAb CR3022 in *N. benthamiana*. However, this plant-produced mAb was found to bind to SARS-CoV-2 but fail to neutralize the virus in vitro [34]. These findings provide proof-of-concept for using plants as an expression system to produce SARS-CoV-2 antibodies.

Plant-made H4 was then examined in greater detail by being expressed in the four human IgG subclasses present in human serum (IgG1–4) [55]. Four constructs, each with the same variable region but different heavy chain regions, were adapted for expression in glyco-engineered *N. benthamiana*. H4-IgG3 demonstrated an up to 50-fold superior neutralization ability compared to the other three IgG against live SARS-CoV-2 virus in vivo. Complete protection from cytotoxic effects of infection (NT_{100}) using Vero cells was attained with an H4-IgG3 concentration of 5.91 nM.

Using a cocktail of mAbs that bind to complementary neutralizing epitopes represents a strategy to prevent escape of the SARS-CoV-2 mutant from mAb treatment [51]. To develop mAb cocktail-based therapeutics against SARS-CoV-2 in plants, two neutralizing mAbs, CA1 and CB6 were expressed in *N. benthamiana*. The effectiveness of plant-produced mAbs against the original SARS-CoV-2 virus and a member of the Delta lineage was tested in vitro. Both mAbs retained target epitope recognition and neutralized multiple SARS-CoV-2 variants [51]. The half maximal inhibitory concentration (IC_{50}) of CA1 was 9.29 nM for the original strain and 89.87 nM against the Delta strain. The IC_{50} of CB6 was 0.93 nM for the original strain and 0.75 nM for the Delta strain [51]. Both also demonstrated neutralizing potential against a mouse adapted strain of SARS-CoV-2 in vitro. It was also shown that one plant-made mAb has neutralizing synergy with other mAbs developed in hybridomas by the authors. A third neutralizing mAb, 11D7, which was a chimeric human IgG, was then expressed in DeltaXFT *N. benthamiana* to produce a mAb with human-like, highly homogenous *N*-linked glycans [56]. Plant-produced 11D7 was found to maintain recognition against the RBD of original, Delta and Omicron strains and neutralizing activity. Because 11D7 neutralizes SARS-CoV-2 through a mechanism not typical among currently developed mAbs, it may be useful in providing additional synergy to existing mAbs cocktails.

4. Plant-Produced Angiotensin-Converting Enzyme 2-Based Biologics

4.1. Plant-Produced Angiotensin-Converting Enzyme 2-Immunoadhesins

Although vaccines and antibodies have been developed to effectively combat COVID-19 worldwide, the rapid emergence of SARS-CoV-2 variants with altered RBD can severely affect the efficacy of such immunotherapeutic agents [57]. This problem seems to be especially pronounced with the Omicron variants that resist many of the previously isolated monoclonal antibodies [58]. Immunoadhesins, which are antibody-like molecules, make another class of immunotherapeutic agents that may complement the current therapy issue with vaccines and antibodies [59]. Immunoadhesins consist of an engineered binding domain fused to an Fc region of an antibody [60]. In the case of SARS-CoV-2, the viral cellular receptor ACE2 (extracellular domain) can serve as a binding domain for constructing such immunoadhesins, which can then function as a decoy to block the interaction of the virus with cellular ACE2 receptors [13][61]. Fusing ACE2 with the Fc region offers advantages over the treatment with ACE2 alone. This is because the Fc domain can provide effector functions, allowing the recruitment of some phagocytic immune cells and facilitating the activation of the host antiviral immune response through triggering antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). Furthermore, the Fc domain can prolong the half-life, binding affinity and neutralization efficacy of the binding domain [13][62][63]. So far, more than 13 Fc fusion proteins have been approved by the FDA.

In the past 3 years, many ACE2-based immunoadhesins, including the enhanced ACE2 for binding to S protein of SARS-CoV-2 were developed [59][61][64][65][66][67][68][69][70][71]. These ACE2-immunoadhesins were effective in neutralizing multiple SARS-CoV-2 variants, including the Delta and the Omicron variants, suggesting that immunoadhesins-based immunotherapy is less prone to escape by the virus [59]. Again, plants can provide an economic platform to rapidly produce these biologics.

With transient expression in *N. benthamiana*, ACE2-Fc was produced at up to 100 µg/g fresh leaf. The recombinant ACE2-Fc exhibited potent anti-SARS-CoV-2 activity in vitro, and dramatically inhibited SARS-CoV-2 infectivity in Vero cells with an IC_{50} value of 0.84 µg/mL. Furthermore, treating Vero cells with ACE2-Fc at the pre-entry stage suppressed SARS-CoV-2 infection with an IC_{50} (half maximal inhibitory concentration) of 94.66 ug/mL [63].

Because ACE2 is heavily glycosylated and its glycans impact on binding to the S protein and virus infectivity, the ACE2-Fc was also expressed in glycol-engineered *N. benthamiana*. It was found that the recombinant dimeric ACE2-Fc was glycosylated with mainly complex human-type *N*-glycans and showed function in peptidase activity, binding to the RBD of the virus and neutralizing the wild-type SARS-CoV-2 virus [72].

4.2. Plant-Produced Angiotensin-Converting Enzyme 2 and Angiotensin-Converting Enzyme 2-Based Chewing Gum

Besides the ACE2-based immunoadhesins, ACE2 alone could also be developed as a therapeutic to inhibit the virus spread, though there are limitations, such as short circulating half-life [13]. Human soluble (truncated) ACE2 was reported to express in *N. benthamiana* with a high-level yield (about ~750 µg/g fresh leaf). Plant-produced ACE2 could bind to the SARS-CoV-2 S protein. Both glycosylated and deglycosylated forms of ACE2 demonstrated strong anti-SARS-CoV-2 activities in vitro, with an IC_{50} being ~1.0 and 8.48 µg/mL, respectively [12].

Of special interest is the ACE2-based chewing gum developed by Dr. Henry Daniell and his colleagues at the University of Pennsylvania [73][74][75]. This virus-trapping gum contains plant-made CTB-ACE2, which is ACE2 fused with non-toxic cholera toxin subunit B (CTB). CTB-ACE2 is made in chloroplasts of transgenic lettuce. The lettuce was then powdered and blended with cinnamon-flavored chewing gum. The CTB-ACE2 can efficiently bind to both GM1 and ACE2 receptors, effectively blocking binding of the S protein and viral entry into human cells. As oral epithelial cells are enriched with both receptors, this gum was designed to trap and neutralize SARS-CoV-2 in the saliva and diminish the amount of virus left in the mouth. The Phase I/II clinical trial of the chewing gum started in June 2022 (ClinicalTrials.gov Identifier: NCT05433181). If the gum proves safe and effective, it could be given to patients whose infection status is unknown or even for dental check-ups to reduce the likelihood of passing the virus to caregivers [75].

5. Plant Produced Antiviral Lectins

Lectins from plants and algae, which are carbohydrate-binding proteins of non-immune origin, were earlier found to inhibit several viral diseases, such as HIV, hepatitis C, influenza A/B, herpes, Japanese encephalitis, Ebola, and SARS coronavirus that occurred in 2003 [76][77][78][79]. Recently, some lectins have shown significant activity against SARS-CoV-2

[80][81][82]. For example, Griffithsin, a red algae-derived lectin of 121 amino acids, is a high mannose-specific lectin that has been recognized as a potential viral entry inhibitor [83]. Griffithsin was tested for SARS-CoV-2 entry and found that it could significantly inhibit the SARS-CoV-2 infection in a dose-dependent manner. Remarkably, the IC_{50} of griffithsin was 63 nmol/L, which is about 11-fold more potent than Remdesivir [14]. Other research demonstrated that griffithsin could block the entry of SARS-CoV-2 and its variants, Delta and Omicron, into the Vero E6 cell lines and $IFNAR^{-/-}$ mouse models by targeting the S proteins of the virus [84]. Similarly, recent molecular docking studies have shown that a banana-derived mannose-specific lectin could also neutralize SARS-CoV-2 infectivity [85]. Lectins are natural proteins which are cheap and easily accessible. They have been proven to be active against SARS-CoV-2. However, their clinical application is still hampered by several obstacles. These include the high-cost purification, short stability in the body, potential cytotoxicity and mitogenicity, and the possibility for eliciting deleterious responses in the immune system [86]. Future investigations are needed to develop plant lectins as a new antiviral agent against COVID-19.

6. Challenges in Commercialization of Plant-Produced Biologics against SARS-CoV-2

Numerous anti-SARS-CoV-2 biologics, including vaccines, antibodies, and other biologics against the virus have been expressed in plant systems, as mentioned above. However, compared with other production systems, such as bacterial and mammalian cell culture, plant systems suffer from a major disadvantage: low production levels of the desired proteins [6]. Additionally, isolation and purification of the recombinant proteins from plant tissues is quite expensive [1]. Although plant systems have proven effective in performing glycosylation required for complex proteins [6][87], there is a major difference in the plant and mammalian glycan structure. The N-linked glycans produced by plants carry two plant specific residues, β-1,2-xylose and core α-1,3-fucose, which are absent from mammalian cell produced proteins [88]. The immunogenicity and allergenicity of plant-specific N-glycans has been a key concern in human therapy [88]. So far, there is only one plant cell produced biopharmaceutical, taliglucerase alfa (Elelyso®), approved by FDA. Concerted research efforts based on molecular biology strategies, such as enhancing gene transcription and translation, minimizing post-translational degradation, and glycoengineering to humanize glycosylation, and engineering strategies, such as improving bioreactor design and operation and optimizing the protein purification procedure, are still needed for the commercial success of plant-based production platforms.

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