Universitat de Lleida

BACHELOR'S THESIS

Development of plant-based vaccines and diagnostic kits for SARS-CoV-2: A literature review

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Agrotecnio, Universitat de Lleida

Bachelor's Degree in Biotechnology June 2022

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ABBREVIATIONS

ACE2	Angiotensin Converting Enzyme 2
CatB/CatL	Cathepsin B/Cathepsin L
CTB	Cholera Non-Toxic Subunit B
DDP4	Dipeptidyl peptidase 4
DMVs	Double Membrane Vesicles
E	Envelope protein
Fc	Fragment crystallizable region
HR1/HR2	Hepeptide Repeat 1/Hepeptide Repeat 2
LMIC	Low- and Middle-Income Countries
Ly6E	Lymphocyte antigen 6 family member E
mAb	Monoclonal antibody
MERS	Middle East Respiratory Syndrome
N	Nucleocapsid protein
ORF	Open Reading Frame
RBD	Receptor Binding Domain
RGD	Arginine-glycine-aspartic motif
RTC	Replication and Transcription Complex
S	Spike protein
SARS	Severe Acute Respiratory Syndrome
TMPRSS2	Transmembrane protease serine 2
VLP	Virus-like Particle

ACKNOWLEDGMENTS

I would like to thank my tutor, Dr. Teresa Capell, as well as Dr. Paul Christou and the personnel of the Applied Plant Biotechnology laboratory, especially to Andrea and Hugo, in supporting and encourage me to participate in the paper this thesis inspired; and to my family and friends, who have been always by my side.

ABSTRACT

COVID-19 is the disease caused by the SARS-CoV-2 virus responsible for the ongoing pandemic. Despite the fact that most industrialized countries appeared to have already gone through the health emergency precipitated by the disease, developing countries are still struggling to cope with the disease and its health and socio-economic consequences. Therefore, in order to end the virus' spread and prevent mutations that might render the current vaccines ineffective, humanity needs to provide vaccines and diagnostic test kits to developing countries. Plant biotechnology offers a suitable solution to accomplish this challenge: in addition to providing low cost production systems and scalability, SARS-CoV-2 antigens can be stored in planta without the requirement of a cold chain. Because of these advantages, several companies have invested in the development of plant-based vaccines against SARS-CoV-2. The first commercial plant-produced COVID-19 vaccine was produced by transient expression in *Nicotiana benthamiana*, a tobacco relative, by the Canadian company Medicago Inc. This vaccine received the approval of the Canadian Government in February, 2022. Furthermore, extensive research has been carried out in the production of different types of vaccines and therapeutic for COVID-19, demonstrating that plant-produced SARS-CoV-2 antigens and therapeutics are efficiently and economically produced. Several experiments in vitro and in vivo demonstrated effective binding of the plant produced antigens to host cells, comparable to mammalian-produced antigens, and are capable inducing immunogenicity. Therefore, plant expression systems provide a viable and economic option for developing country use.

RESUM

La COVID-19 és la malaltia causada pel virus SARS-CoV-2 el qual és responsable l'actual pandèmia. Malgrat els països del primer món aparentment han superat l'emergència sanitària que ha comportat la malaltia en qüestió, els països en vies de desenvolupament encara estan patint la malaltia i les seves conseqüències socioeconòmiques. Per tant, per tal d'acabar amb la transmissió del virus i prevenir mutacions d'aquest que podrien comprometre l'efectivitat de les actuals vacunes, cal que la humanitat proporcioni vacunes i tests de diagnosi de la malaltia a aquests països. La biotecnologia vegetal ofereix una solució adequada per a complir aquest objectiu: a més de proporcionar sistemes de baix cost i escalabilitat, els antígens del SARS-CoV-2 es poden emmagatzemar in planta sense el requisit d'una cadena de fred. Degut a aquests avantatges, moltes empreses han invertit en el desenvolupament de vacunes contra el SARS-CoV-2 produïdes en plantes. La primera vacuna comercial contra la COVID-19 fabricada en plantes es va produir mitjançant expressió transitòria en *Nicotiana benthamiana*, de la família del tabac, per la farmacèutica canadenca Medicago Inc. Aquesta vacuna rebé l'aprovació del govern del Canadà al febrer de 2022. A més a més, s'ha dut a terme una recerca extensiva en la matèria, la qual ha demostrat que els antígens del SARS-CoV-2 fabricats en plantes poden ser produïts eficient i econòmicament. Diversos experiments in vitro i in vivo han demostrat una activitat d'unió a la cèl·lula hoste dels antígens produïts en plantes comparable als antígens produïts en mamífers i són capaços de induir immunogenicitat. Així doncs, els sistemes d'expressió vegetals proporcionen una opció viable i rentable per als països en vies de desenvolupament.

RESUMEN

La COVID-19 es la enfermedad causada por el virus SARS-CoV-2, que es responsable la actual pandemia. A pesar de que los países del primer mundo aparentemente han superado la emergencia sanitaria que ha conllevado la enfermedad en cuestión, los países en vías de desarrollo todavía están lidiando con la enfermedad y sus consecuencias socioeconómicas. Por lo tanto, con tal de acabar con la transmisión del virus y prevenir mutaciones de éste que podrían comprometer la efectividad de les actuales vacunas, la humanidad necesita proporcionar vacunas y test de diagnosis de la enfermedad a estos países. La biotecnología vegetal ofrece una solución adecuada para lograr este objetivo: además de proporcionar sistemas de bajo coste y escalabilidad, los antígenos del SARS-CoV-2 se pueden almacenar in planta sin el requisito de una cadena de frío. Debido a estas ventajas, muchas empresas han invertido en el desarrollo de vacunas contra el SARS-CoV-2 producidas en plantas. La primera vacuna comercial contra la COVID-19 fabricada en plantas se produjo mediante la expresión transitoria en *Nicotiana benthamiana*, de la familia del tabaco, por la farmacéutica canadiense Medicago Inc. Esta vacuna recibió la aprobación del gobierno del Canadá en febrero de 2022. Además, se ha llevado a cabo una investigación extensiva en la materia, la cual ha demostrado que los antígenos del SARS-CoV-2 fabricados en plantas pueden ser producidos eficiente y económicamente. Diversos experimentos in vitro e in vivo han demostrado una actividad de unión a la célula hospedadora de los antígenos producidos en plantas comparable a los antígenos producidos en mamíferos y son capaces de inducir immunogenicidad. Así pues, los sistemas de expresión vegetales proporcionan una opción viable y rentable para los países en vías de desarrollo.

1. INTRODUCTION

1.1. General context and data

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus causing the COVID-19 human disease, which was first detected in Wuhan, China, in December 2019 and led to the ongoing pandemic. More than 83 million positive cases and 3 million deaths were recorded in the year following the initial outbreak, rising to more than 350 million cases and 5.6 million deaths by February 2022 (Johns Hopkins Coronavirus Resource Center 2022). Besides the global health crisis generated by this pandemic, a deep socioeconomic recession is currently going on as a direct consequence. These concerning data underlined the need of releasing prophylaxis treatments against the disease, fact that was reflected at the end of 2020 by the vaccine race in pharmaceutical corporations. By that time, the first vaccines were released and thousands of lives were estimated to be saved as a consequence of vaccination. Moreover, in order to control the epidemiologic evolution of the virus within the global population, and therefore develop mathematical predictions for the nearest future, detection is essential. Thus, the need for diagnostic tests and kits also exists.

1.2. Virus structure, characteristics and infection mechanism

Despite SARS-CoV-2 is causing a pandemic, this virus is not the first coronavirus affecting human beings. While SARS-CoV produced an endemic in China in the early 2000s, the Middle East respiratory syndrome (MERS-CoV) outbreak occurred in 2012, transmitted by camels. In fact, coronaviruses are a highly diverse family of viruses. They do not only infect bats and humans, but other mammals and avian species, including livestock and companion animals. The *Coronaviridae* family, which lies within the suborder of *Coronavirineae* and the order of *Nidovirales*, consists of four genera: *alphacoronavirus*, *betacoronavirus*, *gammacoronavirus* and *deltacoronavirus*. While alphacoronaviruses and betacoronaviruses exclusively infect mammalian species, gammacoronaviruses and deltacoronaviruses have a wider host range, including avian species. Coronavirus infections mainly result in respiratory and enteric diseases. From 7 known to affect humans, 4 cause mild colds and 3 can be lethal: SARS-CoV, MERS-CoV and SARS-CoV-2 (Cohen 2021). By infecting bronchial epithelial cells, pneumocytes and upper respiratory tract cells in humans, SARS-CoV, MERS-CoV and SARS-CoV-2 infections can develop into severe, life-threatening respiratory pathologies and lung injuries (V'kovski et al. 2021).

All coronaviruses have a single-stranded, positive-sense RNA and similar structural proteins: an envelope (E) protein, a membrane (M) protein, a nucleocapsid (N) protein

and a Spike (S) protein, the latter one being the crown's jewel: besides creating the distinctive crown-like appearance that earned the name, this protein is involved in the virus' infection mechanism and most of designed vaccines are focusing on this protein to develop protection against SARS-CoV-2. This S protein, consisting of 1,273 amino acids (Uniprot.org 2020) is cleaved in different domains: the S1 domain is the head part and the S2 domain is the "stem". Spike varies between coronaviruses and the most conserved region is the S2 domain. Within the S1 domain there is a region known as Receptor Binding Domain (RBD), which has been discovered to be crucial for host cell entry (Cohen 2021), thereby determining virus cell tropism and pathogenicity. The transmembrane S2 domain contains heptad repeat regions, particularly the hepeptide repeat 1 (HR1) and hepeptide repeat 2 (HR2), and the fusion peptide, which mediate the fusion of viral and cellular membranes upon extensive conformational rearrangements (Li et al. 2021).

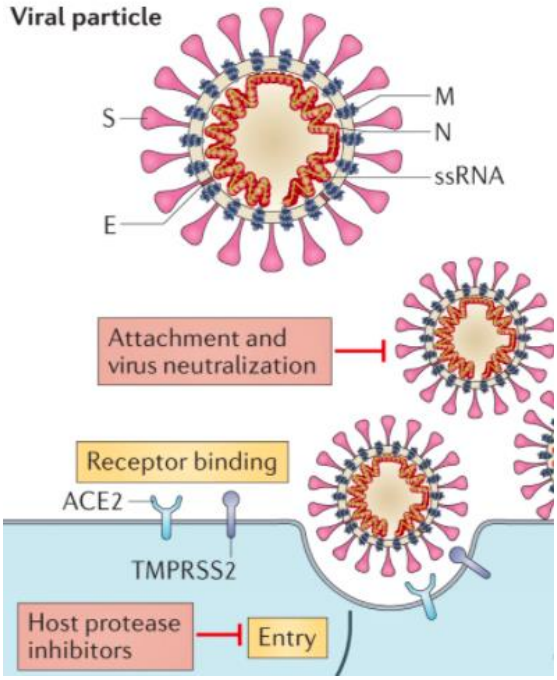

Figure 1: SARS-CoV-2 cell entry mechanism and key proteins involved in the process. Adapted from V'kovski. et al.. 2021

On the other hand, human angiotensin converting enzyme 2 (ACE2) was identified as the functional receptor that enables infection by SARS-CoV, as shown in Figure 1. (Lupala et al. 2022). The high genomic and structural homology between the S proteins of SARS-CoV and SARS-CoV-2 (76% amino acid identity) supported the identification of ACE2 as the cell-surface receptor for SARS-CoV-2. Remarkably, essential SARS-CoV contact residues that interact with ACE2 were highly conserved in SARS-CoV-2 as well as in members of the species SARS-related coronavirus that use ACE2 or have similar amino acid side chain properties. However, several residue changes in the SARS-CoV-2 RBD stabilize two virus-binding hotspots at the RBD-ACE2 interface, and therefore increasing its ACE2 binding affinity when compared with SARS-CoV (Shang et al. 2020b). SARS-CoV primarily targets pneumocytes and lung macrophages in lower respiratory tract tissues, where ACE2 is predominantly expressed, consistent with the lower respiratory tract disease resulting from SARS-CoV infection and the limited viral spread. By contrast, SARS-CoV-2 replicates abundantly in upper respiratory epithelia, where ACE2 is also expressed, and is efficiently transmitted.

The proteolytic cleavage of coronavirus S proteins by host cell-derived proteases is essential to permit fusion. SARS-CoV has been shown to use the cell-surface serine protease TMPRSS2 for priming and entry, although the endosomal cysteine proteases cathepsin B (CatB) and CatL can also assist this process. Concordantly, the simultaneous inhibition of TMPRSS2, CatB and CatL prevents SARS-CoV entry into the host cell in vitro. TMPRSS2 is expressed in the human respiratory tract and thus strongly contributes to both SARS-CoV spread and pathogenesis. Notably, SARS-CoV-2 entry relies mainly on TMPRSS2 rather than on CatB and CatL, as inhibition of TMPRSS2 was sufficient to prevent SARS-CoV-2 entry in lung cell lines and primary lung cells (V'kovski et al. 2021). In fact, cell entry of SARS-CoV-2 is preactivated by proprotein convertase furin, reducing its dependence on target cell proteases for cell entry (Shang et al. 2020a). In addition, more receptors are involved in the adhesion and invasion of SARS-CoV-2 in the human host, such as arginine-glycine-aspartic (RGD) motif found in cell surface integrins, as well as dipeptidyl peptidase 4 (DPP4), which might interact with the S1 domain (Gu et al. 2022).

The importance of coronavirus S protein-mediated receptor binding and temporally coordinated conformational rearrangements that result in membrane fusion make this process a prime target of innate and adaptive antiviral responses. Notably, a screen involving several hundred interferon-stimulated genes identified lymphocyte antigen 6 family member E (Ly6E) as a potent inhibitor of coronavirus fusion. Ly6E-mediated inhibition of coronavirus entry was demonstrated for various coronaviruses, including SARS-CoV-2, and seems to have decisive importance in protecting the hematopoietic immune cell compartment. Moreover, the exposure of S protein on the surface of the virion results in the induction of specific neutralizing humoral immune responses (V'kovski et al. 2021).

Coronavirus accessory proteins are highly variable sets of virus-specific proteins that display limited conservation even within individual species, but they are principally thought to contribute modulating host responses to infection and are determinants of viral pathogenicity. Nevertheless, the molecular functions of many accessory proteins remain unknown due to the lack of homologies to accessory proteins of other coronaviruses or to other known proteins (V'kovski et al. 2021).

These data suggest that, much like during the evolution of SARS-CoV, frequent recombination events between severe acute respiratory syndrome-related

coronaviruses that coexist in bats probably favored the emergence of SARS-CoV-2. Indeed, predicted recombination breakpoints divide the S gene into three parts. The middle part of the S protein (amino acid 1,030-1,651, encompassing the RBD) is most similar to SARS-CoV, which use human ACE2 as a cellular entry receptor (He et al. 2020). These observations highlight the importance of recombination as a general mechanism contributing to coronavirus diversity and might therefore drive the emergence of future pathogenic human coronaviruses from bat reservoirs. This emphasizes the need for surveillance to determine the breadth of diversity of SARS-related coronaviruses, to evaluate how frequently recombination events take place in the field and to understand which virus variants have the potential to infect humans (Cohen 2021).

Since the discovery of the first coronavirus (avian infectious bronchitis virus) in the 1930s and the discovery of the first human coronaviruses (HCoV-229E and HCoV-OC43) in the 1960s, the coronavirus research field has made substantial progress in understanding the basic principles of coronavirus replication and pathogenesis. This advancement was accelerated after the emergence of SARS-CoV in 2002 and MERS-CoV in 2012 and has broadened our view on coronaviruses as zoonotic pathogens that can severely affect human health. Moreover, the unprecedented speed and technical progress of coronavirus research that has become evident in a few months after the appearance of SARS-CoV-2 at the end of 2019 has led to a rapidly growing understanding of this newly emerging pathogen and of its associated disease, COVID-19 (V'kovski et al. 2021).

1.3. The need for vaccines and detection kits' reagents and their target proteins

Currently, 140 candidate vaccines are in clinical trials and 194 candidate vaccines are in preclinical trials (World Health Organisation (WHO) 2021). Produced vaccines elicit neutralizing antibodies against the S protein, and specifically against RBD (Jiang et al. 2020), therefore blocking virus uptake and achieving up to 96% protective efficacy against morbidity and mortality in phase 3 trials (Williams and Burgers 2021). The S protein/RBD is also used as a component of diagnostic kits for the detection of antibodies in vaccinated, infected and/or convalescent individuals and for the quantification of antibody titers, resulting in a requirement for inexpensive recombinant S-protein/RBD on a large scale. While first world countries arrange third and fourth doses of the available vaccines, more than 130 countries have yet to receive a single dose (He et al. 2021). In addition to this imbalance, to brought the disease under control, most of global population needs to be vaccinated or tested, thus leading to a

deep crisis for diagnostic reagents and therapeutics, revealing a critical shortage in the corresponding reagents and the means to produce them (Capell et al. 2020), as well as placing a massive pressure on the supply and distribution network worldwide (Shohag et al. 2021).

1.4. Possibility of plants as system to produce COVID-19 therapeutics

It is fair to say that the world has coped with the need for vaccines and diagnostics without plants so far, so it would be a stretch to claim that plants are needed to meet the short-term challenge of COVID-19 pandemic. Plants had a trivial role in SARS-CoV and MERS-CoV outbreaks, which never reached the pandemic status as in SARS-CoV-2 case. Despite of this, some studies displayed the expression of the SARS-CoV S protein in a plant expression system (Pogrebnyak et al. 2005; Li et al. 2006). Accordingly, it is necessary to focus on long-term production of vaccines and reagents to maintain coverage against current strains. Therefore, we need less expensive source of boosters and ensure we have capacity and preparedness for any new strains with emphasis and focus on Low- and Middle-Income Countries (LMIC).

Plant biotechnology offers a solution to overcome this production issues. Molecular farming advantages include low cost, greater scalability and intrinsic safety in comparison with fermented-based bacteria, yeasts and mammalian cells system (Rybicki 2020). Moreover, it permits proper eukaryotic protein modification and decreased cost for improved product accessibility to developing countries (Hemmati et al. 2022). Specifically, the ease of transporting seeds combined with the inexpensive cultivation, increasing availability of disposable equipment and portable infrastructure (Lobato Gómez et al. 2021) enables the provision of emergency testing infrastructure to the manufacture of vaccines, therapeutics and diagnostic reagents (Capell et al. 2020; McDonald and Holtz 2020; Rosales-Mendoza 2020; Tusé et al. 2020; Webb et al. 2020), which means that plants can be grown where needed to provide a local source of research even in LMIC settings. Such local production facilities could be paired with portable downstream processing suites when the product is intended for purification, as shown in Figure 2. Oral or topical application of plant tissues releases plants from the demands of extensive product purification, in some cases allowing the consumption of medicines as unprocessed or part-processed edible tissues (Sharma et al. 2021).

It is important to ensure that the technology and product can be made available to local stakeholders without the encumbrance of IP restrictions and onerous licensing

requirements, therefore allowing these valuable medicines to be delivered to those most in need (Drake and Thangaraj 2010). It requires almost five to six weeks to produce a plant-based vaccine compared to a five to six-month period preparing the vaccine in chicken eggs, which the various vaccine manufacturers are currently practicing (Kumar et al. 2021). Transient transformation approaches offer rapid methods (expression within a week) for the efficient expression of the target antigen or antibody (Rosales-Mendoza 2020) and would provide rapid access to antiviral proteins and the process can be scaled up to provide gram amounts within a few weeks (Capell et al. 2020). This will help to address the massive demand for research reagents, and could ultimately be used to produce vaccines and therapeutics on a sufficient scale for global distribution (Capell et al. 2020; Tusé et al. 2020). Concretely, tobacco is widely used to develop transgenic lines expressing pharmaceutical proteins, but leafy crops have the disadvantage of product instability during storage, meaning that immediate extraction and downstream purification is necessary unless the biomass can be frozen or dried (Hoelscher et al. 2018). Instead, transgenic plants could be used to provide a more permanent resource for even larger-scale production (Capell et al. 2020).

Despite it takes longer to develop products in transgenic plants, they allow the continuous, large-scale production of proteins, which is ideal for slowly spreading or established pandemic diseases and widely prevalent endemic diseases. In addition, the use of any edible tissue enables oral delivery with minimal processing or topical application as a crude extract, thus eliminating up to 80% of the costs of production (Lobato Gómez et al. 2021).

The world needs to go from zero production to making available multiple billions of tests in developed and developing country settings, which is where transgenic plants could provide a solution (Capell et al. 2020).

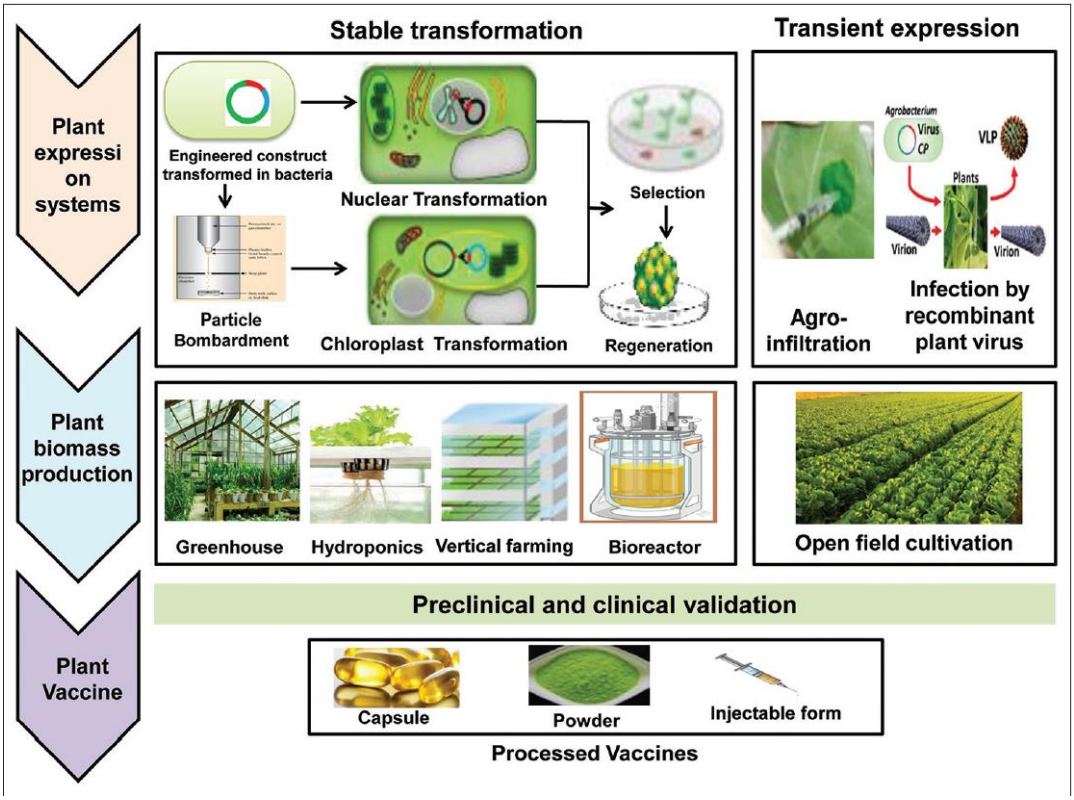

Figure 2: An overview of expression systems and down-stream processing for plant-based vaccines. Adapted from Sharma, et al., 2021.

2. OBJECTIVES

The aim of this bibliographic search is to acquire a deep understanding of SARS-CoV-2 infection mechanism and, therefore, accomplish an extensive comprehension on strategies used in vaccines and diagnostic kits production. Moreover, this project aims to display solid reasons to exhibit plants as a suitable expression and manufacturer system that can solve the production and infrastructure limitations when delivering these pharmaceuticals in low- and middle-income countries.

3. METHODOLOGY USED FOR THE REVISION

In order to develop this thesis, a strategic, extensive, bibliographic search was carried out. Certified databases such as PubMed, Scopus, Web of Science and Google Scholar were consulted to reach the information of interest. Applying different search filters depending on the search and criteria phase, diverse multidisciplinary research articles and reviews were obtained.

1. First stage of search: general overview on the topic; reviews and research articles regarding SARS-CoV-2 infection mechanism and background.
2. Second step: research articles regarding just expression of the SARS-CoV-2 molecules in plants
3. Third step: Research articles regarding other objectives such as scale up, yield increase and serological assays.
4. Fourth and Last step: Current stage of commercial plant-produced vaccines and their corresponding articles.

Some information was also gathered through the references of the reviews and papers found in a first search.

During the search' first step, the words *SARS-CoV-2 mechanism* were used in the mention databases. On the other hand, the words *SARS-CoV-2* plants were used in order to complete the second stage of the bibliographic search. Moreover, the words *SARS-CoV-2 Nicotiana benthamiana*' were also used since it is known that most of the research done in the plant biotechnology field is carried out in this species, as well as the use of *RBD plants*, *Spike protein plants* and *ACE-2 plants*, which are the main molecules involved in SARS-CoV-2 infection mechanism. These words were also useful to complete the objectives of the third phase of the search. In the last step, the words *vlp*[1] *SARS-CoV-2 plants* and *plant-based SARS-CoV-2* were useful to complete the information gathering. As for the Boolean operators, only *AND* was used to look for multiple keywords in the same article, but others such as *NOT* or *OR* can also be applied.

When regarding the articles' selection, abstracts were carefully read in order to achieve the objective of each step from the bibliographic search.

1 Vlp is the abbreviation of *Virus-like particle*, which is a useful methodology to develop a vaccine since it is based in the administration of small viral, fragmented proteins to immunize an individual.

Note that, since COVID-19 is a novel disease in humans, most publications concerning this topic were published no more than two years ago.

To finalise this section, it is also relevant to emphasise that other servers such as UniProtKB were used in order to understand the structure of the different proteins involved in SARS-CoV-2 infection mechanism.

4. STATISTICAL RESULTS

First of all, it is interesting to emphasize in the increasing interest in plant biotechnology among the years. The first results, around 1980s decade, match the invention of genetically engineering, the generation of the first genetically modified organisms, and therefore the utilisation of the term “biotechnology”. Since then, the interest on plant biotechnology research is increasing over the years, since it has been shown all the benefits it can provide.

Focusing on SARS-CoV-2-related research, plant biotechnology has also been relevant, as it can be shown in Figure 3. Regarding databases, the search engine that displayed the highest number of results was always Google Scholar, although most of these results can also be misleading and in most cases, the results did not match the matter of interest. On the other hand, PubMed and Scopus offered more results than Web of Science, being these three databases more accurate and specific than Google Scholar.

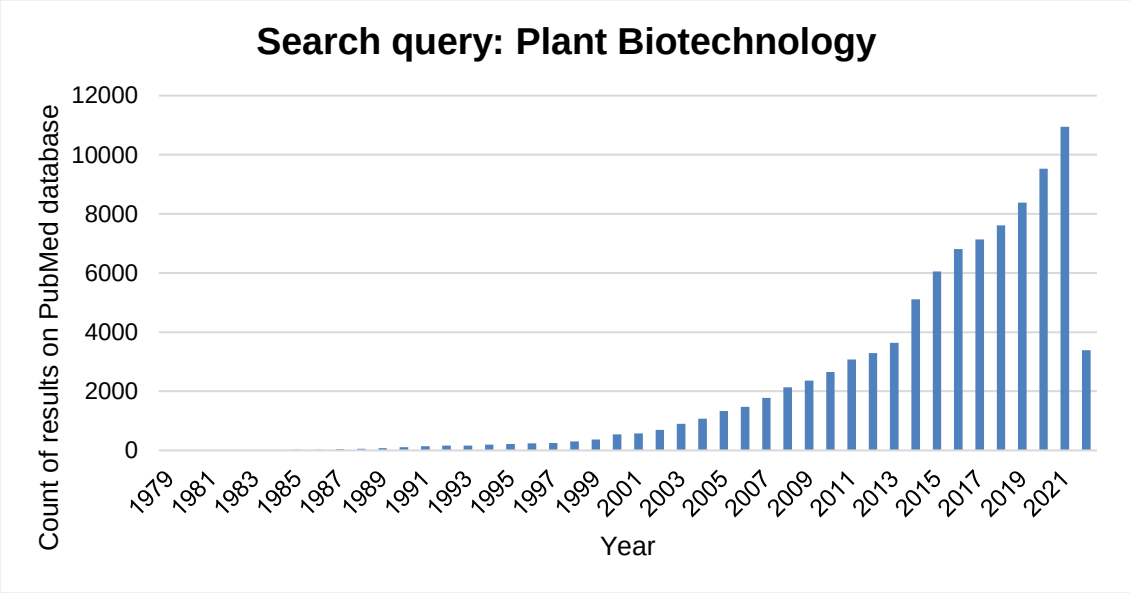

Figure 3: Graphic showing the evolution of publications containing the Plant Biotechnology topic since 1979 up to now concerning NCBI’s PubMed database

Concerning the type of molecule researched, Web of Science has been used to compare their different incidence on scientific literature. The results are displayed in Figure 4, emphasizing in the SARS-CoV-2 Spike protein that, due to the fact that is the key protein involved in host cell entry during virus infection. This fact is also demonstrated by the high results concerning RBD research, which is the specific domain in the Spike protein involved in infection. On the other hand, Angiotensin converting enzyme 2 (ACE-2) is also relevant for being the host cell receptor which binds the Spike’s RBD and allowing virus entry in the host cell. However, this result can

be misleading since ACE-2 is also the receptor for other coronaviruses such as SARS-CoV, which was studied hence the endemic in 2002, as well as being involved in many respiratory diseases such as pulmonary hypertension, that have been also studied before SARS-CoV-2 outbreaks. Finally, N protein and the specific anti-SARS-CoV-2 monoclonal antibodies (B38 and H4) are found in the ranking's last positions. The reason is probably because the main research on SARS-CoV-2 molecules is focused on a vaccine approach; in this case, RBD and Spike protein become more suitable vaccine candidates than N protein because of their specificity and immunogenicity.

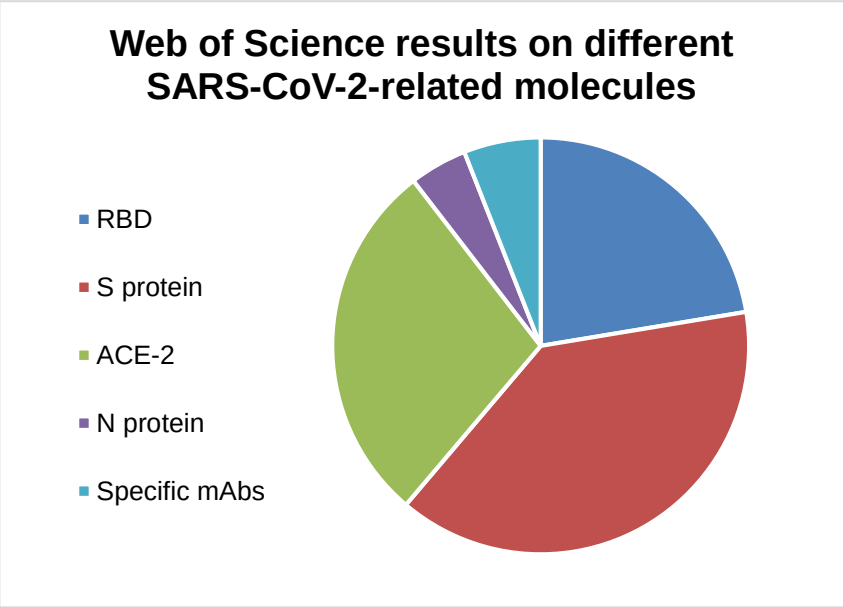

Figure 4: Pie chart that displays the different number of total publications on Web of Science database concerning each SARS-CoV-2 related proteins. Accordingly, there is more interest in S protein, ACE-2 and RBD proteins.

Moreover, plant-produced monoclonal antibodies are less suitable because do not have the same characteristics (post-translational modifications mainly) than the human-produced ones, and are more complex to produce for therapeutic purposes.

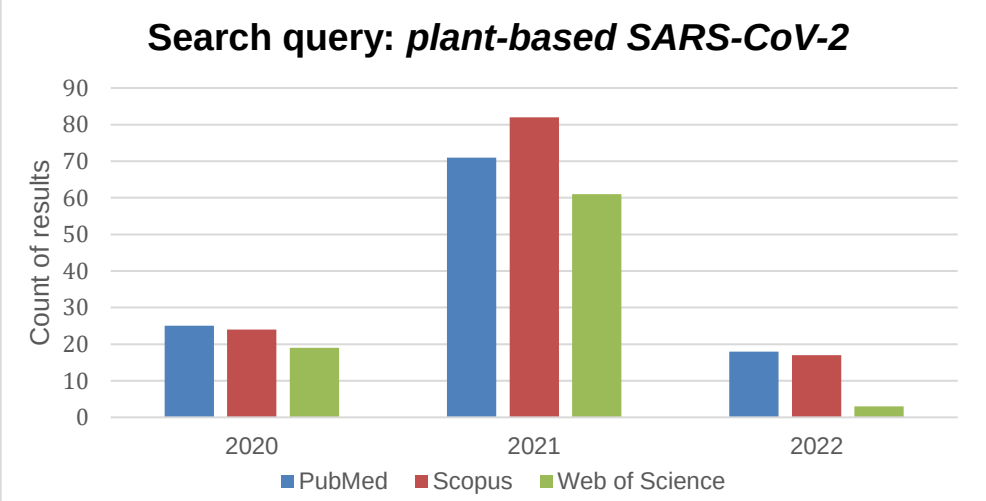

Figure 5: Graphic displaying the evolution of publications referred to plant-based products related to SARS-CoV-2 from three different databases across these last two years and up to now.

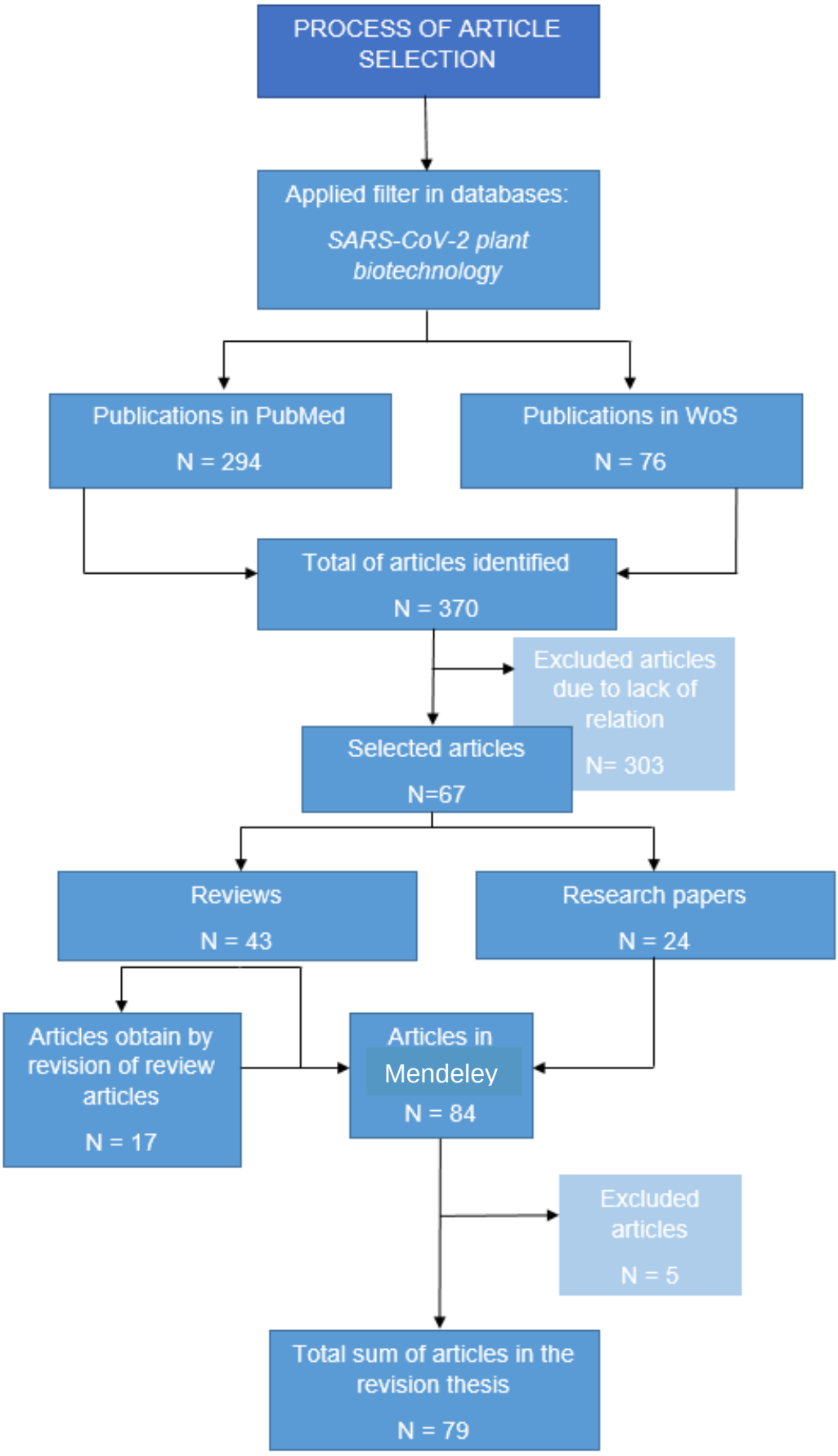

Figure 6: Schematic representation of the process of article selection

5. SCIENTIFIC RESULTS

5.1. Plants as a suitable system of production of therapeutics against SARS-CoV-2

Human beings have applied a selection of crop plants since the very beginning of the agricultural practice. Sorting the most suitable characteristics for human consumption and crop adaptation, people have been choosing specific random mutations in each plant across history. Once the genetic manipulation and engineering was developed, humanity became able to select the characteristics of interest in a faster and more accurate manner. This genetic engineering and editing methodology to select the plant of interest in order to produce a protein of interest is called “molecular farming”.

Why are plants necessary for vaccine production against SARS-CoV-2? Indeed, plants did not play a relevant role in past endemics or diseases, even in coronavirus diseases such as SARS-CoV and MERS-CoV outbreaks. Notwithstanding, SARS-CoV-2 outbreak resulted in a pandemic that humanity is still dealing with; while most of the first world countries had successful vaccination rates across the population, most of inhabitants of developing regions do not have the opportunity to obtain the vaccine. Despite of that, even if these countries were able to afford vaccines, they do not dispose of equipment to keep these vaccines in stock. This is a serious logistical issue, since if this problem is not solved it cannot be assured that humanity will avoid more SARS-CoV-2 outbreaks. If the virus keeps spreading across the population, the chances of it developing mutations that change proteins involved in the infection mechanism and therefore inactivating the current vaccines become higher and higher. Thus, the highest possible number of global populations must be protected against the virus in order to eliminate this thread in an effective manner.

According to this data, LMIC countries are the main target to stop the pandemic at this stage. Since the equipment in these areas is very limited, a solution should be found to make the vaccines arrive to the people. Therefore, the vaccine in question has to be resistant to the conditions in these countries, capable of keeping intact without a cold chain supply. At this point is where plants can be useful. By expressing the antigen proteins in the appropriate plant system, there would be no need for a cold chain or special equipment. The only requirement could be a purification system to obtain just the protein of interest to make an intravenous vaccine, but this could be optional if an edible vaccine is developed. Therefore, this is one of the greatest advantages of plants as an expression system. People from LMIC countries could just plant these vaccine crops and wait until the tissue carrying the protein is fully developed, such as the seeds or leaves, and get vaccinated.

Crop plants can be grown in diverse environments; therefore, biopharmaceuticals could be produced using already established infrastructures for agricultural production and the same distribution networks that exist for the supply of food and cereals seeds, without the need for a cold supply chain (Shohag et al. 2021); this approach is the most practical to perform large vaccination campaigns at low cost and under easy-to-implement logistics (Rosales-Mendoza 2020). For these many advantages plant biotechnology offers, several molecular farming companies specialize in the development of plant-derived proteins. As an example, Medicago, Inc. (Québec, Canada) has developed a platform using *Nicotiana benthamiana* as the production host and achieved the important milestone of producing more than 10 million doses of vaccine against H1N1 influenza in one month (D'Aoust et al. 2010). Equivalently, a passive immunotherapy approach was established by Mapp Biopharmaceutical (San Diego, CA, USA) during the 2014 outbreak of Zaire ebolavirus in West Africa, a cocktail of three neutralizing antibodies known as ZMapp. Moreover, Fraunhofer IME produced the HIV neutralizing antibody 2G12 in tobacco for testing in a first-in-human Phase I clinical trial (Capell et al. 2020). In addition, a study by (Marques et al. 2020) expressed a plant-produced Dengue Virus NS1 antigen with promising results for use as a diagnostic antigen.

Recent studies expressed SARS-CoV-2 proteins in plants, especially S glycoprotein and RBD, which displayed different binding activity depending some S specific antibodies (Rattanapisit et al. 2021), possibility of rapid production (Diego-Martin et al. 2020; Rattanapisit et al. 2020), production efficiency and immunogenicity depending on specific *N*-glycosylations (Mamedov et al. 2021c; Schwestka et al. 2021; Shin et al. 2021) and immunogenic properties on mice and non-human primates (Mamedov et al. 2020; Siriwattananon et al. 2021b), also along different adjuvant formulations (Siriwattananon et al. 2021c). Moreover, it has been tested the ease of using these plant-produced antigens not only as a vaccine, but as a COVID-19 detection kit (Makatsa et al. 2021; Pietschmann et al. 2021). Hence the characterization of SARS-CoV-2 antigen expressed in plants, some molecular farming companies announced their plant-produced pharmaceutics will reach the market soon. The Canadian company Medicago will use their platform for the rapid production of vaccines against SARS-CoV-2 composed of recombinant S glycoprotein and is estimated to be produced at a rate of 10 million doses per month (Philip Morris International 2020). Medicago Inc is currently at the forefront of SARS-CoV-2 vaccine development and its vaccine candidate has shown to be successful in phase I human clinical trials and is

currently undergoing phase 2 and 3 clinical trials (Ward et al. 2021). Similarly, iBio (TX, USA) are developing a vaccine in tobacco plants as well as in *Arabidopsis thaliana*, the latter being in the pre-clinical status (iBio 2022). Simultaneously, Kentucky Bioprocessing (Ownsboro, KT, USA) is developing a vaccine based on tobacco-expressed SARS-CoV-2 protein subunits (British American Tobacco 2020; Royal et al. 2021), which is in second phase of clinical trials (Maharjan and Choe 2021). There are four other candidate vaccines from Akdeniz University (Turkey), Shiraz University (Iran), and Baiya Phyto-pharm/Chula Vaccine Research Center (Thailand) that are in the pre-clinical stage and have used the plant as an expression system (Kumar et al. 2021). Additionally, other biopharma products are being designed, such as Medicago's COVID-19 Antibody 1 and Antibody 2 in *Nicotiana benthamiana*, John Innes Centre's RT-PCR based diagnostic control reagent by agroinfiltration in *Vignan unguiculota* and Leaf Expansion System's SARS-CoV-2 N Protein diagnostic reagent in *N. benthamiana* (Medicago.com). Several molecular farming companies specialize in the development of plant-derived proteins as diagnostic reagents, for example Agrenvec (Madrid), Diamante (Verona), ORF Genetics (Kópavogur, Iceland), and Ventria Bioscience/Invitria (Fort Collins, CO, USA) (Ortega-Berlanga and Pniewski 2022). As an example of the former approach, the Italian Biotechnology company Diamante is using tobacco to express antigens based on the SARS-CoV-2 RBD for use in ELISA tests for the detection of serum antibodies (Capell et al. 2020).

Most previous studies concerning expression of antigen/antibody proteins in plants used *Nicotiana benthamiana*, which is suitable in terms of speed of production. However, using rice as expression host means less infrastructure is needed when compared with *N. benthamiana* in front of certain weather conditions, which casually are often found in developing countries. Moreover, stable expression in rice enables reuse of land additional to food and is a cheaper system since no cold chain is needed. In addition, rice has recently proven versatile as a means to produce 2G12 along with two antiviral lectins (Vamvaka et al. 2018). These advantages make transgenic rice as an excellent candidate to cope with the long-term challenge of COVID-19 and future pandemics.

5.2. Plant-produced SARS-CoV-2 molecules for therapeutic applications

The general strategy for plant transformation to obtain plant-produced SARS-CoV-2-related molecules involves mainly the use of *Nicotiana benthamiana* as an expression

host. It has been also described the use of a glycosylation mutant *N. benthamiana*, the ΔXT/FT mutant, which lacks the core fucose and xylose residues that are typical from post-translational modifications in plant-produced proteins, and therefore generating a more accurate human-targeted protein. Indeed, such an expression host is suitable for the transformation method *Agrobacterium tumefaciens* by agroinfiltrating the bacteria containing the plasmid of interest in *N. benthamiana* leaves, although other expression systems such as virus-mediated expression have been used to produce SARS-CoV-2 accessory protein ORF8 (Imamura et al. 2021). Agroinfiltration transformation method obviously involves a transiently expressed product, since *A. tumefaciens* does not integrate its plasmid into the plant genome. This method allows rapid expression and results within days, which is suitable for research purposes.

The general strategy for analytical and clinical methods used to detect the expressed products consists of SDS-PAGE in reducing and non-reducing conditions, therefore comparing the native and denaturalized conformations of the protein; Western Blot analysis to confirm the protein is being expressed in the plant tissue; measurement of binding activity in order to check the appropriate folding and functionality of the plant-produced protein; purification by liquid chromatography column to obtain the yield and compare with the expression yield to calculate the recover and check whether this expression system can be used for commercialization; neutralization capacity against SARS-CoV-2, meaning a significant population has been immunized with the plant-produced protein and to check if the sera contains antibodies capable of neutralizing the virus, and therefore if the plant-produced product is immunogenic (is able to generate and immune response). Finally, there are few studies of serological assays with plant-produced products in order to detect how is the sera reacting.

5.2.1. Spike protein

SARS-CoV-2 Spike (S) protein has been a protein of interest in producing therapeutic treatments against COVID-19 since it is the main antigen that interacts with host cell receptor ACE-2. Many studies attempted to express the Spike protein in plants and knowing they could succeed do to the antecedents of expressing S protein of different coronaviruses in plants before, such as SARS-CoV, Infection Bronchitis Virus and TGEV, which affected humans or other mammalians such as swine (Gómez et al. 1998; Tuboly et al. 2000; Bae et al. 2003; Zhou et al. 2003; Lamphear et al. 2004; Pogrebnyak et al. 2005; Li et al. 2006).

These studies have confirmed that plant-produced SARS-CoV-2 S protein has a binding affinity to commercial ACE2, confirming its functionality and had the capability of induction of high levels of IgG specific antibody in mice (Mamedov et al. 2020), findings that confirm its immunogenic properties and the potentiality of prophylaxis.

On the other hand, since glycosylation is a key process in protein expression and very diverse patterns across species and kingdoms, Spike protein has been studied as in a glycosylated form and the deglycosylated form, this last one generated through the digestion with an EndoH enzyme which cleaves the glycosylation. The results found higher stability of glycosylated S1 (gS1) than deglycosylated S1 (dS1) protein (Mamedov et al. 2020).

As well as for serological assays and testing across a population, the development of a serological assay for the determination of SARS-CoV-2-specific antibodies in patient sera was carried out (Pietschmann et al. 2021).

Moreover, the development of optimized indirect ELISA with sensitivity and specificity as high as in the commercial S1 IgG ELISA kit (Makatsa et al. 2021) was also displayed, as well as the production of Spike VLPs with similar biological properties as to those of the parent virus (Jung et al. 2022).

5.2.2. Receptor Binding Domain

Spike's Receptor Binding Domain has been showed to be one of the most crucial elements involved in SARS-CoV-2 entry to the host cell and one of the most genetically variable proteins across coronaviruses. Therefore, it is the most studied SARS-CoV-2-related molecule by the moment, which has allowed a decent characterization of the protein.

Some studies showed that the plant-produced RBD is capable of the Production of humoral immunity in mice. The molecule could be properly expressed in tobacco plants regarding functionality and folding, as it was demonstrated by ACE-2 binding by ELISA. Sera from immunized mice protected Vero E6 cells from live SARS-CoV-2 (Maharjan et al. 2021). Moreover, another study suggested that plant-produced RBD fused to a mucosal adjuvant such as bacterial flagellin could be used for intranasal mucosal vaccines against COVID-19 (Mardanova et al. 2021).

Plant-produced RBD was also able to be recognized by CR3022 antibody, which is SARS-CoV-specific, and polyclonal antibodies from sera of SARS-CoV-2-infected individuals (Rattanapisit et al. 2020). Moreover, RBD binding to the ACE2 receptor was efficiently neutralized by antibodies from sera of SARS-CoV-2 infected patients, fact that demonstrated the correct recognition of plant-produced RBD to the antibodies (Demone et al. 2021).

In another study RBD alpha and beta variants fused with Fc protein were produced in *N. benthamiana*; its binding capacity to ACE2 had similar affinity than mammalian-produced RBD variants. Also, both variants exhibited the ability to bind to anti-SARS-CoV and anti-SARS-CoV-2 monoclonal antibodies, displaying a correct protein conformation and functionality (Rattanapisit et al. 2021).

When focusing on the impact of posttranslational modification in RBD transiently produced in *N. benthamiana*, a truncated RBD variant was generated. This RBD portion comprises from R319 to F541 AA from the original sequence and resolved the homodimerization issue that existed in full-length RBD, which was observed in Western Blotting and complicated the purification process. Because of this, a higher expression level was displayed compared to original RBD. Moreover, this RBD portion reacted with convalescent sera and was able to bind to commercial ACE2 receptor and CR3022 mAb. These results concluded that *N*-glycans are important for proper RBD folding, but differences in *N*-glycan processing had no effect on protein expression and function. N-glycosylation is crucial for RBD folding when transiently produced in *N. benthamiana*, but the degree of N-glycan processing and type of attached *N*-glycans are not important for RBD production and secretion (Shin et al. 2021).

Following the last study, truncated RBD-215 glycoforms were also generated. These glycoforms lacked a cysteine at position 538, which is believed to form disulphide bonds within monomers and therefore contributes in dimerization. According to the study results, RBD-215 glycoforms had similar binding affinity to ACE2 compared with the original RBD. In addition, the glycoforms were also tested as antigens in serological assays and concluded that the glycoforms were highly suitable to detect IgG and IgM antibodies in convalescent sera (Schwestka et al. 2021). Recently, plant-produced RBD glycoforms carrying blood group antigens were generated in a study, resulting in non-infected RBD-negative blood group O individuals have antibodies that strongly bind to RBD modified with blood group A antigen structures, suggesting that these

antibodies could provide some degree of protection from SARS-CoV-2 infection (König-Beihammer et al. 2022).

Lastly, RBD was also expressed in *N. benthamiana* as fully glycosylated, known as gRBD, and deglycosylated, known as dRBD (Mamedov et al. 2021c). This study resulted in deglycosylated variant RBD showing stronger binding activity to ACE2 than its glycosylated counterpart, although gRBD was more stable when expressed in *N. benthamiana*. Additionally, these glycoforms were tested in serological assays. The administration of both plant-produced antigens in mice induced high levels of IgG specific antibodies against RBD. Interestingly, mice sera immunized with the plant-produced deglycosylated RBD had a greater neutralizing activity against live SARS-CoV-2.

5.2.3. Nucleocapsid protein

Since the role of the Nucleocapsid (N) protein is not as relevant as the Spike protein in SARS-CoV-2 infection mechanism, less research has been made to express this protein in plants. However, some studies successfully expressed functional N protein in *N. benthamiana* and got higher purification yields when compared with plant-produced S and RBD antigens, confirming the difficulty in purifying the last ones (Diego-Martin et al. 2020; Lindsay et al. 2020; Mamedov et al. 2020; Williams et al. 2021).

5.2.4. Angiotensin Converting Enzyme 2

A study reported the expression of ACE2-Fc fusion protein in *N. benthamiana* (Castilho et al. 2021), which had better binding activity with RBD than ACE2-Fc produced in human cells. Moreover, the peptidase activity of ACE-2 was as efficient as human-produced ACE2-Fc. This study also confirmed that *N*-glycans from HEK293-produced ACE2-Fc are more processed and display increased microheterogeneity. The neutralization capacity of ACE2-Fc in pre-entry and post-entry treatments was also tested and resulted in better inhibition in the post-infection treatment (Siriwattananon et al. 2021a).

Glycosylated and deglycosylated forms of ACE2 were also generated in *N. benthamiana* according to another study (Mamedov et al. 2021b, a). gACE2 displayed higher neutralization potency than dACE2. However, dACE2 bound strongly to the S protein than the gACE2.

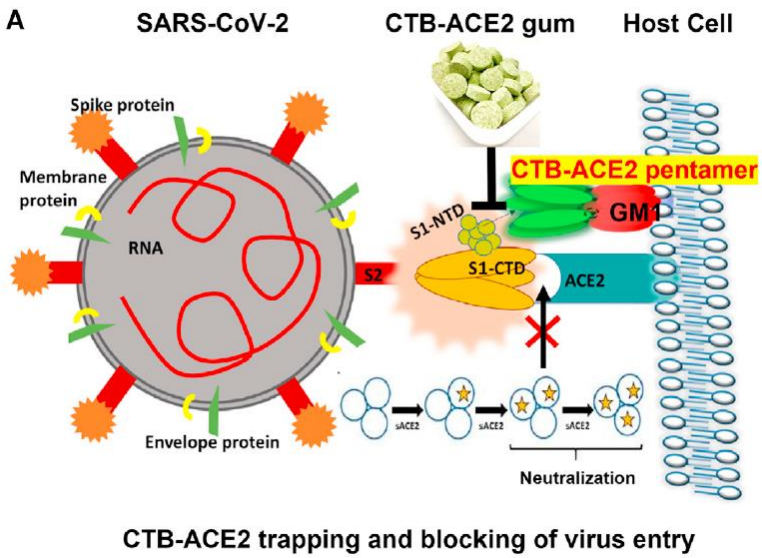

Figure 7: Visual representation of debulking and blocking of viral entry using ACE2 chewing gum. Adapted from Daniell, et al., 2021.

Interestingly, Daniell et al., (2022), expressed ACE2 fused with Cholera Non-Toxic subunit B (CTB) in lettuce, generating CTB-ACE2 fusion protein, similarly to their work on Angiotensin (Ang1-7) to attenuate pulmonary hypertension (Daniell et al. 2021). The role of CTB is to allow oral delivery by protecting ACE2 from degradation. The aim of their work was to lyophilize the product and to generate a gum able to minimize SARS-CoV-2 transmission by capturing viral particles with the binding of the ACE2 from the gum with the virus (Figure 7). In this case, the fusion protein was successfully stably expressed in lettuce and a chewing gum based on this plant-produced protein was developed. The study resulted a decreased amount of N antigens, a high neutralization activity against spike-mediated viral infection, thus confirming effectiveness in avoiding virus transmission. In fact, CTB-ACE2 could inhibit viral particles by 85%. However, ACE2 activity in presence of SARS-CoV-2 infected saliva was completely inhibited: spike protein could bind directly to ACE2 through the RBD, concluding that the gum could not avoid the virus once the individual is infected.

5.2.5. Human antibodies against SARS-CoV-2: B38 and H4

Specific antibodies against SARS-CoV-2, B38 and H4, have also been expressed in plants because of their potentiality as cheap diagnostic kits and testing for COVID-19 across the population. H4 antibody was isolated from a convalescent COVID-19 patient and was discovered to specifically neutralize SARS-CoV-2, *in vitro,* by blocking the interaction between RBD and ACE2. Early in the pandemic, a SARS-CoV neutralizing mAb (CR3022) against RBD was found to cross-react with SARS-CoV-2 (Meng et al. 2020). However, CR3022 does not neutralize SARS-CoV-2 because, unlike clone H4,

it may not target the ACE2 binding motif in the RBD. Interestingly, synergistic neutralization ability was observed when clone H4 was used in combination with a different epitope-targeting antibody, clone B38, identified in the same study. This makes them a potentially promising virus-targeting antibody cocktail for therapeutic and/or vaccine purposes (Tian et al. 2020).

The first reported study to express these SARS-CoV-2-specific antibodies in a plant system (Shanmugaraj et al. 2020) obtained functional proteins, with proper binding activity and neutralization capacity against SARS-CoV-2 in vitro. However, in contrary to the present results, the plant-produced CR3022 can effectively bind with RBD of SARS-CoV-2 but did not neutralize SARS-CoV-2 in vitro, which could be due to the fact that epitope of CR3022 does not overlap with the ACE2 binding site (Rattanapisit et al. 2020; Tian et al. 2020). Unlike CR3022, mAbs B38 and H4 exhibit competition with ACE2 for RBD binding and recognize different epitopes on the RBD of SARS-CoV-2, allowing them to effectively neutralize SARS-CoV-2 by preventing the virus binding with the cellular receptor ACE2 (Yan et al. 2020). In order to effectively control the spread of the infection, a therapeutic approach based on mAbs should provide sufficient breadth of protection against different strains of SARS-CoV2. In such cases, a single mAb might not be sufficient to prevent viral escape and extend the breadth of protection. Hence, the application of two or more mAb cocktails for effective passive immunoprophylaxis is needed, which requires a cost-effective platform to manufacture such mAbs, so that it can be accessible outside the developed world.

Sun et al., (2021) transiently expressed both of these antibodies in *N. benthamiana*, including the different isotypes IgG1, IgA1 (monomers) and IgA1 (dimers), all of them exhibiting appropriate binding activity and functionality and resulting in H4 displaying higher binding activity than B38. Moreover, higher neutralization potency was detected in H4-IgA1 dimers and B38-IgA1 dimers compared with their monomeric counterparts, suggesting that structural features such as the long hinge region in dimeric form and multivalency of IgA1 positively impact neutralization potency.

On the other hand, H4-IgG1-4 isotypes were also expressed in *N. benthamiana* and the four antibodies displayed highly homogeneous Fc glycosylation profiles (Kallolimath et al. 2021), contrary to their mammalian-produced counterparts. However, when looking at neutralization potency, H4-IgG3 outstands among the other antibodies, suggesting that superior neutralization might be a consequence of cross-linking the Spike protein on the viral surface.

6. DISCUSSION

Whereas in first world countries seem to be done going through the COVID-19, the disease is still a threat in developing countries, given that they cannot afford treatments. Allowing the virus spreading also means permitting SARS-CoV-2 to be able to develop some kind of mutation that, in the end, could lead to the current vaccines' ineffectiveness; besides this, plant-based vaccines can offer a more economical solution to these countries, as well as solving the logistical problem of maintaining millions of doses in highly expensive installations and waiting to be administered.

Findings of the bibliography search proved that this is a topic of interest and several research groups are working on the issue. Most of the groups are focused on SARS-CoV-2 receptor binding domain, since it is the main element interacting with host cell receptor ACE2, and thus allowing virus entry to the cell. Interestingly, modifying the protein sequence in very specific amino acids let to an important increasing of both expression and purification yields, and accomplished to gain similarity to mammalian-produced proteins by improve post-translational processing. Indeed, it is a relevant fact due to the several complications in purifying RBD and obtaining a decent yield which could allow the system to scale up and demonstrate it validity for commercial purposes.

Indeed, this vaccine is produced from a genetically modified (GM) plant, which might be seen as controversial by some social groups. This vaccine, therefore, represents one of the controversies in bioethics: genetically modified organisms or GMO. This debate, however, is pointless, since several pharmaceutics products are generated in GMOs, being bacteria, plants and animals, among others. Unfortunately, organisms such as the EU, which has one of the most restrictive legislatures in GMO regulations, avoid this matter. While countries such as the US, Brazil and India make great economical profit of GM products, the EU still resist and still has to import most GM products, therefore spending money on products that could be produced locally.

This tactical bibliographic search has evidenced several efforts in producing SARS-CoV-2 proteins in order to demonstrate that plants are a suitable system for industrial production of vaccines and diagnostic kits for COVID-19 disease. However, it remains to be seen whether another pharmaceutical company will get behind plant-based vaccines and diagnostic tests against COVID-19. Additionally, the impact that this first plant-made vaccine will have is a matter of interest in fighting against the pandemic and SARS-CoV-2 evolution in the future

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