Analysis of N-Glycans linked to receptor binding domain of SARS-Cov-2 produced in *Nicotiana benthamiana*

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Abstract

The COVID-19 pandemic caused by the SARS-CoV-2 coronavirus continues to affect millions of people around the world. To combat the spread of COVID-19, it is urgent that the scientific community and pharmaceutical companies implement technological platforms for vaccines production. The receptor binding domain (RBD) present in the viral surface spike protein was transiently expressed in *Nicotiana benthamiana*, with the signal peptide from the sporamine protein of *Ipomea batata* L. N-glycosylation of RBD is critical for the virus infectivity. The characterization of the RBD N-glycosylation produced in *N. benthamiana* showed high content of paucimannoside N-glycans, which contain fucosylated and xylosylated structural motifs, typical of plant secretory pathway protein trafficking. This glycosylation pattern did not affect RBDr reactivity in serological assays of immunized mice according to the previous study. Therefore, the transient expression system in plants can be considered as a safe, fast and valuable alternative to produce antigens of pharmaceutical interest allowing a prompt response to virus evolution during a pandemic.

Resumen

La pandemia de COVID-19 causada por el coronavirus SARS-CoV-2 sigue afectando a millones de personas en todo el mundo. Para combatir la propagación de COVID-19, es urgente que la comunidad científica y las empresas farmacéuticas implementen plataformas tecnológicas para la producción de vacunas. El dominio de unión al receptor (RBD) presente en la proteína de espiga de la superficie viral se expresó transitoriamente en *Nicotiana benthamiana*, con el péptido señal de la proteína esporamina de *Ipomea batata* L. La N-glicosilación de RBD, es crítica para la infectividad del virus. La caracterización de la N-glicosilación de RBD producida en *N. benthamiana* mostró un contenido alto de N-glicanos paucimanosídicos, que contienen motivos estructurales fucosilados y xilosilados, típicos del tráfico de proteínas de la vía secretora de plantas. Este patrón de glicosilación no afectó la reactividad de RBDr en los ensayos serológicos de los ratones inmunizados según el estudio anterior. Por lo que se puede considerar el sistema de expresión transitorio en plantas como una alternativa segura, rápida y de valiosa contribución para la producción de antígenos de interés farmacéuticos que permiten responder con prontitud a la evolución de los virus durante una pandemia.

INTRODUCTION

The current global pandemic of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causing COVID-19 continues to affect public health worldwide. Efforts to find an effective vaccine to limit the transmission of the virus involve different technologies, including plant-based vaccines (Moon et al., 2022; Ortega-Berlanga & Pniewski, 2022). N-glycosylation plays a significant role in the structure and functions of membrane and secreted proteins respectively. The spike protein on the surface of SARS-CoV-2 is a major target for the development of vaccines, therapeutic drugs and diagnostic tests. The SARS-CoV-2 spike trimer contains 66 N-glycosylation sites that are highly occupied with N-glycans (Watanabe et al., 2020). The S1 subunit comprises the head of the molecule containing the receptor binding domain (RBD) that serves to bind angiotensin-converting enzyme 2 (ACE2) on the human cell surface. The RBD has two N-glycosylation sites (N331 and N343) that are fully glycosylated when expressed in heterologous expression systems (Shajahan et al., 2020; Watanabe et al., 2020; Antonopoulos et al., 2021; Limonta-Fernández et al., 2021). For coronaviruses, N-glycans are important for spike protein folding, modulating the accessibility to host proteases, shielding to avoid the detection by the immune response of infected individuals, and interaction with cellular receptors (Guo et al., 2021).

During the last decades, plant-based expression systems have emerged as a new productive alternative for recombinant proteins due to several advantages when compared to traditional systems (bacteria, yeast species, insect or mammalian cells, and transgenic animals). Including rapid production, high scalability, low cost, safety, and the ability to produce multimeric and glycosylated proteins (Obembe et al., 2011). It is worth highlighting the example of the recombinant enzyme produced in carrot cells that was approved by the Food and Drug Administration (FDA) for the treatment of Gaucher disease (Tekoah et al., 2015).Also cited are the plant-manufactured scFV mAb used in the production of a recombinant HBV vaccine in Cuba (Ramírez et al., 2003), and the Newcastle disease virus (NDV) vaccine for poultry approved by the Department of United States Agriculture (USDA) (Naderi & Fakheri, 2015). Recombinant SARS-CoV-2 RBD has recently been expressed in *N. benthamiana* using different transient expression systems (Diego-Martin et al., 2020; Mamedov et al., 2020; Makatsa et al., 2021; Ceballo et al., 2022; Rattanapisit et al., 2022).

Currently, new generation vaccines, known as subunit vaccines, are also being investigated in plants. These are designed from components of viruses or bacteria, which stimulate the immune system and have advantages over existing conventional vaccines. Subunit vaccines are more stable and safer, with no danger of both disease and adverse effects due to the presence of only the specific antigens, thus rejecting other components that may be pathogenic. In addition, by using only a part of the infectious microorganism, they cannot replicate in the host and there is no risk of pathogenicity. Also, since only a few components are included in the vaccine, they present less antigenic competition. They allow vaccines to be obtained from pathogens that cannot be grown in the laboratory, since only the genetic material is required. This means a low production cost and an increase in safety, since risks for production personnel are avoided by not handling live pathogenic organisms. As they are purified preparations of certain components of the microorganism, they guarantee the absence of contamination with foreign proteins or nucleic acids, and help us to obtain less reactogenicity (de Pinho Favaro et al., 2022).

There are plant-derived COVID-19 subunit vaccine candidates, specifically coronavirus virus-like particles (CoVLP) and Kentucky Bioprocessing (KBP)-201 that are in clinical trials and preclinical stage. Results from the interim phase 2 clinical trial have revealed the high safety and efficacy of the CoVLP vaccine, with 10-fold higher neutralizing antibody responses compared to those present in convalescent patient plasma (Maharjan & Choe, 2021). In addition, Medicago's vaccine mimics the surface structure of the natural

SARS-CoV-2 virus with an antigenic moiety, is safe, and can stimulate an immune response against the virus when administered to humans (Ward et al., 2021).

The expression of glycoproteins in plants is characterized by the formation of complex, paucimannosidic and Lewis A structures. Complex-type glycans containing α1,3-fucose and β1,2-xylose are the most abundant glycan species, and are generated in the Golgi apparatus by α1,3-fucosyltransferase and β1,2-xylosyltransferase, respectively (Wilson et al., 2001). Paucimannosidic glycans, comprising truncated structures in which the terminal N-acetylglucosamines have been removed from the glycan core, are also observed in vacuolar and extracellular glycoproteins due to processing by β-hexosaminidases that localize to these sites (Shin et al., 2021).The Lewis A epitope, is characterized by the presence of β1,3-galactose and α1,4-fucose extensions of the glycan-terminal N-acetylglucosamines (Fitchette-Lainé et al., 1997; Wilson et al., 2001; Shin et al., 2021).

In this report, the N-glycans associated with the RBD SARS-COV2 protein produced in *N. benthamiana* (RBDr) were studied. It is novel to emphasize that the N-glycans identified were mainly of the paucimannoside type, with no studies in the literature reporting the association of these glycans with RBD. In addition structural motifs, β1,2 xylose and α1,3 fucose characteristic of plant glycoproteins, were identified. These results are particularly useful, since they will allow the prediction of sugars bound to recombinant proteins that transit through the secretory pathway of the plant and their implication in immunogenicity.

Materials and Methods

Expression of the RBDr

Nicotiana benthamiana plants were obtained and transiently vacuum transformed as described by Ceballo et al. (2022), using the pCambiahis-RBDapo vector, which carried the sequence of the RBD (amino acids 331-530 of the Spike protein from SARS-CoV-2, strain Wuhan-Hu-1 (NCBIAcc. No. YP_009724390)) and contained the sweet potato sporamine signal peptide. The expression levels of the RBDr were determined following the protocol reported by Ceballo et al. (2022). The transcriptional unit also carried the 5' and 3' untranslatable sequences (UTR) of Cowpea mosaic virus, provided by Sainsbury & Lomonossoff (2008).

Preparation and analysis of N-glycans

The RBDr purified by IMAC was concentrated and separated using SDS-PAGE in polyacrylamide gel at 12.5% under reducing conditions (Figure 1). The purity and molecular size of RBDr were estimated by a GS-800 densitometer (Calibrate Densitometer, Bio-Rad, USA) and the Imagen Lab 6.1 software. The bands of protein (corresponding to RBDr confirmed by Western blot) that were stained using Coomassie blue (Figure 1a, b), were cut from the gel and divided into small cubes of approximately 1 mm^3. The fragments of the gel were then washed with Milli-Q water for 5 min and incubated at 37 ºC with acetonitrile (Sigma-Aldrich, USA) at 50% in ammonium bicarbonate 1%, pH 8.3, until the fragments of the gel were completely decolorized. These fragments were dried in a vacuum evaporation centrifuge (Concentrator Plus, Eppendorf, USA) and were rehydrated in ammonium bicarbonate at 25 mM that contained trypsin (sequencing grade) (Promega, USA) at 12.5 ng/µL. The digestion in the gel was performed for 16h at 37 ºC. The resulting proteolytic peptides were extracted in 30 µL of ammonium bicarbonate at 25 mM for 30 min at 24 ºC.

The resulting mixture of peptides and glycopeptides were deglycosilated with 500 units of PNGase A for 24h at 37 °C (Royle et al., 2006). The N-glycans released were purified through GlycoClean H (Glyko, USA) columns, following the manufacturer's instructions. The samples of the N-glycans were dried in a vacuum evaporator and centrifuged without heating for the labeling of 2-aminobenzamide (2AB). The oligosaccharides were separated according to Guile et al. (1994), coupled to a normal-phase liquid chromatography, Amida-80 (TSK gel 250x46 mm, 5 µm, Tosohaas, Japan) and the mobile phase used was ammonium formiate 50 mM pH 4.4 and acetonitrile.

The detection of the 2-AB derivatives was carried out in a fluorescence detector at excitation wavelengths (λexc=330 nm) and emission wavelengths (λem=420 nm). N-glycans structures were confirmed by exoglycosidses digestion using α-fucosidase (in excess), β-galactosidase and β-hexosaminidase. The proposal of the oligosaccharide structure was determined from the experimental values of the glucose units (GU) calculated from the dextran hydrolysate, separated under the same conditions as the problem sample, with the aim of expressing the retention time in glucose units (GU). The values of experimental GU were compared with the values reported in GlycoStore, https://glycostore.org for N-glycans characterized through digestions with exoglycosidases (Zhao et al., 2018).

Results

N-glycosilation of RBDr

The identification of RBDr N-glycans was carried out on an isolated sample from a polyacrylamide gel. The molecular weight of RBDr estimated from SDS-PAGE and Western blot (Figure 1a, b) was approximately 28 kDa, higher than the 24.1 kDa calculated from the amino acid sequence. This increase (4.5 kDa) could be related to the presence of N-glycans. After the sample was processed, the mixture of 2AB oligosaccharide derivatives was analyzed by normal phase high performance liquid chromatography (Figure 2). Taking as a reference a partial hydrolysate of dextran, we calculated the GU value of the peaks identified in the chromatograph. GU is a parameter that indicates the size of the N-glycans. The structural assignment was made through the comparison of the experimental GU values with the theoretical values reported in the GlycoStore database (Zhao et al., 2018).

The relative abundance of each one of the structures is shown in Table 1. The N-glycans identified were mainly of the complex type (91.5%) which can contain both structural motifs, characteristic of the plants glycoproteins, β1,2 xylose and α1,3 fucose. The fucosylated structures comprise 66.6%, the xylosylated structures comprise 73.1% and 61.3% of the complex structures contain β1,2 xylose and α1,3 fucose. The oligomanosidic structures (M3-M9) are a minority (5.3%). The majority abundance corresponds to F(3)XM3 (28.8%) and 2.4% are related to structures only fucosylated in the antennas. Only a 3.2% of the structures detected could not be assigned by the GU values.

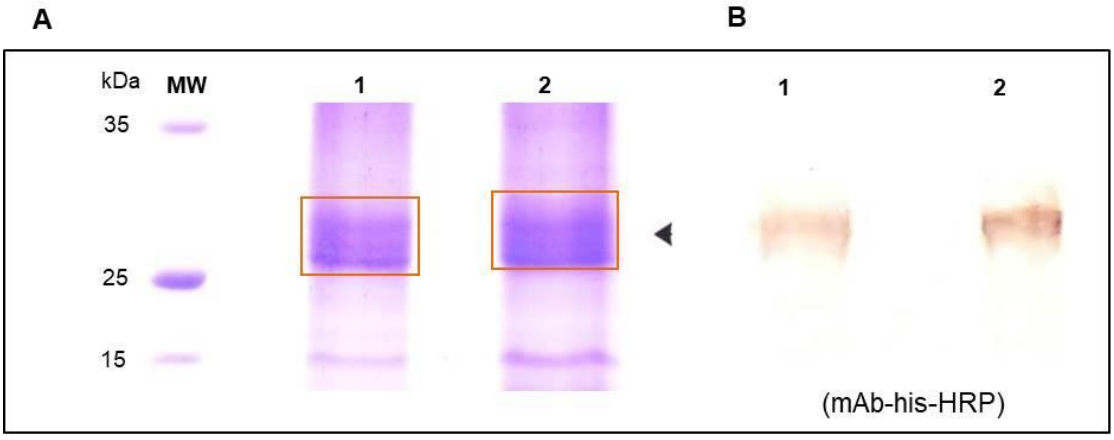

Figure 1. *RBDr sample from SDS-PAGE gel to proceed for N-glycans determination.* ***A:*** *Separation of the proteins in 12.5% polyacrylamide gel under reducing conditions. 1 and 2: purified RBDr. MW: molecular weight standard (Broad Range Protein Molecular Markers, cat V89A, Promega, USA). The arrowhead points to the RBDr in the sample analyzed.* ***B:*** *Western blot with anti-histidine peroxidase antibody (1:2000) to detect the protein of interest*

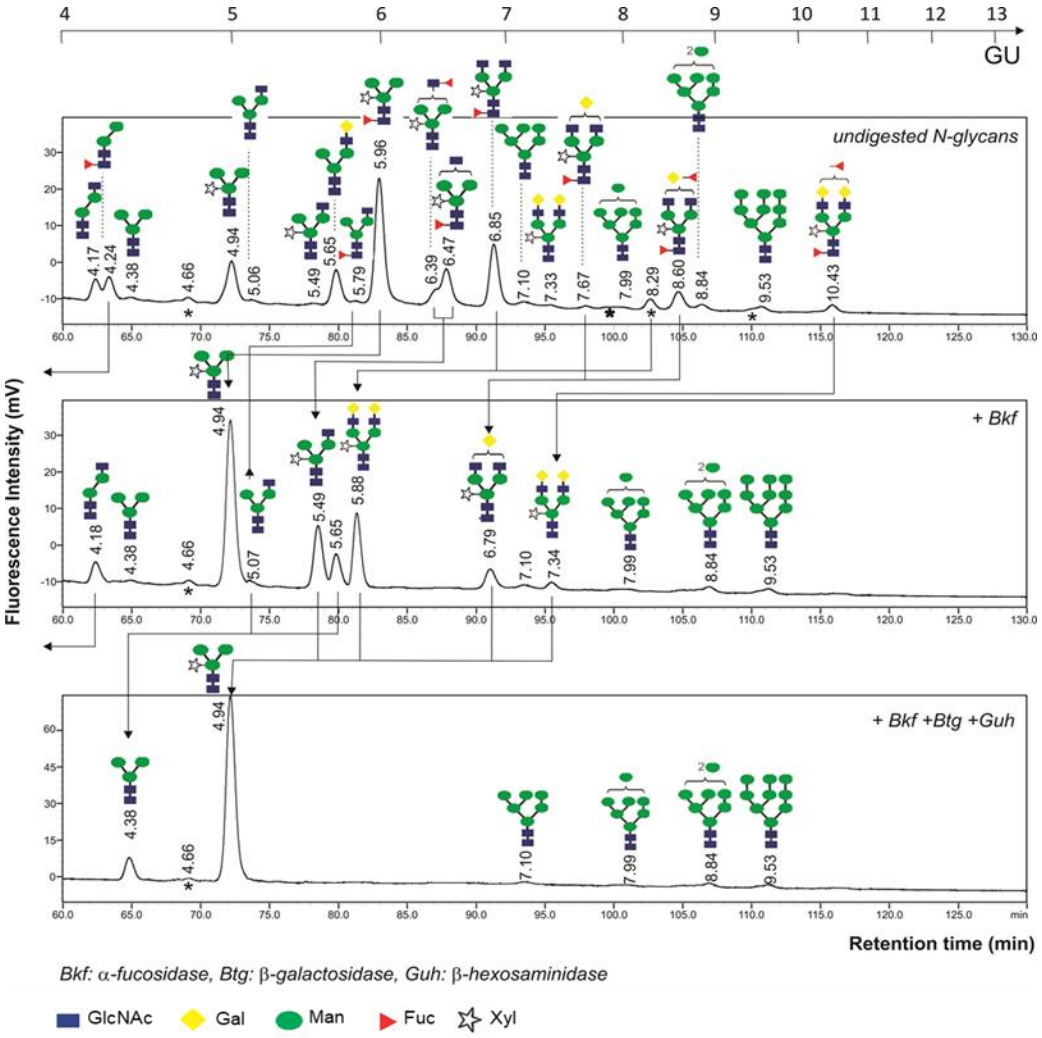

Figure 2. *Profile of N-glycans of the purified RBDr. Separation in normal phase high performance liquid chromatography using an Amide 80 column of the N-linked RBDr oligosaccharides derivatized with 2AB. The values of the experimental GU (top profile) of each one of the fractions of the chromatogram were calculated from the retention time, using a partial hydrolyzate of dextran as the reference. The nomenclature used to represent the N-glycans is shown in the legend. The asterisks represent unidentified structures. GlcNAc: N-acetyglucosamine; man: mannose; gal: galactose; xyl: xylose; fuc: fucose. The symbols described by (Harvey et al., 2009)*

Discussion

The plant-derived RBD showed a band around 28 kDa in SDS-PAGE assays. This electrophoretic migration of RBDr could be related to the N-glycosylation of the recombinant protein, in its transit through the secretory pathway. This phenomena is common when a recombinant protein containing consensus sequence N-X-S/T (where X is not proline), context is produced in host like yeast (Wang et al., 2020), mammalian cells (Sinegubova et al., 2021) or plants (Hamorsky et al., 2015; Ponndorf et al., 2021);, where the N-glycosylation machinery is present, while bacteria such as *E. coli* does not have this modification machinery. However, it was not possible to confirm that presence through the electrophoretic separation of the N-deglycosylated RBDr, because the release of the N-glycans from the plants glycoproteins with PNGase A requires

a previous digestion step using trypsin or pepsin. This technique is time consuming and requires complicated purification steps of the released N-glycans (Strasser et al., 2004).

Table 1. Estimated N-glycan structures corresponding to RBDr. Each oligosaccharide structure is represented with its respective relative abundance. NI: Unidentified structures. The most represented structure is shaded. The letters: A: antenna; M: mannose; X: xylose; F: fucose; G. galactose; RT: retention time

RT	Exp. GU	Theor. GU	Structure	Representation	Area	Abundance (%)
62.4	4.17	4.18	A1M2		235809	4.2
63.3	4.24	4.29	F(3)M2		262093	4.7
64.9	4.36	4.41 ±0.13	M3		23525	0.4
68.7	4.66	NI	NI	NI	13733	0.2
72.2	4.94	4.92±0.021	M3X		612505	10.9
73.7	5.06	5.05±0.112	A1[3]		49737	0.9
75.4	5.23	5.28±0.111	M4		4052	0.1
78.2	5.49	5.47±0.042	XA1		13287	0.2
79.8	5.65	5.66±0.072	A1G1		443620	7.9
81.3	5.78	5.72±0.028	F(3)A1		35605	0.6
83.1	5.96	5.91±0.006	F(3)XM3		1625023	28.8
87.1	6.39	6.39	XA1F(4)1		134308	2.4
87.8	6.47	6.42 ±0.026	F(3)XA1		529885	9.4
91.3	6.85	6.85	F(3)XA2		811872	14.4
93.4	7.10	7.07	M6		120777	2.1
95.3	7.33	7.3	XA2G(3)2		48216	0.9
97.9	7.67	7.65	F(3)XA2G(3)1		23023	0.4
99.8	7.91	NI	NI	NI	26524	0.5
100.5	7.99	7.94±0.05	M7		23655	0.4
102.6	8.30	NI	NI	NI 	119896	2.1
104.7	8.60	8.6	F(3)XA2F(4)1G(3)1		250859	4.5
106.4	8.85	8.85±0.169	M8		67936	1.2
110.2	9.46	NI	NI	NI	22033	0.4
110.7	9.54	9.52±0.069	M9		57815	1.0
115.8	10.43	10.45	F(3)XA2F1G2		79859	1.4

Furthermore, large amounts of starting plant material are needed to perform the deglycosylation protocol (Karg et al., 2009).

N-glycosylation is one of the main post-translational modifications essential for the structure and function of proteins, playing an important role for proteins stability and structure by avoiding proteolytic degradation and aggregation (Zhou & Qiu, 2019). Plant glycans may contain an α(1,3)-fucose linked to the proximal N-acetylglucosamine (GlcNAc) residue and/or a β(1,2)-xylose residue. These glycans differ in structure from those of mammals (Aviezer et al., 2009), however correlation between the presence of plant glycans specific IgE and clinical effects has been not demonstrated (van Ree, 2002; Foetisch et al., 2003). In this paper, in order to study the sugars bound to RBDr, we carried out an analysis of the profile of the N-glycans, where there was a major prevalence of complex structures containing α1,3 fucose and β1,2 xylose. Similar evidence, with a preponderance of xylosyled and xylosyled-fucosyled glycoforms, was also found in the expression of secretory IgA antibodies in wild type *N. benthamiana* (Teh et al., 2021). These results confirm those described in the transport of recombinant proteins through the Golgi apparatus, where xylosylation and fucosylation occur mainly in the medial and trans cisterns, respectively. Moreover, the transfer of β1,2 xylose to the core of the glycan occurs before adding α1,3 fucose (Fitchette-Lainé et al., 1994; Pagny et al., 2003; Sourrouille et al., 2008). Specificity studies of the substrate of the α1,3 fucosyl transferase and the β1,2 xylosyl transferase have demonstrated that the presence of at least one N-acetyglucosamine (GlcNAc) residue is required to transfer xylose and/or fucose residues to N-glycan. The α1,3 fucosyl-transferase and the β1,2 xylosyl-transferase are located in the secretory route after the mannosidase II of Golgi and glucosaminyl transferase II. If the mannosidase II does not act, the N-glycan substrate would be of hybrid type, in which fucose and xylose could be assigned, as well as mannose residues (Strasser et al., 2006; Liebminger et al., 2009; Kajiura et al., 2010).

Plant glycoproteins contain amounts of paucimannosidic N-glycans (truncated glycans) that lack terminal GlcNAc residues at their non-reducing ends (Lerouge et al., 1998). It is suggested that this is due to the action of β-hexosaminidases during the later stages of N-glycan processing or during of N-glycan turnover. The site for this trimming reaction is most likely in a post-Golgi compartment or in the apoplast. The biosynthesis and physiological importance of this type of N-glycans have not been fully explained (Strasser et al., 2007; Liebminger et al., 2011).

The highest abundance of N-glycans associated with RBDr was paucimannosidic structures (28%), residues that do not contain the native RBD of SARs-CoV-2. In this sense, several reported recombinant proteins showed considerable amounts of paucimannosidic structures, such is the case of the human antitrypsin protein produced in *N. benthamiana* (Castilho et al., 2014), the bovine follicle stimulating hormone (Dirnberger et al., 2001), recombinant glucocerebrosidase directed to the apoplast and human lactoferrin (Samyn-Petit et al., 2003). In the case of glucocerebrosidase, the paucimannosidic structures confer an advantage in production over that produced in CHO cells, which require in vitro deglycosylation to expose the terminal mannoses necessary to achieve the desired therapeutic effects (Limkul et al., 2016). It has been shown that one way to increase protein yield in plants is to direct them to vacuoles where the presence of pausiminosides does not affect the activity of immunoglobulins, complement proteins, transglutaminases and others (Marin Viegas et al., 2017). There are several examples of organisms rich in N-glycans of the paucimannoside type: the *Drosophila melanogaster* insect, human myoloperoxidase, and human cathepsin G. Structural and functional characterization studies of paucimannose-rich N-glycans have revealed new insights into the potential immunomodulatory roles of paucimanoside N-glycans (Loke et al., 2017). There are differences in the glycosylation pattern between plant obtained RBD and other expression systems, for example,

RBD association of sialic acid with said protein in mammalian cells (Gstöttner et al., 2021) RBDr, which lacks this monosaccharide, which is not synthesized in plants. In contrast, *P. pastoris* glycosylation pattern is characterized by higher mannose content. Mannosylation enhances activation of antigen-presenting cells like macrophages and dendritic cells, functioning as immunopotentiator, while increasing antigen immunogenicity (Limonta-Fernández et al., 2021).

Particularly, in our case the most abundant peak of the N-glycans (F(3)XM3) presumably indicates apoplastic and/or vacuolar localization (Lerouge et al., 1998; Bosch et al., 2013). The presence of this structure in the total N-glycans pool do not allows elucidating if RBDr was stored in vacuolar or apoplastic compartment. Nevertheless, in the RBDr N-glycans profile was identified several structure bearing antennary fucose, forming Lewis-A motif. Lewis structure only has been identified in plant glycoprotein located in apoplast (Bosch et al., 2013) suggesting that RBDr is likely to follow intracellularly traffic into this compartment. Nevertheless, in our case the localization of RBDr is still an open question that could be investigated using immunolocalization tools. However, the glycosylation pattern of the RBDr obtained does not seem to affect its function, since immunized mice were able to generate an immune response (Ceballo et al., 2022). Plant-specific N-glycan epitopes may probably be exploited as a target of the host immune response and may be beneficial for immune protection. The presence of these carbohydrates as 'cis-adjuvants' can help in antigen uptake through lectin receptors, subsequent degradation and presentation of antigens from dendrite cells (DC) to T cells.

Conclusions

In this article, the presence of N-glycan structures associated with RBDr, produced in leaves of N. benthamiana, was confirmed. These oligosaccharides were mainly of the paucimanocidic type, in addition structural motifs β1,2 xylose and α1,3 fucose, characteristic of plant proteins that travel through the secretory pathway, were identified. Glycosylated RBDr induced neutralizing antibodies in mice, according to a study published by Ceballo et al. (2022). These results confirm the use of plant-produced vaccines, specifically subunit vaccines.

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