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Cloning an RBD-T4-LINKER-C5a sequence encoding the SARS-CoV-2 antigen into a plant expression vector

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Abstract. This study aims to make a plant expression vector with an RBD-T4-Linker-C5a sequence that codes for the SARS-CoV-2 antigen and an A3Dsp signal peptide from the rice 3D amylase gene located before the RBD. Methods of molecular cloning were applied in this study. The plant expression vector pNHL22 harboring the RBD-T4-Linker-C5a sequence was successfully established and conjugated into *Agrobacterium tumefaciens* LBA4404 by triparental mating. Bacteria *A. tumefaciens* containing the RBD-T4-Linker-C5a sequence are now ready for genetic transformation into the *Nicotiana benthamiana* plant for future applications.

Keywords: Cloning, SARS-CoV-2 antigen, RBD, C5a

1 Introduction

Coronavirus disease (COVID-19), an infectious disease caused by the SARS-CoV-2 virus, has alerted many countries because of the high fatality rate it has produced [1, 2]. Due to how quickly it spread across continents, the World Health Organization has called it a pandemic. This led to the urgent hunt for effective vaccines and the development of medicines and quick, affordable testing methods. Several vaccine candidates based on nucleic acids, viral vectors, and subunit vaccines are used and tested in clinical trials [3]. Spike (S) glycoprotein (sometimes also called spike protein) is the largest of the four major structural proteins found in coronaviruses. The spike proteins assemble into trimers that form large structures, called spikes or peplomers, that project from the surface of the virion. There are two components of this protein called the S1 and S2 subunits [4, 5]. The S1 sequence, which contains the receptor-binding domain (RBD) located between amino acids 319 and 591, plays a decisive role in the viral infection process [4]. The RBD contains eight cysteines and two N-glycosylation sites (N331 and N343). According to Shin *et al.* (2021), the addition of glycans in *in vitro* conditions can significantly affect the folding, dynamics, stability, and solvent accessibility of the RBD [6].

The RBD-hACE2 complex comprises the angiotensin-converting enzyme 2 (hACE2), located on the exterior surface of the membrane of several types of human cells. Thus, viral particles can enter the host cells and release their genetic material into the cytoplasm of the infected cells [7]. The abovementioned factors have prompted researchers to examine the RBD as a potential protein candidate for a subunit vaccination. Various biotechnological platforms have been used to create medications; however, most pharmaceuticals and recombinant vaccines have been produced by microbial or mammalian cells. These productive systems have many benefits, but

they also have drawbacks, such as high production costs [8], which may encourage the use of alternative production environments with significant cost savings. Using plants to make recombinant proteins has many advantages over using conventional platforms. For instance, they are inexpensive and easy to cultivate and scale up production [9, 10]. Generally, plants can be used as bioreactors to make permanent or temporary pharmaceuticals. The latter is a great way to quickly get heterologous proteins with medical value [11, 12]. In this study, we cloned the sequence encoding a novel SARS-CoV-2 antigen called RBD-T4-Linker-C5a, fused with rice amylase 3D signal peptide (A3Dsp). This sequence was then transferred into *A. tumefaciens* LBA4404 to prepare for the antigen's production in *N. benthamiana* plants.

2 Materials and methods

2.1 Gene design

RBD-T4-Linker-C5a sequence, which encodes SARS-CoV-2 antigen, is the combination of 4 distinct sequences, which are the spike S1 subunit's RBD sequence (NC_045512.2), the spike S2 subunit's T4 peptide sequence [5], a flexible linker, and a DNA sequence encoding human C5a protein **(NC_000009.12)**. A rice amylase 3D signal peptide (A3Dsp) was placed at the 5' end of RBD to excrete protein extracellularly. Additionally, Kozak and SEKDEL sequences were added to the 5′ and 3′ ends of the A3Dsp-RBD-T4-Linker-C5a sequence, respectively, to enhance protein production.

All of them are synthesized precisely according to the previously mentioned order. Also, three restriction sites for *Xba*I, *Kpn*I, and *Sac*I were added to the 5′ end, the site between the T4 peptide sequence and the flexible linker region, and the 3′ end of the Kozak-A3Dsp-RBD-T4-linker-C5a-SEKDEL sequence, respectively (Fig. 1). This full-length sequence was synthesized and cloned into the pJET1.2 vector (named pNHL22A) by Nanning GenSys Biotechnology Co., Ltd. (China).

2.2 Construction of plant expression binary vector

The Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence from the pNHL22A vector was inserted into the pMYV719 plant expression vector, to form the new vector, pNHL22B.

In the first step, the Kozak-A3Dsp-RBD-T4 sequence was cut out of the pNHL22A vector by *Kpn*I and *Xba*I enzymes (NEB, UK) and then was inserted into the pMYV719 vector, which had been linearized by the same two enzymes, using T4 DNA ligase (Fig. 2). Next, the Linker-C5a-SEKDEL sequence was digested from the pNHL22A vector using *Kpn*I and *Sac*I (NEB, UK) and put into the vector made above, which had already removed the old SEKDEL fragment in the pMYV719 vector using the same enzymes. The new pNHL22B vector containing the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence was transformed into *E. coli* TOP10 using the heat-shock method (Bergkessel, 2013). The cloning efficacy was checked by restriction digestion.

2.3 Triparental mating

A. tumefaciens LBA4404, *E. coli* TOP10 with the plant expression vector pNHL22B, and *E. coli* TOP10 with the helper plasmid pRK2013 were all grown in LB medium with the right antibiotics added. Rifamycin (100 μg/mL) was used for *A. tumefaciens* LBA4404 culture, whereas kanamycin (100 μg/mL) was used for 2 strains of *E. coli* TOP10 culture. Triparental mating was initially conducted by the co-culture of three bacteria in solid LB medium. After 3 days of growth, *Agrobacterium* with the vector pNHL22B was selected in solid LB medium with 100 μg/mL

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rifamycin and 100 μg/mL kanamycin. Screening for transformed *A. tumefaciens* colonies by PCR and digestion with restriction enzymes to confirm the pNHL22B vector had been conjugated successfully [13].

2.4 PCR amplification

PCR amplification was performed with specific primers for the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence (Table 1). The composition of the reaction consists of template DNA (a single *E. coli* colony or 20 ng of plasmid DNA extracted from *A. tumefaciens*), 10 pmol of each primer, 1x of Green Master Mix (Thermo Scientific), and nuclease-free water (Thermo Scientific) to a final volume of 20 μL. The PCR reaction began with template DNA denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were then analyzed on a 1% agarose gel.

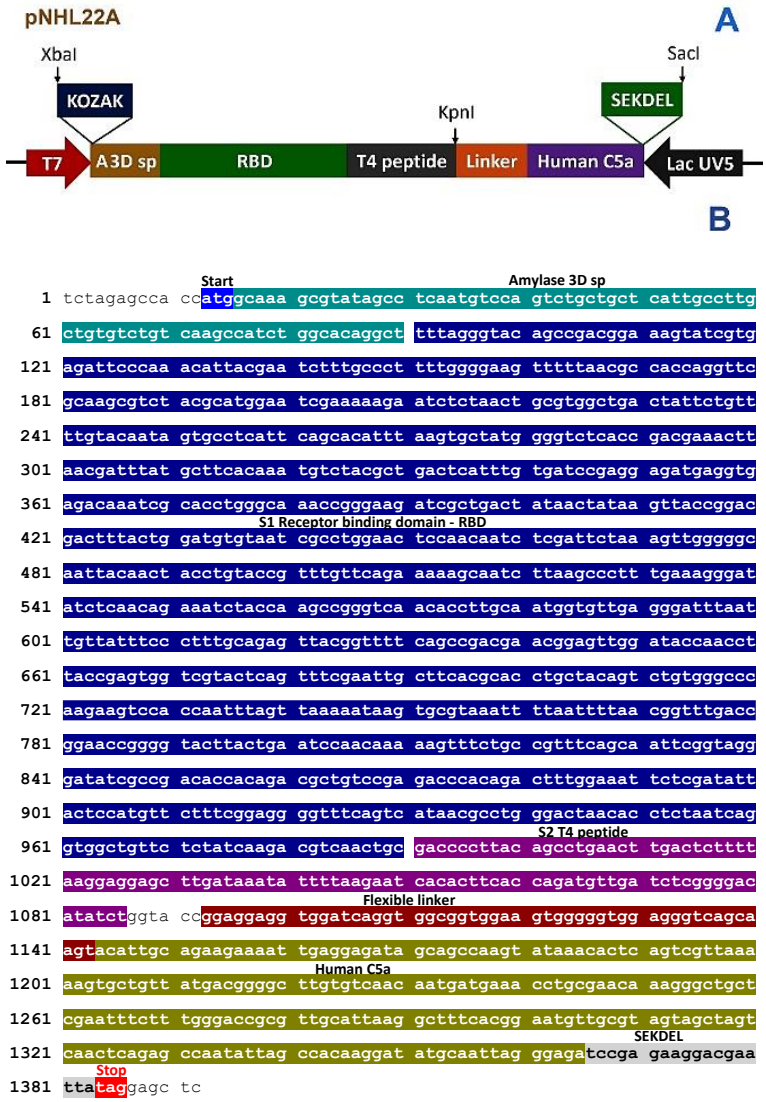

Fig. 1. A: Map of pNHL22A vector. B: DNA sequence of Kozak-A3Dsp-RBD-T4-linker-C5a-SEKDEL. The different colored DNA sequences are underlined together with their various functions

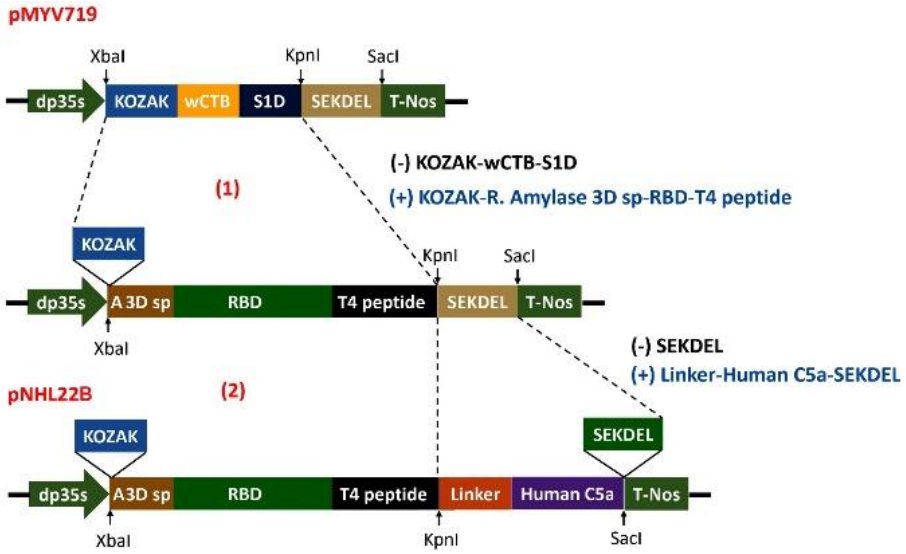

Fig. 2. Strategy for cloning the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence into pMYV719 vector

Table 1. RBD-T4-Linker-C5a specific primers for PCR amplification

Name of primers	Nucleotide sequence 5′-3′	Size of PCR product
RBD-T4-linker-C5a (F)	GCGCTCTAGAAA AACTAAAAGTAG AAG	1392 bp
RBD-T4-linker-C5a (R)	GCGCGAGCTCTT AATTCAAACCAG AT	

3 Results and discussion

3.1 Construction of pNHL22B plant expression vector

A 3 kb open-loop vector and a 1.1 kb Kozak-A3Dsp-RBD-T4 sequence were obtained from the pNHL22A vector after being cut by the restriction enzymes *Xba*I and *Kpn*I. The Kozak-A3Dsp-RBD-T4 sequence was then ligated into the pMYV719 vector (approx. 11 kb), ring-opened with the same two abovementioned enzymes using T4 DNA ligase. The ligation mixture was transformed into competent *E. coli* TOP10. To determine the transformation efficiency, the plasmid vector containing the gene was obtained from *E. coli* TOP10 and digested with *Xba*I and *Kpn*I. Two DNA bands from digestion with sizes of approximately 11 kb and 1.1 kb found on the gel confirmed that the Kozak-A3Dsp-RBD-T4 sequence has been successfully cloned into the vector pMYV719 (Fig. 3A).

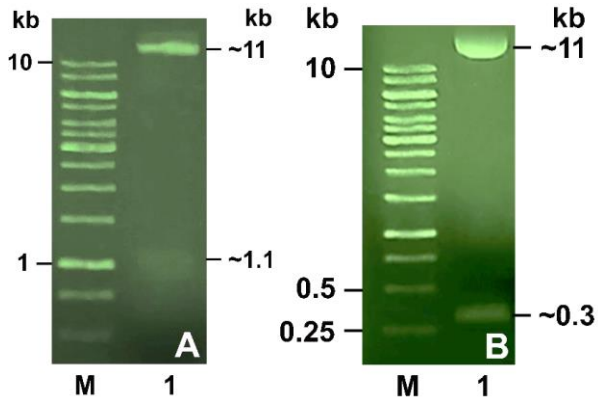

Fig. 3. Digestion of plasmid DNA with restriction enzymes. A: Digestion with *Kpn*I and *Xba*I, 1: pMYV719 vector harboring Kozak-A3Dsp-RBD-T4 sequence. B: Digestion with *Kpn*I and *Sac*I, 1: pMYV719 vector harboring Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence. M: Gene Ruler 1 kb DNA Ladder (Thermo Scientific)

The first recombinant pMYV719 vector harboring Kozak-A3Dsp-RBD-T4 sequence was further cloned with the Linker-C5a-SEKDEL sequence. The approximately 0.3 kb Linker-C5a-SEKDEL sequence was obtained by opening the

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pNHL22A vector with *Sac*I and *Kpn*I. This sequence was then inserted flanking the 3′ end of the Kozak-A3Dsp-RBD-T4 sequence in the first recombinant pMYV719 vector by T4 DNA ligase and transformed into *E. coli* TOP10. The transformation results were verified by restriction cleavage of the second recombinant pMYV719 vector (named pNHL22B) with the *Kpn*I and *Sac*I enzymes. Two DNA bands of approximately 1.1 kb (vector) and 0.3 kb (insert) in size found on the gel demonstrated success in transformation (Fig. 3B).

Digestion with *Xba*I and *Sac*I showed that the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence was successfully inserted into the pMYV719 vector between the dp35s promoter and T-Nos terminator to form a new recombinant vector, pNHL22B. Two DNA bands with sizes of approximately 11 kb (vector) and 1.4 kb (insert) from digestion were found on the gel (Fig. 4A). The insertion of the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence in vector pMYV719 was also demonstrated by PCR amplification with specific primers; the amplicon with the expected size of about 1.4 kb appeared after electrophoresis (Fig. 4B).

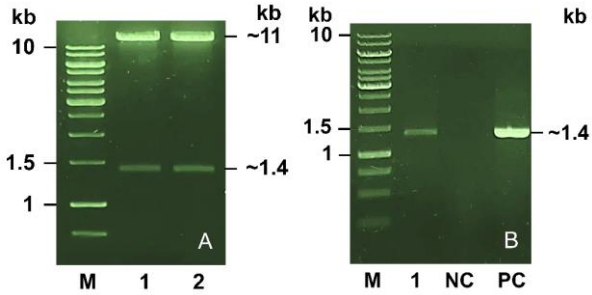

Fig. 4. Verification of the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence in vector pNHL22B by digestion and PCR amplification. A: Digestion with *Xba*I and *Sac*I, 1 and 2: Plasmids from two single *E. coli* colonies. B: PCR amplification with specific primers, 1: PCR product, PC: pNHL22A vector was used as a positive control, NC: non-transformed *E. coli* was used as a negative control. M: Gene Ruler 1 kb DNA Ladder (Thermo Scientific)

3.2 Triparental Mating

A pNHL22B vector containing the sequence encoding the SARS-Cov-2 antigen was transferred into *A. tumefaciens* LBA4404 according to the triparental mating method. Some *Agrobacterium* colonies grown on selection medium were then verified by PCR amplification and restriction digestion with *Sac*I and *Xba*I to determine the presence of the pNHL22B vector. As a result, a DNA band from PCR and digestion of approximately 1.4 kb in size found on the gel is matched with the true size of the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence (Fig. 5A and B). Therefore, it can be confirmed that the vector pNHL22B containing the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence has been successfully conjugated into *A. tumefaciens* LBA4404 and is ready to be transferred into *N. benthamiana* plants.

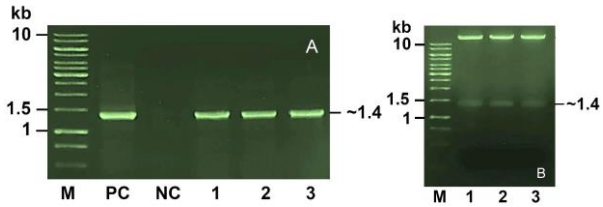

Fig. 5. Verification of pNHL22B vector harboring the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence in *A. tumefaciens* LBA4404. A: PCR amplification with specific primers, PC: pNHL22A vector was used as a positive control; NC: non-conjugated *Agrobacterium* was used as a negative control; 1-3: conjugated *Agrobacterium* colonies. B: Digestion of pNHL22B vector with *Xba*I and *Sac*I. 1-3: plasmid DNA from conjugated *Agrobacterium* colonies. M: 1 kb DNA Page Ladder (Thermo Scientific).

4 Conclusion

We successfully cloned the Kozak-A3Dsp-RBD-T4-Linker-C5a-SEKDEL sequence into the plant expression vector pNHL22B, which is expected to produce SARS-CoV-2 antigen that binds to the nasal cavity and enhances recipients' mucosal immune response, and are now ready to transfer

this expression construction into the *N. benthamiana* genome.

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Conflicts

The authors have no conflicts of interest to declare.

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