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Recombinant proteins of spike protein of SARS-CoV-2 with the Omicron receptor-binding domain induce production of highly Omicron-specific neutralizing antibodies

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Various vaccines have been developed to fight severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for the coronavirus disease 2019 pandemic. However, new variants of SARS-CoV-2 undermine the effort to fight SARS-CoV-2. Here, we produced S proteins harboring the receptor-binding domain (RBD) of the Omicron variant in plants. Plant-produced S proteins together with adjuvant CIA09A triggered strong immune responses in mice. Antibodies in serum inhibited interaction of recombinant human angiotensin-converting enzyme 2 with RBD of the Omicron variant, but not RBD of other variants. These results suggest that antibodies induced by RBD of the Omicron variant are highly specific for the Omicron RBD, but not for that of other variants.

Keywords: SARS-CoV-2, Vaccine, Omicron variant, *Nicotiana benthamiana*, Recombinant S protein

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus responsible for the coronavirus disease 2019 (COVID-19) pandemic, has caused serious health problems worldwide since its emergence in 2019 in Wuhan, China; indeed, it remains an ongoing health issue in every country on the globe [1]. Since the most effective way to combat virus infection is vaccination, there was a rush to develop effective vaccines against SARS-CoV-2 soon after its identification [2-4]. With unprecedented speed, several vaccines were developed; these include mRNA-based vaccines (BNT162b2 and mRNA-1273), virus-vector vaccines (ChAdOx-1, Ad26COVs1, and Sputnik V), recombinant protein-based vaccines (NVX-CoV373 and Co-VLP), and inactivated virus-based vaccines (BBIBP-CorV and CVAXIN) [5]. After emergency approval was granted, these vaccines were quickly introduced into the field. More vaccines are on the way.

The degree of protection afforded by these vaccines varied, but BNT162b2 and mRNA-1273 provided >90% protection against SARS-CoV-2 [6,7]. However, one continuing challenge is the emergence of new variants, particularly vaccine-resistant variants [8]. After identification of the original SARS-CoV-2 strain in Wuhan, China, in 2019, numerous variants have emerged, including B.1.1.7 (Alpha), B.1.351 (Beta), P1 (Gamma), B.1.617.2 (Delta), and Omicron (B.1.1.529) [9]. These variants harbor a different number of mutations from the original SARS-CoV-2 strain. Omicron harbors 34

mutations within the spike protein. Those mutations occur in the S1 subunit, in particular the receptor-binding domain (RBD). These variants show varying degrees of interaction with neutralizing antibodies induced by current vaccines [10,11]. Indeed, the new variants can cause breakthrough infections in people who have been vaccinated or infected before [12]. Therefore, a new vaccine targeting new variants has to be developed.

In this study, we aimed to investigate the characteristics of antibodies induced by an S protein containing the RBD of the Omicron variant. First, we generated a recombinant construct comprising the S protein (amino acids 14–1162) from the D614G variant of SARS-CoV-2 and the RBD of the Omicron variant. We did this by introducing 15 Omicron-specific mutations into the RBD (Fig. 1A). In addition, we introduced three additional mutations (A942P, K986P, and V987P) to increase the stability of the S protein, and deleted the furin cleavage site (ΔPRRA) to maintain the S protein in its prefusion form. To express the S protein as a trimer, we fused the foldon motif of T4 fibritin to the C-terminus [13]. Additionally, we added histidine and HDEL residues to the C-terminus as an affinity tag (for purification) and an endoplasmic reticulum (ER) retention signal, respectively (Fig. 1A). Finally, a leader sequence (NB) from Arabidopsis BiP1 was added to the N-terminus (*NB:S(rOmi3P)delFnL:Fd:7H:HDEL*, referred to as *pSrOmi*) for ER targeting. Thus, recombinant protein pSrOmi was designed to be produced as a trimer in the ER of *Nicotiana benthamiana*. The construct *pSrOmi* was introduced into the leaf tissues of *N. benthamian* via *Agrobacterium*-mediated infiltration, and leaf tissues were harvested 4 days later [14,15]. First, we examined expression of *pSrOmi* in the plants. Total soluble protein extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS/PAGE) and analyzed by western blotting using anti-His antibody. As a reference, we loaded 100 ng of the recombinant D614G variant S1 protein tagged with a His tag; this was

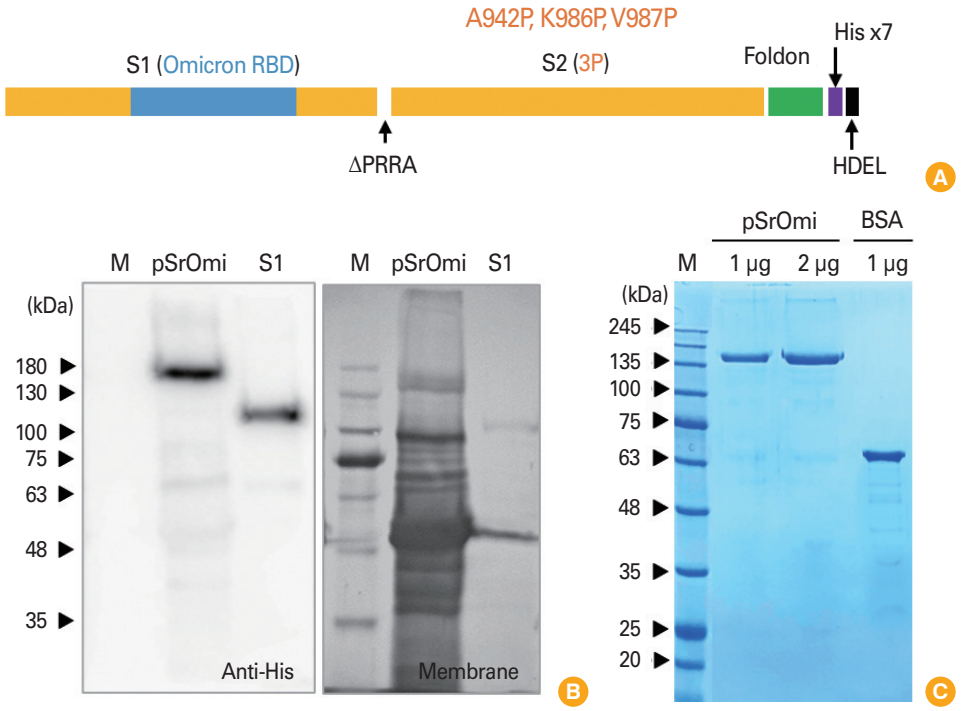

Fig. 1. Expression of pSrOmi in *Nicotiana benthamiana* leaves and its purification from total soluble protein extracts. (A) Schematic presentation of pSrOmi. The spike (S) protein of the severe acute respiratory syndrome coronavirus 2 D614G strain was mutated such that it contained the receptor-binding domain (RBD) of the Omicron variant. In addition, the S protein lacked the furin cleavage site (PRRA) and contained three P substitutions (A942P, K986P, and V987P). The mutated S protein was fused to the foldon motif, a histidine tag, and an endoplasmic reticulum (ER) retention signal (HDEL) at the C-terminus. The leader signal (NB) of Arabidopsis BiP1 is not shown because it is removed after translocation to the ER. (B) Expression of the pSrOmi protein. Total soluble protein extracts from infiltrated *N. benthamiana* leaf tissues were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blotting using an anti-His antibody. His-tagged S1 protein (100 ng) was used as a loading control. The immunoblot was stained with Coomassie brilliant blue (CBB). (C) Analysis of purified pSrOmi by SDS-PAGE. Purified pSrOmi proteins (1 and 2 μg) were separated by SDS-PAGE and stained with CBB. BSA (1 μg) was used as a loading control. M, protein standard marker.

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done to estimate the expression level of *pSrOmi*. The results showed that 40 μg of protein was produced by 1 g of leaf tissue (Fig. 1B). Thus, expression was considered to be very high. Next, we purified pSrOmi from total soluble protein extracts of *N. benthamiana* to use to induce immune responses in mice. pSrOmi was purified from total soluble protein extracts using an Ni^{2+}-IDA affinity column followed by further purification using a prepacked Q-HP column. The purity and quantity of the protein were examined by SDS/PAGE and Coomassie brilliant blue staining. The purity was high, with almost no visible contaminating proteins (Fig. 1C).

We examined the immunogenicity of pSrOmi in mice. Mice (BALB/c, female, 16–20 g, 5 weeks old) were intramuscularly injected 2 times at an interval of 3 weeks with pSrOmi proteins (0.1, 1, 5, or 10 μg) and CIA09A as an adjuvant [16,17]. All animal experiments were performed in compliance with the Pohang Technopark IACUC (approval no., ABCC2022004). We included 10 μg of pSrOmi alone or phosphate-buffered saline as controls. Serum was obtained at 14 days after the second immunization. Antigen-specific antibody titers in sera were measured by enzyme-linked immunosorbent assay after serial dilution (from 1:150 to 1:11,718,750). The antibody titers induced by 1, 5, or 10 μg of pSrOmi in the presence of CIA09A were similar (Fig. 2A), indicating that CIA09A increases the immunogenicity of pSrOmi. Mice that received 10 μg of pSrOmi alone also showed strong immune response. However, the immune response to 10 μg of pSrOmi alone was weaker than that to 0.1 μg pSrOmi plus CIA09A. Next, we examined the end-point titer of these vaccines doses. The end-point titer ranged from 4.41 $\log_{10}$ for 10 μg pSrOmi alone to 5.53 $\log_{10}$ for 10 μg pSrOmi plus CIA09A (Fig. 2B).

To access the degree of protection against SARS-CoV-2 infection afforded by pSrOmi-induced neutralizing antibodies, we examined the extent to which the neutralizing antibodies inhibit the interaction between the RBD and human angiotensin-converting enzyme 2 (hACE2), the human receptor for SARS-CoV-2 [18]. Current COVID-19 vaccines are thought to provide a certain degree of protection against newly emerged variants, including the Omicron variant [19,20]. Therefore, we set the cutoff value at 20%. Neutralizing antibodies in sera obtained from mice injected with 1–10 μg pSrOmi plus CIA09A inhibited interaction between the RBD of Omicron and hACE2 by >90% (Fig. 3A). However, this fell to 79% at a dose of 0.1 μg pSrOmi plus CIA09A, and to 33% when using 10 μg pSrOmi alone, indicating that the titer must be >5 $\log_{10}$ to effectively inhibit interaction between RBD and hACE2 by >90%. Next, we accessed the cross-reactivity of pSrOmi-induced neutralizing antibodies with other variants of SARS-CoV-2. mRNA-based vaccines such as BNT162b2 and mRNA-1273 provide >90% protection against serious disease after infection by the Delta variant [9,21]. However, other reports show that the majority of the current vaccines cannot neutralize the Omicron variant effectively [10,21]. Only 20% and 24% of BNT162b2 recipients had detectable neutralizing antibodies against Omicron variants HKU691 and HKU344-R346K, respectively [9]. Therefore, we next measured the ability of pSrOmi-induced neutralizing antibodies to inhibit the interaction between the RBD of variants and hACE2. The percentage inhibition of the RBD of the Beta and Delta variants was close to the cutoff value (20%), or

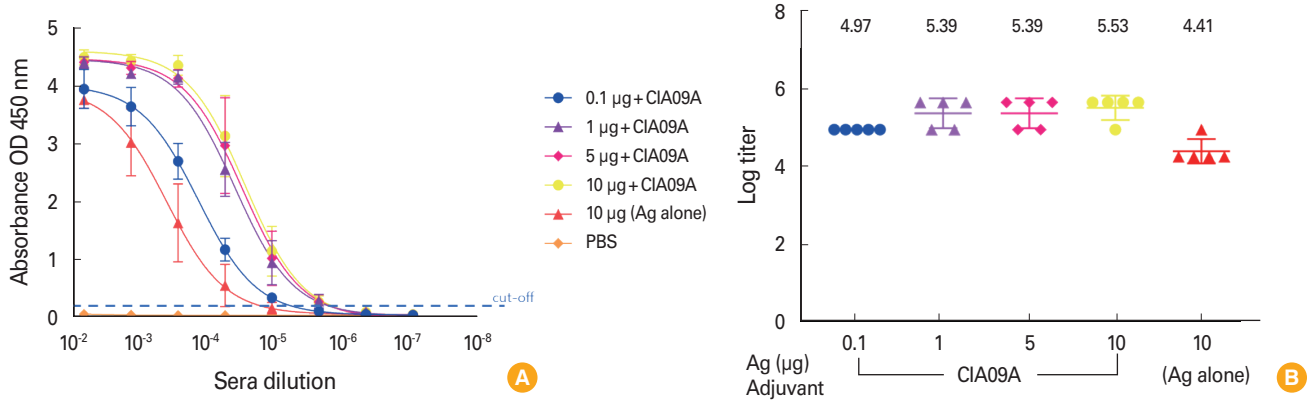

Fig. 2. Analysis of antigen (Ag)-specific serum immunoglobulin G (IgG) from immunized mice at 14 dpi. (A) Antibody titration curve based on sera obtained from mice at 14 dpi (diluted from 1:150 to 1:11,718,750). The optical density (OD) value of the lowest three dilutions of serum from phosphate-buffered saline (PBS)-injected mice was multiplied by 4 to yield the titration cutoff threshold. (B) The end-point titration values. The end-point titer of IgG antibodies was measured in enzyme-linked immunosorbent assay coated with recombinant pSrOmi. The end-point titration value was expressed as $\log_{10}$ values.

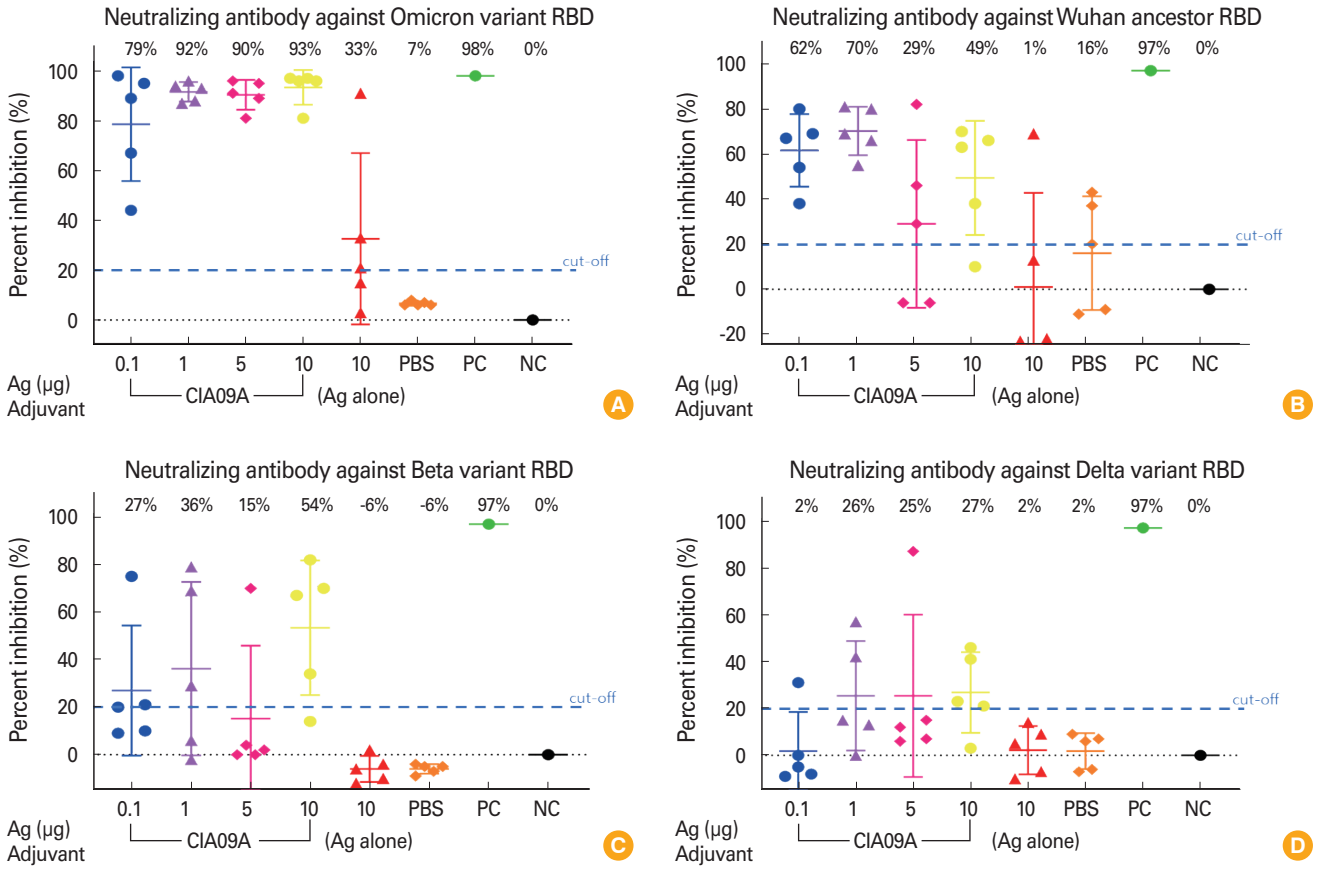

Fig. 3. Omicron-induced antibodies in mice are highly specific for the Omicron receptor-binding domain (RBD), and show no or greatly reduced reactivity with the RBDs of other variants. Mice sera were diluted 1:10 (in duplicate) and applied to the competition enzyme-linked immunosorbent assay plates (ACROBiosystems); the assays were based on recombinant human angiotensin-converting enzyme 2 and spike proteins from different variants: (A) Omicron variant, (B) Wuhan ancestor, (C) Beta variant, and (D) Delta variant. Ag, antigen.

slightly higher (Fig. 3C, D); however, inhibition of the RBD of the Wuhan strain ranged from 60% to 70% (Fig. 3B). These data suggest almost no inhibition of the Beta and Delta variants. Thus, pSrOmi-induced neutralizing antibodies are highly specific for the Omicron RBD and may not provide any protection against other variants. One possible explanation for this would be that the heavily mutated RBD of the Omicron variant induces Omicron RBD-specific antibodies that do not react with the RBD of other variants. However, this does not agree with data showing that three doses of mRNA-based vaccines provide a high level of protection against the Omicron variant [19,20]. In animal models and humans, neutralizing antibodies generated by mRNA vaccines appear to be the primary correlate of COVID-19 protection [22]. Thus, one possible explanation is that neutralizing antibodies induced by the original RBD recognize the RBD of the Omicron variant, but not vice versa. Here, we examined inhibition of the RBD/hACE2 interaction only *in vitro*, but did not examine the degree of cross-protection against other variants *in vivo*. Thus, further studies are necessary to understand the behavior of neutralizing antibodies induced by the S protein of the Omicron variant. However, despite this limitation, our results provide valuable information that may be useful for future development of vaccines against SARS-CoV-2 in a world where more variants will emerge in the future.

In conclusion, we observed that antibodies raised by S proteins with RBD of the Omicron variant can inhibit interaction of hACE2 with the RBD of Omicron variant but not with that of other variants. These results suggest that vaccine generated using the Omicron variant may be specific for the protection of Omicron variant, but not other variants.

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