Rapid and Scalable Production of Functional Anti-Coronavirus Monoclonal Antibody CR3022 in Plants

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Abstract
Severe acute respiratory syndrome coronavirus-2 is responsible for an ongoing global outbreak of coronavirus disease (COVID-19) and represents a significant public health threat. The rapid spread of COVID-19 necessitates the development of cost-effective technology platforms for the production of diagnostic reagents/biopharmaceuticals for COVID-19. We explored the possibility of producing an anti-SARS-CoV monoclonal antibody (mAb) CR3022 and the receptor binding domain (RBD) of SARS-CoV-2 in Nicotiana benthamiana. Both RBD and the mAb were transiently expressed with the expression of 8μg/g and 130μg/g leaf fresh weight respectively. The plant-purified mAb binds to SARS-CoV-2, but fails to neutralize it in vitro. This is the first report showing the functional characterization of an anti- SARS-CoV mAb CR3022 in plants. Overall these findings showed that plants are a promising platform to produce anti-SARS-CoV mAb to use as a research reagent or a biotherapeutic in a cost-effective manner, which is especially important to developing economies during epidemics.

Introduction
An outbreak of coronavirus disease 2019 (COVID-19) was reported very recently in December 2019 in one of the largest cities in China, Wuhan, Hubei province which was later confirmed to be caused by the betacoronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2; formerly known as 2019-nCoV). This zoonotic virus is believed to have originated from animals and was transmitted to humans by an animal-to-human spillover event linked with a local seafood and animal market in Wuhan. The infection spread in mainland China rapidly and subsequently expanded to multiple countries mainly through international travel. Many confirmed cases of COVID-19 have been reported worldwide, with more than 2 million infected cases and >1,00,000 deaths altogether in 6 continents, with a variable mortality rate 1, 2, 3, 4.

Outbreaks of other similar coronaviruses including severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2003 and 2012 respectively also caused severe fatal illness in humans. Although the pathogenicity of SARS-CoV-2 might be similar or higher as compared to SARS-CoV and MERS-CoV, it is inappropriate to predict the
pathogenicity of the virus at this stage. Human- to-human transmission has been reported via., respiratory droplets or close contacts with an infected person, which has caused widespread fear and concern over this disease 5, 6, 7. Currently, SARS-CoV-2 has emerged as global public health concern, with many people being infected around the world, hence the WHO declared this coronavirus outbreak as a Public Health Emergency of International Concern and characterized COVID-19 as a pandemic 8. To date, there is no specific treatment or vaccines available to treat COVID-19 infections, as research in these areas is in the preliminary stages of development. Hence, there is an urgent need to develop rapid diagnostic methods, vaccines and therapeutics to tackle the COVID-19 outbreak.

Over the past two decades, biopharmaceuticals have been produced in a number of different expression systems such as yeast, mammalian cells, and plants, but currently most of the commercially available recombinant vaccines or biopharmaceuticals are produced in mammalian or microbial cell cultures. One of the major issues with the biopharmaceuticals produced in mammalian system is the requirement of initial capital investment and the high production costs associated with it 9, 10. While each expression system has its own advantages, they have all proven to possess distinct shortcomings, and the limitations of each expression system led to development of alternative production platforms that could significantly reduce the production costs. Recently, plants have emerged as an effective recombinant protein production platform, as they offer many advantages over conventional platforms such as economy, flexibility, rapid scalability and safety. Previous reports have demonstrated the potential of plant transient expression systems for the rapid production of proteins of pharmaceutical importance 11, 12, 13, 14, 15, 16, 17, 18. Several groups have characterized potent antibodies targeting the coronavirus spike protein and effectively neutralizing SARS-CoV in vitro and in vivo 19, 20, 21, 22, 23, 24, 25, 26, 27. Based on the recent report by Tian and colleagues (2020), a SARS coronavirus-specific human monoclonal antibody CR3022 was reported to potently bind with SARS-CoV-2 RBD 26 and therefore represents an important candidate mAb with potential as a therapeutic molecule alone or in combination with other potential candidates for COVID-19. Given the ability of plants to assemble functional antibodies, the
ease and speed of functional protein production, we employed a plant expression system for the rapid production of mAb CR3022 in order to use as a biotherapeutic agent to address the global COVID-19 threat.

The present study demonstrates the rapid production of anti-SARS-CoV monoclonal antibody CR3022 and the RBD of SARS-CoV-2 in plants by using a transient expression system. The mAb CR3022 was efficiently expressed and assembled in *Nicotiana benthamiana* plants. The purified antibody was tested for its specificity and binding with recombinant RBD protein of SARS-CoV-2, as well as its ability to neutralize SARS-CoV-2 *in vitro*. The *in vitro* testing showed that the plant purified CR3022 mAb did not neutralize the virus. Although the plant produced antibody didn’t neutralize the virus *in vitro*, this study provides a spotlight for the rapid production of protein reagents in a plant expression system for the diagnosis of SARS-CoV-2 during epidemics. Moreover, a specific neutralizing antibody, if identified against SARS-CoV-2 in near future can be produced in a plant system inexpensively in a short time which could be rapidly translated into clinical trials.

**Results**

**Expression mAb CR3022 and the RBD of SARS-CoV-2 in *N. benthamiana* plants**

The schematic representation and the timeline for recombinant protein production in plant systems are given in Fig. 1a and 1b. The codon-optimized heavy chain (HC) and light chain (LC) expression cassettes of mAb CR3022 and the RBD domain of SARS-CoV-2 were cloned into the geminiviral plant expression vector pBY2e (Fig.2) and transformed into *Agrobacterium tumefaciens*. *N. benthamiana* plants were infiltrated with an *Agrobacterium* culture containing the RBD domain of SARS-CoV-2 cloned in pBY2e, or co-infiltrated with heavy chain and light chain expression cassettes of mAb CR3022. The expression of recombinant RBD antigen and mAb CR3022 was evaluated by SDS-PAGE and western blot analysis. The results showed that the RBD antigen and the fully assembled mAb CR3022 were detected in the respective infiltrated plant samples with the expected molecular weights. The expression level of RBD antigen of SARS-CoV-2 and mAb CR3022 was estimated to be 8 μg and 130 μg per gram leaf fresh weight, respectively.

**Purification of RBD antigen and mAb CR3022 from *N. benthamiana* leaves**
The expressed mAb CR3022 was extracted and purified from plant leaf extracts using Protein A affinity chromatography. The recombinant RBD was purified from the clarified total protein extract using Amintra® Ni-NTA resin. The purity of the purified antigens and antibodies were analyzed by SDS-PAGE and InstantBlue™ staining (Fig 3 and 4). The gel analysis showed that the plant can produce fully assembled antibody and intact mAbs with >90% homogeneity and can be purified from the crude plant extracts by using Protein A affinity chromatography.

**Functional Characterization by ELISA**

ELISA was performed to determine the biological activity of plant produced RBD antigen and mAb. The results showed that the plant produced mAb CR3022 retains the ability to specifically recognize and bind efficiently to the plant-derived RBD antigen of SARS-CoV-2 and no interaction was detected when IgG1 was used as a negative control (Fig. 5).

**Binding and neutralization activity of plant-derived mAb CR3022 against SARS-CoV-2**

An immunofluorescence assay was performed to determine whether mAb CR3022 recognized SARS-CoV-2. SARS-CoV-2 was inoculated onto Vero cells and infected cells were incubated with known positive serum, CR3022, and a negative control antibody before detection with an anti-human IgG conjugated with FITC. The results showed that plant produced CR3022 could bind to SARS-CoV-2 in infected cells, similar to the positive control serum (Fig. 6). To test the neutralizing activity of mAb CR3022, Vero cells infected with SARS-CoV-2 developed cytopathic effects at 3 days post infection. Positive serum had a high viral neutralization titer against SARS-CoV-2 (Table 1). In contrast, mAb CR3022 and negative serum had no neutralizing activity against SARS-CoV-2.

**Discussion**

The frequent outbreaks of emerging or re-emerging infectious diseases threaten global health security, as they can have devastating health and economic impact especially in the developing world. The fear and panic over the spread of epidemic diseases can disrupt economy, travel, social activities and tourism, as well as decrease trade which in turn can affect whole societies, economies and political systems. Thus, infectious diseases create a massive burden for the global economy 29. The recent emergence and rapid spread of the novel coronavirus SARS-CoV-2 that causes COVID-19
has attracted the attention of the whole world. Tens of thousands of infected cases have been reported and death toll is escalating daily. The continued spread of SARS-CoV-2 in many countries demands the development of cost-effective rapid diagnostic assays and therapeutics for COVID-19. The receptor binding domain (RBD) located within the spike region of SARS-CoV mediates virus entry into the host cell by interacting with host receptor angiotensin converting enzyme 2 (ACE2). The new virus SARS-CoV-2 is genetically related to SARS-CoV which also utilizes the ACE2 receptor on human cells for its cell attachment and entry. The RBD region located in the spike glycoprotein is essential for membrane fusion and is regarded as a major target of the host antibody response. As a result, antibodies targeting the RBD region have been extensively explored as potential coronavirus therapeutic candidates, and these may additionally be utilized for the development of SARS-CoV-2 diagnostics.

In the current study, we used a plant expression system for the production of the RBD antigen of SARS-CoV-2 and a mAb CR3022 that is specific to the RBD of SARS-CoV. We used a geminiviral replicon vector derived from the bean yellow dwarf virus for the production of both RBD and mAb CR3022. Our results showed that both RBD antigen of SARS-CoV and mAb CR3022 could be produced rapidly in a large scale in N. benthamiana within a time frame of less than 2 weeks after the gene construct delivery. Recent developments in plant expression strategies using viral vectors and transient expression has increased protein yield, significantly reduced the upstream production cost, simplified the downstream processing of plant recombinant proteins which improves the commercial viability of the system. Our results indicated that RBD of SARS-CoV-2 was expressed in N. benthamiana plant as a soluble protein rapidly and accumulated to 8 µg/g leaf fresh weight, whereas mAb CR3022 accumulated at high levels at 130 µg/g leaf fresh weight in N. benthamiana leaves. The expression level is comparable with the expression level of other recombinant proteins produced by geminiviral vectors.

Interestingly, the plant-derived mAb exhibit potent binding against SARS-CoV-2, but failed to neutralize SARS-CoV-2 in vitro. Plant-made antibodies developed for West Nile virus, HIV, rabies lyssavirus, dengue virus and chikungunya virus have shown potent neutralization activity which
demonstrating that plants are a suitable platform for mAb production 41, 42, 43, 44, 45. Though mAb CR3022 cannot neutralize SARS-CoV-2 in vitro, the synergistic effect of CR3022 with other SARS-CoV-2 RBD-targeted monoclonal antibodies needs to be studied. Moreover, the earlier reports showed that the antibodies against alphavirus, cytomegalovirus and influenza virus did not show in vitro neutralization activity but confer protection in in vivo studies which highlights the importance of in vivo evaluation of non-neutralizing antibodies 46, 47, 48, 49, 50. Additionally, this mAb could also be utilized in the development of diagnostic assays for SARS-CoV-2, and the results of this study could contribute towards the low-cost development of mAbs specific diagnostic tools for SARS-CoV-2.

Altogether, our results convincingly demonstrate the practicability of using a plant expression system for the rapid and large-scale production of antibodies with diagnostic or therapeutic potential. In particular, this methodology is scalable to a commercial basis without a high capital investment, and is therefore suitable for use in developing economies. Furthermore, this study proved the robustness of plant transient expression system for the production of anti-SARS-CoV mAb CR3022 with high yield and low cost which can likely improve the affordability of mAb-based diagnosis in the developing world.

Conclusion

In summary, we have demonstrated the rapid production of mAb CR3022 in Nicotiana benthamiana. The expressed antibody was purified and analyzed for antigen-binding and SARS-CoV-2 virus neutralization activity in vitro. The results showed that plant-produced CR3022 mAb could bind to recombinant RBD protein of SARS-CoV-2, but that it did not neutralize the virus in vitro. Our study indicated that plant transient expression systems can greatly reduce the production cost and could be adapted for the low cost, rapid large-scale production of recombinant antibodies that could be used as detection/diagnostic reagents to detect COVID infection.

Materials And Methods

Construction of expression vectors of mAb CR3022 and RBD

The Institutional Review Board of Chulalongkorn University approved the present study. The coding gene fragments of variable heavy chain (VH) and variable light chain (VL) regions of mAb CR3022
(Accession Nos.: DQ168569.1 and DQ168570.1) were codon optimized for expression in *N. benthamiana* and commercially synthesized (Genewiz, Suzhou, China). The VH and VL were fused with human IgG1 CH and CL regions respectively. The resulting full length coding sequences of CR3022 HC and LC were cloned into geminiviral vector (pBY2e) as described previously.10 by a three fragment ligation: the backbone from pBY2e was obtained from XbaI-Sacl digestion; VH and CH were obtained by XbaI-Nhel and Nhel-Sacl digestion, respectively while VL and CL were obtained by XbaI-AfII and AfII-Sacl digestion, respectively to create the expression cassette pBY2e-CR3022-HC and pBY2e-CR3022-LC.

The receptor binding domain (RBD) located in spike protein of SARS-CoV-2 (SARS-CoV-2-RBD) (Accession No.: YP_009724390.1; F318-C617) was produced to test the mAb CR3022 binding. The coding nucleotide sequence of the RBD region of SARS-CoV-2 was codon optimized for *N. benthamiana* and commercially synthesized (Genewiz, Suzhou, China). The RBD was fused with an 8XHis tag at the C-terminus and cloned into geminiviral vector (pBY2e) with XbaI and Sacl restriction enzymes to create pBY2e-SARS-CoV-2-RBD.

**Transient expression of SARS-CoV-2-RBD and mAb CR3022 in *N. benthamiana* leaves**

The expression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 via electroporation, and the resulting strains were confirmed by PCR. Wild type *N. benthamiana* plants were grown in a green house with a suitable light/dark cycle at 28°C for 6-8 weeks. For mAb CR3022 expression, recombinant *Agrobacterium* containing pBY2e-CR3022-HC and pBY2e-CR3022-LC were pelleted and resuspended in infiltration buffer to an OD600 of 0.4 and mixed at a 1:1 ratio prior to vacuum infiltration. Similarly, an *Agrobacterium* strain harboring pBY2e-SARS-CoV-2-RBD was delivered into tobacco leaves by agroinfiltration.

**Extraction and purification of recombinant monoclonal antibody and RBDs from plant leaves**

Briefly, agroinfiltrated leaves were harvested at 3 days post infiltration (d.p.i) and proteins extracted in extraction buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.47 mM KH2PO4) at pH 7.4 using a previously developed method with some modifications10 for CR3022 mAb. The crude leaf extract was
obtained by homogenization and clarified by centrifugation at 15,000 g for 30 min at 4 °C. The recombinant proteins in the clarified protein extract were purified using Amintra® protein A resin affinity chromatography (Expedeon, Cambridge, UK). The recombinant purified antibody was analyzed by SDS-PAGE and the bands were visualized by InstantBlue™ staining (Expedeon, Cambridge, UK). For western blot analysis of mAb CR3022, the separated proteins were transferred onto nitrocellulose membranes and detected with human kappa light chain or gamma heavy chain conjugated with horseradish peroxidase (HRP).

For RBD antigen, leaves were harvested 3 d.p.i. and extracted with extraction buffer (5 mM imidazole, 20 mM Tris-HCl pH 8.8, 50 mM NaCl) as described previously with some modifications. The crude leaf extract was obtained by homogenization and clarified by centrifugation at 15,000 g for 30 min at 4 °C. The crude extract was purified by Amintra® Ni-NTA affinity resin (Expedeon, Cambridge, UK). Then the purity of the recombinant protein was analyzed by SDS PAGE and the bands were visualized by InstantBlue™ staining and detected by western blotting. The concentration of the purified RBD-His protein was determined by the Bradford assay.

**Binding assay of plant produced CR3022 antibody by ELISA**

ELISA was performed as described previously to examine the binding of CR3022 antibody to RBDs with some modifications. Briefly, 50 μl of the purified SARS-CoV-2 RBD (2 μg/ml) was coated on 96-well microplates (Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated at 4 °C overnight. After washing, the plates were blocked with 5% skim milk (BD, Franklin Lakes, NJ) in 1X PBS for 2 hours at 37 °C. Then, the plant-produced CR3022 antibody was added in triplicate 2-fold serial dilutions to the plate. After 2 hours incubation at 37 °C, sheep anti-human kappa light chain conjugated with HRP (The Binding Site, Birmingham, UK) at a dilution of 1:1000 in 1X PBS was added and samples were incubated for 1 hour at 37 °C. The plate was then washed three times with PBST, developed with TMB substrate (R&D system, Minnesota, USA) and the absorbance read at 450 nm.

**Virus and cells**

The Institutional Review Board of Mahidol University approved the present study. Vero and Vero E6 cells were incubated at 37°C and 5% CO2 in a humidified incubator. Cells were grown in DMEM
medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco, NY, USA), 100 U/mL of penicillin and 0.1 mg/mL of streptomycin. A SARS-CoV-2 isolate (SARS-CoV-2/01/human/Jan2020/Thailand) isolated from a confirmed COVID-19 patient at Bamrasnaradura Infectious Diseases Institute, Nonthaburi, Thailand was grown in Vero cells. The virus stock used in the experiments had undergone one passage in Vero E6 cells. Virus titers were quantitated as TCID50/ml in confluent cells in 96-well microtiter plates and stored at -80°C before use. All the experiments with live SARS-CoV-2 virus were performed at a certified Biosafety level 3 facility, Department of Microbiology, Faculty of Science, Mahidol University. The experimental protocol was approved by Mahidol University and all methods were performed in accordance with the relevant guidelines and regulations.

**Binding and neutralization of plant-derived mAb CR3022 against SARS-CoV-2**

Neutralizing titers were determined by a microneutralization assay. Positive convalescent serum of a COVID-19 patient was approved to use as clinical specimen by Faculty of Medicine Ramathibodi Hospital. The informed consent was waived by the Institutional Review Boards that approved the present study. The mAbs or positive serum were serially diluted 2-fold and incubated with 100 TCID50 of the SARS-CoV2 virus for 1 h at 37°C. The virus and antibodies were then added to a 96-well plate with 1 × 104 Vero E6 cells/well in DMEM supplemented with 2% FBS, 100 U/mL of penicillin and 0.1 mg/mL of streptomycin in quadruplicates. Wells were observed for cytopathic effect (CPE) at 3 days post infection, and the 50% neutralization titer was determined as the mAb concentration at which at least 50% of wells revealed no CPE. Anti-SARS-CoV mAb binding was detected by immunofluorescence. Vero E6 cell monolayers in 96 wells were inoculated with 10TCID50 SARS-CoV2 and incubated for 3 days. Uninfected and infected cells were washed three times with PBS, then incubated with ice-cold 1:1 methanol/acetone fixative for 20 minutes at 4°C then washed 3 times with PBST. Blocking reagent (2% bovine serum albumin, BSA) was added to the wells, and plates were incubated for 1 hr at room temperature. After washing, the mAbs or the positive serum at dilution factor 1:40 were added and samples were incubated at 37°C for 1 hr. The antibodies were detected by a 1:1000 dilution of an anti-human IgG antibody conjugated
with FITC (Santa Cruz Biotechnology, Inc.). After incubation at 37°C for 1 hr, the plate was washed three times and DNA staining dye, Hoechst33342 was added. The plate was then subjected to automated image acquisition and analysis using Operetta (PerkinElmer). All sera were heated inactivated in 56°C for 30 mins before use.

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Declarations

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Author Contributions

A.T., D.R.S., and W.P. designed all experiments. K.R., B.S., K.S., N.K., O.H., and W.B. performed protein expression, protein purification, and antigen binding by ELISA. S.M. and P.P. performed the viral binding and neutralization assay. All authors analyzed the data and contributed to paper preparation.

Conflict of Interest

The authors declare no conflict of interest.

Table

[Please see the supplementary files section to view the table.]

Figures
Figure 1

Schematic representation (a) and timeline (b) for the production of recombinant protein production in plants by transient gene expression (Agroinfiltration).
Figure 2

Schematic diagram of the T-DNA region of the plant expression vector used in the present study. 35S: cauliflower mosaic virus 35S promoter; Est 3’ FL: expressed sequence tags-full length at the 3’ end of transcription; Rb 7: tobacco RB7 promoter; C2/C1: bean yellow dwarf virus (BeYDV) ORFs C1 and C2, which encode the replication initiation protein (Rep) and RepA; LIR: long intergenic region of the BeYDV genome; SIR: short intergenic region of the BeYDV genome; P19: P19 gene from tomato bushy stunt virus (TBSV); LB: the left border, RB: the right border.
SDS-PAGE and western blot analysis of RBD protein of SARS-CoV-2 produced in *N. benthamiana*. The crude proteins were extracted from plants, and the RBD antigen was purified and analyzed on SDS-PAGE gels and visualized with InstantBlue™ (a). Lane M: protein ladder; Lane 1: total soluble protein of *N. benthamiana* agroinfiltrated with pBY2e-SARS-CoV-2-RBD; Lane 2: purified SARS-CoV-2-RBD. For western blot analysis, proteins on the blot were probed with a rabbit anti-His conjugated with HRP (b). Lane 1: crude extract from non-infiltrated *N. benthamiana*; Lane 2: total soluble protein of *N. benthamiana* agroinfiltrated with pBY2e-SARS-CoV-2-RBD; Lane 3: purified SARS-CoV-2-RBD.
SDS-PAGE and western blot analysis of plant-produced mAb CR3022. The crude proteins were extracted from plants, and the antibody was purified and analyzed on SDS-PAGE gels and visualized with InstantBlue™ (a). For western blot analysis, proteins on the blot were probed with anti-human IgG gamma chain conjugated with HRP (b) and anti-human IgG kappa chain conjugated with HRP (c) under non-reducing conditions. Lane M: protein ladder; Lane 1: Total soluble protein of N. benthamiana agroinfiltrated with pBY2e-CR3022-HC and LC; Lane 2: purified plant-produced mAb CR3022. Arrow head indicates full-length antibody.

Coomassie staining of purified antibody (d) Lane 1: commercial human IgG1 antibody (abcam); Lane 2: purified plant-produced mAb CR3022; Lane 3: purified plant-produced anti PD1 antibody.
Figure 5

Specific binding ELISA of plant-produced mAb CR3022 and SARS-CoV-2-RBD. The plant-produced mAb CR3022, standard human IgG1, and plant-produced anti PD1 antibody (as negative control) were incubated on plates coated with plant-produced SARS-CoV-2-RBD and detected with an HRP-conjugated anti-human kappa antibody. The data are the mean values of triplicate assays from each concentration.
Specific binding of plant-produced mAb CR3022 to SARS-CoV-2 in infected Vero E6 cells using immunofluorescence. The plant-produced mAb CR3022, positive serum, and plant-produced anti-PD1 antibody (as negative control) were incubated with SARS-CoV-2-infected and uninfected Vero E6 cells and signal detected with an FITC-conjugated anti-human IgG antibody (green color). Hoechst33342 was used for counterstaining (blue color). The data are the representative images of triplicate assays.

Supplementary Files
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Table1.docx