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Boosted expression of the SARS-CoV nucleocapsid protein in tobacco and its immunogenicity in mice

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ABSTRACT

Vaccines produced in plant systems are safe and economical; however, the extensive application of plant-based vaccines is mainly hindered by low expression levels of heterologous proteins in plant systems. Here, we demonstrated that the post-transcriptional gene silencing suppressor p19 protein from tomato bushy stunt virus substantially enhanced the transient expression of recombinant SARS-CoV nucleocapsid (rN) protein in *Nicotiana benthamiana*. The rN protein in the agrobacteria-infiltrated plant leaf accumulated up to a concentration of 79 μg per g fresh leaf weight at 3 days post infiltration. BALB/c mice were intraperitoneally vaccinated with pre-treated plant extract emulsified in Freund's adjuvant. The rN protein-specific IgG in the mouse sera attained a titer about 1:1,800 following three doses of immunization, which suggested effective B-cell maturation and differentiation in mice. Antibodies of the subclasses IgG1 and IgG2a were abundantly present in the mouse sera. During vaccination of rN protein, the expression of IFN-γ and IL-10 was evidently up-regulated in splenocytes at different time points, while the expression of IL-2 and IL-4 was not. Up to now, this is the first study that plant-expressed recombinant SARS-CoV N protein can induce strong humoral and cellular responses in mice.

1. Introduction

Severe acute respiratory syndrome (SARS) is an infectious disease caused by a newly evolved coronavirus (SARS-CoV) [1]. SARS was first detected in the Guangdong Province of China in late 2002. It rapidly spread across the world, and more than 8000 cases have been recorded thus far, with 10% mortality [2]. SARS-CoV infection may cause fever, pneumonia, diarrhea, respiratory lesions, or even death [3]. SARS-CoV was a zoonotic coronavirus in bats [4] or civets [5,6] that transcended the species barrier and then infected humans [7]. The SARS-CoV genome is a polyadenylated RNA of 29,727 nucleotides which encodes the 5′-replicase (rep) and at least 7 structural proteins, namely, the spike (S), 3a, envelope (E), membrane (M), 7a, 7b, and nucleocapsid (N) proteins [8–10]. Among these structural proteins, the S, E, M, and N proteins are common in all known coronaviruses [1]. The S protein of SARS-CoV binds to the cellular receptor ACE2 and then mediates viral entry and cell fusion; thus, the S protein is expected to be the most important candidate for vaccines [11–13]. In animal models, only S protein but not N, M, or E proteins can induce a high titer of SARS-CoV-neutralizing antibodies [14,15]. The N protein is the structural component of the helical nucleocapsid, and it plays an important role in the viral pathogenesis, replication, RNA binding, cell cytokinesis and proliferation [16–18]. Antibodies against N and S proteins were evidently detected in SARS patients on the early stage of SARS infection [19,20], which indicated that N protein of SARS-CoV was highly immunogenic in human. DNA vaccination of animal with SARS-CoV N gene led to the production of N-specific antibodies and specific cytotoxic T lymphocytes (CTLs) response, or even the replicon inhibition of vaccinia virus expressing N protein [21–23]. A DNA vaccine encoding N epitope stimulated cytotoxic T cells to kill N protein-expressing cell [24]. N protein expressed from recombinant measles and baculovirus resulted in strong humoral and cellular immune response in mice [25,26]. Furthermore, the SARS-CoV N protein induced not only a temporal specific T-cell response but also a long-term memory T-cell response that persisted for 2 years in recovered SARS patients [27–29]. Although N protein alone cannot elicit the production of neutralizing antibodies against SARS-CoV, the fused peptide of N and S protein was more efficient in stimulating the production of neutralizing antibodies than S peptide alone in BALB/c mice [30]. All these data suggested that N protein was an effective elicitor of humoral and cellular response.

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In addition, the pathological changes in SARS patients were associated with complicated cytokines dysregulation; moreover, the Th1/Th2 cytokine imbalance was closely correlated with the severity and outcome of SARS-CoV infection [31–34]. Zhang et al. had reported that IL-6 was positively correlated with SARS severity while IL-8 and TGF-β were negatively correlated with SARS severity in patient serum, and furthermore, IL-4, IL-10, and IFN-γ only showed changes in convalescent SARS patients [31,35]. However, another report showed that IFN-γ, IL-1, IL-6, IL-12 but not IL-2, IL-10, and IL-4 were up-regulated in SARS patients before and after treatment [35]. A serum analysis of acute SARS patients ($n = 98$) showed substantial elevation of IFN-γ, IL-6, IL-8, IL-10, IL-12, but not IL-2, IL-4, and TNF-α before any treatment [34]. As a key immunogen of SARS-CoV, the regulatory function of N protein on cytokines also showed complex pattern in animal system. For example, the vaccination of mice with recombinant baculovirus expressing SARS N protein resulted in augment of IFN-γ- and IL-4-secreting CD4+ cells, though IFN-γ secretion level was much high than that of IL-4, which indicated predominant Th1 response [25]. However, prior immunization of mice with recombinant vaccinia virus expressing SARS-CoV N protein and later SARS-CoV virus challenge caused significant expansion of both Th1 (IFN-g, IL-2) and Th2 (IL-4, IL-5) cytokines, reduction of anti-inflammatory cytokines (IL-10, TGF-β), as well as severe pneumonia [36,37]. The induction of IFN-γ [25,28,29], IL-6 [37], IL-11 [38] and other cytokines was also observed to related with SARS-N protein. Therefore, it is worthy to evaluate the cytokine changes in our system specially regarding of Th1 and Th2 response.

Plants are now considered as promising bioreactors for pharmaceutical protein due to their safety, low cost, high output, simple storage requirement, and benefits of eukaryotic posttranslational modifications [39]. Transient expression of target protein can be easily achieved by agrobacteria infiltration, thereby saving the time spent on generation of transgenic plants. Recombinant strains of *Agrobacterium tumefaciens* can be intermediator of transient expression of exogenous protein in plant by inserting the gene of interest into the T-DNA region of modified *A. tumefaciens* plasmid and then introducing the transformed agrobacteria into plant leaves [40]. However, the utility of this system was limited because ectopic protein expression was often down-regulated by post-transcriptional gene silencing (PTGS), which operates as a natural defense mechanism against viral accumulation or foreign DNA invasion in plants [41]. In plant host, exogenous RNA was likely to be formed into short RNA duplex intermediates (siRNA or miRNA) though multiple step process, and then the duplex RNA was unwound into single strand RNA before incorporation into RISC (RNA-induced silencing complex). Finally, the selected RNA strand bound to cognate host mRNA, resulting in target mRNA cleavage or translational repression [42]. However, for the maximum accumulation of viral proteins in plant, many viruses have developed mechanisms to suppress PTGS in different steps [43–45]. For example, the tomato bushy stunt virus (TBSV) p19 protein dimer and beet yellow virus (BYV) p21 protein can bind to the siRNA or miRNA duplex, and thus block the RNA duplex dissociation and downstream action [46]. Using this strategy, high expression of exogenous proteins had been accomplished in *Nicotiana benthamiana* based on suppression of gene silencing by p19 protein [45].

Plant-based vaccines exhibited immunogenicity and protection against different infectious diseases [47,48]. For example, the potato-expressed S1 protein of the coronavirus infectious bronchitis virus (IBV) protected chickens from virulent IBV infection when administered intramuscularly or orally [48]. However, among the SARS structural proteins, only S protein had been expressed in plants [49,50]. The transgenic tomato expressing S1 protein stimulated IgA secretion in oral vaccination; but IgG secretion was not detected in parental vaccination until commercial S peptide was injected as booster in immunization [50]. In our study, the viral PTGS suppressor p19 protein was enrolled to boost the transient expression of recombinant SARS-CoV N protein (rN) in *N. benthamiana*. The rN protein accumulated up to an average amount of 79 μg per g fresh tobacco leaves at 3 days post infiltration (dpi). Soluble extract of plant leaves containing rN protein was emulsified with Freund's adjuvant and then used to immunize BALB/c mice. Specific IgG (IgG1 and IgG2a) antibodies were effectively induced in mice vaccinated with plant-expressed rN protein. Meanwhile, the expression profiles of IL-10 and IFN-γ were altered in splenocytes of vaccinated mice.

2. Material and methods

2.1. Cloning of the SARS-CoV N gene into plant expression vector

The N gene of SARS-CoV (Urbani strain, GenBank accession no. AY278741) is 1269 bp in length and encodes a 423-aa protein. The cDNA fragment encoding the complete N protein was amplified from a bacterial artificial chromosome (BAC) vector (kindly provided by Dr. Luis Enjuanes, CNB-CSIC, Spain) carrying the whole SARS-CoV cDNA sequence. The following primers were used for the PCR amplification of SARS CoV N gene: forward, 5′-AAGCATGCGTCGACATGTCTGATAATGGACC-3′ and reverse, 5′-ATACGCGTTTATGCCTGAGTTGAATC-3′. The amplified product carried recognition sites for the restriction enzymes *Sph*I and *Mlu*I at the 5′ and 3′ ends, respectively. This N-gene PCR product was cloned into the pGEM-T vector to generate the new pGEM-T-N plasmid. The 1.5 kb fragment containing the SARS-CoV N gene was cleaved from pGEM-T-N with enzymes *Sph*I and *Mlu*I and subsequently inserted into the *Sph*I- *Mlu*I-digested pBAL vector which is a derivative of the empty plant binary vector pBA002 [51]. The new plant expression vector thus obtained was designated as pB-35S-rN. Its expression cassette contained the strong constitutive 35S promoter, the DNA sequence coding for 6 × His fusion tag, the cleavage site for the protease factor Xa, and SARS-CoV N protein. The 47 kDa recombinant N protein expressed from plasmid pB-35S-rN was referred to as rN protein. At the same time, the SARS-CoV N gene was cloned into the prokaryotic expression vector pET-32a (+) and then expressed in *Escherichia coli*. The purified product from *E. coli*, a 64-kDa recombinant N protein with Trx tag, S tag, and 6 × His tags at the N-terminus, was designated as sN protein [52]. The cDNA sequence corresponding to the p19 protein of TBSV (GenBank accession no. AJ288942) was sub-cloned into the binary vector pCambia1300-221 downstream of the 35S promoter, resulting in the new plant binary vector pC-35S-p19.

All the generated plant expression constructs were individually transformed into cells of *A. tumefaciens* EHA105 strain for infiltrating host plants.

2.2. Expression and preparation of recombinant SARS-CoV N protein in tobacco

Wild-type tobacco plants (*N. benthamiana*) were grown until 8–10 weeks old. *Agrobacterium* cells transformed with target plasmid were cultured at 28 °C for 20 h, centrifuged at 3200 × *g*, and resuspended in 10 mM $MgCl_2$ solution supplemented with 200 μM acetosyringone. The density of the agrobacteria suspension was then optimized for infiltration ($OD_{600} = 1.5$ for pB-35S-rN and control vector pBA002; $OD_{600} = 1.0$ for pC-35S-p19). The agrobacterium strain harboring plasmid pB-35S-rN or pBA002 was mixed with that harboring plasmid pC-35S-p19 in a 1:1 ratio, and the mixture was infiltrated into young tobacco leaves with a syringe. The sample leaves were collected at different days post infiltration, immediately frozen in liquid nitrogen, and stored at −80 °C until