Preparation of Polyclonal Antibodies of Rubisco Large and Small Subunits and Their Application in the Functional Analysis of the Genes

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Abstract Spinach Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) large (rbcL) and small (rbcS) subunits were separated by SDS-PAGE, and protein amount and purity were determined by Bradford assay. Polyclonal antibodies against rbcL and rbcS subunit were generated in female BALB/c mice and had no cross-reaction with each other. A total of 81 µg antigens were used and 0.3 ml anti-sera with titer of 1:5000 were yielded. The antibodies were also applicable to study rbcL and rbcS in tobacco plant Nicotiana benthamiana. Potato virus X vector pGR107 induced silencing of rbcS gene by Agrobacterium in Nicotiana benthamiana was performed. The expression level of rbcL and rbcS was lower in rbcS silenced plants than that in control plants as detected by the corresponding antibodies. This implied that the expression of rbcL was regulated by rbcS.

Key words ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); rbcL; rbcS; polyclonal antibody; virus-induced gene silencing (VIGS)

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) located in the chloroplast is the most abundant protein in the leaves of light-grown plants. This enzyme catalyzes the first step in net photosynthetic CO2 fixation and photorespiration. The native Rubisco consists of 8 large (rbcL) and 8 small (rbcS) subunits. In higher plants the large subunits are encoded by the chloroplast genome, while the small subunits are encoded by a gene family in the nuclear genome [1–4]. The primary structure of the large subunit is conservative, while the amino acid composition of the small subunit is highly variable. The large subunit and small subunits have a molecular weight of about 55 kD and 12–14 kD respectively [5]. Polyclonal antibodies against purified Green alga and tobacco Rubisco have been generated in rabbit [6,7].

Virus-induced gene silencing (VIGS) is a type of RNA silencing that is induced by virus vectors carrying homologous fragment of the host genes [8]. Several viruses, including tobacco mosaic virus [9], potato virus X [8] and tobacco rattle virus [10], have been developed as VIGS vectors. The Agrobacterium-mediated transient expression system is a useful tool to introduce genes into plant tissue rapidly [11,12]. This system enables gene expression to be completed in a short period of time without the need to produce transgenic plants. VIGS vectors could be transformed into Agrobacterium for infiltrating the leaves of target plants. In a number of successful cases of VIGS, scientists have conducted VIGS in an Agrobacterium-mediated transient expression system [13–16].

In this paper, the polyclonal antibodies against rbcL and rbcS were prepared in female BALB/C mice using antigens purified from spinach Rubisco. The expression level of rbcL and rbcS in Agrobacterium-mediated rbcS-silenced tobacco plants were studied by using the polyclonal antibodies, and the rbcL expression was regulated at post-
translational level by pGR107-rbcS.

Materials and Methods

Rubisco subunit preparation

Spinach Rubisco (Sigma) was dissolved in 1×PBS (pH 7.8), and run on the 12% SDS-PAGE. The gel was stained by 0.25 mM KCl pre-cooled at 4 °C. The rbcL and rbcS bands of Rubisco were cut from the gel separately and eluted with the electro-elution buffer (20 mM Tris-base, pH 8.3, 150 mM glycine) for 6 h at 100 V. The eluent was mixed with 4–5 times volume acetone and centrifuged at 1000 g for 10 min. and then stored overnight at −20 °C. The precipitate was collected and dissolved in 10 mM Tris-HCl, pH 8.0. Protein amount and purity were determined respectively by Bradford assay [17] and SDS-PAGE.

Immunization of rbcL and rbcS on mice

BALB/c mice received injection in peritoneal cavity with 27 µg/50 µl rbcL on day 1, 16, and 17, respectively. For first injection the Freund’s complete adjuvant was used, and Freund’s incomplete adjuvant was used for the following times. Indirect ELISA (I-ELISA) was performed to test the titer of serum from murine tail on day 27 and 47.

Immunization scheme with rbcS was performed in the same way as the large ones.

Immunogen for rbcL and rbcS by I-ELISA

Microtitration plates (Nunc, Denmark) were coated with 100 µl solution containing 0.5 µg rbcL in Tris buffer (pH 7.0) per well at 4 °C. After washed 3 times with PBST (0.05% Tween-20 in PBS) (each time for 3 min), the plates were blocked with 10% skinned milk in PBS (W/V) for 1 h at room temperature. After being washed several times, each well was incubated with 100 µl primary antibody (1:100) for 1 h at 37 °C, while the control wells were incubated with unimmunized BALB/c murine serum. Then anti-mouse IgG-HRP (1:10,000) was added as the secondary antibody and incubated for 1 h at 37 °C. The plates were washed thoroughly and incubated in dark for 15 min at room temperature. Finally, the substrate o-phenylenediamine was added to each well, and 2 M H2SO4 were added to stop the reaction. The optical density value was measured at 492 nm (A492).

Titer test for Rubisco small subunit antibody was performed in the same way as the large one.

Anti-serum separation

Mice were killed and blood was collected. The blood was incubated for 1 h at 37 °C and overnight at 4 °C, and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was further centrifuged at 10,000 g for 10 min at 4 °C to obtain the anti-serum. The anti-sera was stored at −20 °C.

Agroinfiltration of rbcS into tobacco plants

Agrobacterium strain GV3101 cells carrying the pGR107-rbcS and pGR107 plasmids were grown overnight at 28 °C in 5 ml LB medium containing 5 µg/ml tetracycline and 50 µg/ml kanamycin. 1 ml culture was added into 49 ml LB medium containing 20 µM acetosyringone and 10 mM MES with tetracycline and kanamycin, and incubated overnight at 28 °C. Cells were harvested by centrifugation at 10,000 g for 30 s. Bacterial pellets were suspended in 1 ml solution containing 10 mM MgCl2, 10 mM MES and 100 µM acetosyringone. The 22–25 day-old Nicotiana benthamiana tobacco plant that have been kept overnight in the bench at the room temperature for acclimatization was inoculated in the leaf surface with 20–50 µl bacterial suspension at 24 °C via a 2 ml-syringe.

Western blot

Total proteins were extracted from the leaves of tobacco. The protein concentration was determined using Bradford method with protein assay dye reagent (Sigma). Spinach Rubisco and total protein of Nicotiana benthamiana were separated by 12% SDS-PAGE. Then, proteins were transferred to nitrocellulose membranes with Mini trans-blot electrophoretic transfer cell (Bio-Rad). All membranes were incubated with blocking solution containing 1% skimmed milk in 1×TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) for 1 h at 37 °C. Membranes were then incubated for 1 h at 37 °C with primary antibodies against rbcL (1:500) or antibodies against rbcS (1:250). Following three times washing with 1×TBST (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20) for 20 min, membranes were subjected to peroxidase-conjugated goat anti-mouse IgG (H+L) diluted to 1:5000. After washing with 1×TBST, color reaction was developed with 3-amino-9-ethylcarbazole reagent.

Results

Rubisco subunit separation
Rubisco large and small subunits from spinach Rubisco were separated and purified by using SDS-PAGE and eluted electrically from the gel. The two subunits were considerable pure after gel separation (Fig. 1).

Preparation of polyclonal antibodies against rbcL and rbcS

Two mice were immunized for each subunit and 0.3 ml serum was obtained from each mouse. The antibody concentration was 2.38 mg/ml and 2.15 mg/ml for the large subunit, 2.21 mg/ml and 2.08 mg/ml for the small subunit respectively. Both of them gave satisfactory results. The titer was 1:5000 in ELISA application and the dilution was 500-fold against rbcL and 250-fold against rbcS in Western hybridization.

Phenotypes of rbcS infected tobacco plants

Plants infected with the pGR107-rbcS were shorter in height than the control. The leaves that were infected with pGR107-rbcS in blade tip developed pale green areas in the vein region after 11 day post-infection (dpi). Yellow area appeared on the leaves along the main vein after 22 dpi [Fig. 2(B)]. It indicated that the virus was spread through the leaf vein. After 30 dpi, there was a decrease in the extent of the yellowish areas on upper leaves, and some area of the leave surface curled. After 50 dpi, the phenomena were rarely observed.

Western blot analysis of rbcS silenced plants

Western blot were carried out to determine whether rbcL and rbcS could be identified by prepared polyclonal antibodies. Both 55 kD Rubisco large subunits in partially purified Rubisco from spinach [Fig. 3(B), lane 1] and crude extracts from the leaves of Nicotiana benthamiana [Fig. 3(B), lane 2 and 3] were recognized by anti-rbcL antibodies. The 12–14 kD Rubisco small subunits in partially purified Rubisco from spinach [Fig. 3(A), lane 1] and crude extracts from the leaves of Nicotiana benthamiana [Fig. 3(A), lane 2 and 3] were recognized by anti-rbcS antibodies as well. They did not cross react from each other. The antibodies against Rubisco large and small subunits of spinach also worked with Rubisco from Nicotiana benthamiana leaves.

The amount of rbcS and rbcL in plants silenced by pGR107-rbcS was less than that in control. Western blot
result showed that the band of rbcS gene silenced tobacco was less bright than those of control and pGR107 plants [Fig. 4(A)]. Protein from rbcS gene silenced plant was distinctly shorter in length than those from control plants and pGR107-infected plants [Fig. 4(C)]. The results showed that the amount of rbcL was reduced in rbcS gene silenced plants, though rbcS gene was the target gene of silencing. The expression level of rbcL was somehow regulated by rbcS indicated by the decreased amount of rbcL.

Discussion

In this paper we developed a laboratory-oriented protocol to produce polyclonal antibodies against Rubisco large and small subunits in small-scale. Raising mice polyclonal antibody against Rubisco large and small subunits only needed several dozen micrograms of antigens, which was far less than the amount other mammalian animals needed, i.e. rabbit. However, total sera sometimes contained other antibodies generated from some pathogens like bacteria besides the target antibody. If total proteins of plants contained identical or similar proteins of these microorganisms, these proteins will be recognized by the polyclonal antibodies to yield the extra bands [18]. Polyclonal antibodies differ from the monoclonal antibodies in the number of binding epitopes. Therefore, sometimes the specificity of a polyclonal antibody was not as good as monoclonal...
antibody though the titration was higher [19]. It was important to consider that if polyclonal antibodies were suitable for your own experimental purpose in your study. In our case these antibodies did not cross-react each other between the Rubisco large and small subunits. Furthermore, it was demonstrated the constant reaction to homogeneous proteins was derived from both spinach and tobacco, and indicated that these antibodies had commonly applicable potentials. The amount of antibodies obtained in this protocol could be used for 30 times in Western blot. In some cases proteins of interest were difficult to obtain in a large amount, our protocol might be a choice.

The expression levels of rbcS/rbcL in rbcS-silenced plants were lower than those in control plants as shown by the Western blot result. The rbcL expression level primarily adjusted as the rbcS expression level when the rbcL mRNA translation initiated [20]. Therefore it was concluded that the expression level of rbcL was regulated by rbcS.

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