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ORIGINAL PAPER

A Novel Co-immunoprecipitation Protocol Based on Protoplast Transient Gene Expression for Studying Protein–protein Interactions in Rice

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Abstract Protein–protein interactions constitute the regulatory network that coordinates diverse cellular functions. Co-immunoprecipitation (co-IP) is a widely used and effective technique to study protein–protein interactions in living cells. However, the time and cost for the preparation of a highly specific antibody is the major disadvantage associated with this technique. In the present study, a co-IP system was developed to detect protein–protein interactions based on an improved protoplast transient expression system by using commercially available antibodies. This co-IP system eliminates the need for specific antibody preparation and transgenic plant production. Leaf sheaths of rice green seedlings were used for the protoplast transient expression system which demonstrated high transformation and co-transformation efficiencies of plasmids. The transient expression system developed by this study is suitable for subcellular localization and protein detection. This work provides a rapid, reliable, and cost-effective system to study transient gene expression, protein subcellular localization, and characterization of protein–protein interactions in vivo.

Keywords Co-immunoprecipitation · Commercial antibody · Transient expression · Rice (*Oryza sativa* L.)

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Introduction

The ease and frequency of genome sequencing has motivated the study of protein function in life science research. The transient or stable protein complex of proteins is necessary for proper cellular functions (Uhrig 2006; Tsai et al. 2009). These interactions are fundamental for nearly all biological processes (Kiel et al. 2008; Wu et al. 2009). A large number of methods have been developed to study protein–protein interactions (Berggard et al. 2007). In transient expression systems, fluorescence-based methods such as resonance energy transfer (RET) and bimolecular fluorescence complementation (BiFC) are frequently used for determination of protein–protein interactions (Ciruela 2008). However, these methods are technically and experimentally demanding (Ciruela 2008). Protein co-immunoprecipitation (co-IP), on the other hand, is an effective and technically feasible method for studying protein–protein interactions in living cells, including interactions of subunits within a protein complex. Moreover, the co-IP of proteins from cellular fractions is the most convincing evidence to demonstrate that proteins physically interact in vivo (Berggard et al. 2007; Miernyk and Thelen 2008). However, the co-IP experiment relies on the ability of an antibody to stably and specifically bind to complexes containing the bait protein. The main disadvantage of co-IP is the time and cost associated with the preparation of the specific antibody. Moreover, it is often difficult to obtain an antibody with high specificity (Berggard et al. 2007; Miernyk and Thelen 2008). In animals, a widely used method is based on the co-IP of proteins from cells transiently expressing a tagged bait protein (Masters 2004). The commercially available antibodies against the tags are relatively specific and do not cross-react with the endogenous proteins (Masters 2004; Berggard et al.

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2007). In addition, the co-IP can be performed from cells transfected with different tagged versions of two putative interaction proteins (Berggard et al. 2007). In higher plants (e.g., *Arabidopsis*), transgenic plants with tagged proteins are also commonly used to perform co-IP experiments using an antibody against the tag (He et al. 2009; Gao et al. 2010).

The protoplast transient expression system has been proven to be an important tool for studying protein subcellular localization, RNA interference (RNAi), high-throughput assays, and signal transduction (Sheen 2001; Kagaya 2002; Bart et al. 2006; Kim et al. 2013). Recently, the protoplast transient expression system applying to co-IP assay has been reported in plants such as *Nicotiana tabacum* (Moffett 2011) and *Arabidopsis thaliana* (Krasileva et al. 2010), but has not been reported in rice. In addition, although protoplast transient expression systems based on rice green tissues have been reported (Bart et al. 2006; Chen et al. 2006; Zhang et al. 2011), the efficiency of protoplast isolation and transformation is significantly lower than that of dicot plants. These factors restrict the use of protoplast transient expression systems in rice functional genomics research.

In this study, an improved method for the isolation and transformation of protoplasts from rice green leaf sheaths was developed. Our data suggest that this transient expression system could be used for protein subcellular localization as well as co-IP assay with the commercially available antibodies, i.e., anti-GFP and anti-FLAG antibodies, which provides a useful tool for determination of protein–protein interaction in vivo.

Materials and Methods

Cloning

All transient expression vectors used in this study were constructed based on pUC18 vector. The constitutive Cauliflower Mosaic Virus 35S (CaMV 35S) promoter (p35S), *Green Fluorescent Protein* (*GFP*)/*Yellow Fluorescent Protein* (*YFP*)/*Cyan Fluorescent Protein* (*CFP*) fragments, and the NOS terminator (NOS) were cloned into pUC18 vector. The full-length open reading frames (ORF) from rice gene, i.e., *Ribulose-1,5-bisphosphate carboxylase/oxygenase* (*RuBisCO*) *small subunit* (*RbcS*, *ORF1*, GenBank: NM_001073139.1), *RuBisCO activase* (*RbcA*, GenBank: NM_001075087.2), *ATP synthase gamma chain* (*AtpC*, GenBank: NM_001066303.1), *nuclear protein silent information regulator2* (Os*SRT1*, GenBank: NM_001058878.1), *coatomer protein complex subunit epsilon* (*ε-cop*, *ORF2*, GenBank: NM_001060382.1), *alpha-amylase isoform I-1* (*AmyI-1*, *ORF3*, GenBank: NM_001054755.1), and *Glossy 1-6* (Os*GL1-6*, *ORF4*, GenBank: NM_001055025.1) were amplified and subcloned into the N-terminal of the *GFP*, respectively. For the construction of p35S-RbcS-FLAG, the p35S, 3 tandem repeats of the FLAG sequence and the NOS were cloned into the pUC18, then the ORF of *RbcS* was subcloned between the p35S and 3 tandem FLAG. The primer sequences with corresponding enzyme sites are listed in the supplementary material (Supplementary Table SI).

Plant Growth Conditions

Dehulled rice seeds both Zhonghua 11 (ZH11, *Oryza sativa* L. ssp. *japonica*) and Annong N (AN, *Oryza sativa* L. ssp. *indica*) were surface-sterilized using 2 % (v/v) sodium hypochlorite for 30 min, then germinated and cultured on half-strength Murashige and Skoog medium in the tissue culture room with 12 h light (about 100 μm m^{-2} s^{-1} of intensity)/12 h dark at 26 °C for about 7 days. Seedlings were then grown hydroponically under natural light with 300~400 μm m^{-2} s^{-1} of illumination intensity for 3 days.

Protoplast Isolation

The protoplasts were isolated based on the methods reported by Zhang et al. (2011) with slight modifications. Briefly, leaf sheaths (Fig. 1a) of 30 rice seedlings (about 1.5 g) were cut into 1- to 2-mm pieces using a fresh sharp razor blade on filter paper. The leaf sheath pieces were quickly transferred into 10 ml 0.6 M mannitol and incubated for 30 min at room temperature. Then, the mannitol solution was discarded and replaced with 10 ml digestion solution containing 0.5 M mannitol, 10 mM 4-morpholineethanesulfonic acid (MES), 1.5 % cellulase Onozuka RS (Yakult, Japan), 0.75 % macerozyme Onozuka R-10, 10 mM $CaC1_2$, and 0.1 % Bovine Serum Albumin (BSA), pH 5.7. To optimize the digestion time, the samples were incubated for 1, 2, 3, 4, 5, 6, 8, 10, and 12 h in the dark with gentle shaking (40–50 rpm) at 28 °C, respectively. After incubation, the digestion solution was shaking to release the protoplasts followed by filtration through a 50-μm cell strainer to collect the protoplast suspension. The residues were subsequently re-suspended in 20 ml of modified W5 solution (154 mM NaCl, 125 mM $CaC1_2$, 5 mM KC1, 2 mM MES, pH 5.7) (Bart et al., 2006; Zhang et al., 2011) with shaking to release the remaining protoplasts followed by filtration. Then, the pooled protoplast suspensions were centrifuged at 150*g* for 5 min to collect the protoplasts. The collected protoplasts were gently washed twice with modified W5 solution. The pellet was re-suspended in MMg solution (4 mM MES, pH 5.7, 0.5 M mannitol, and 15 mM $MgCl_2$). Protoplasts were quantified by microscopy using a hemocytometer. The viability of protoplasts was determined by the fluorescein diacetate (FDA) staining method as described (Larkin 1976).

Protoplast Transformation

The protoplasts were re-suspended in MMg solution and adjusted to a concentration of 1×10^7 cells ml^{-1}, and from this, 100 μl

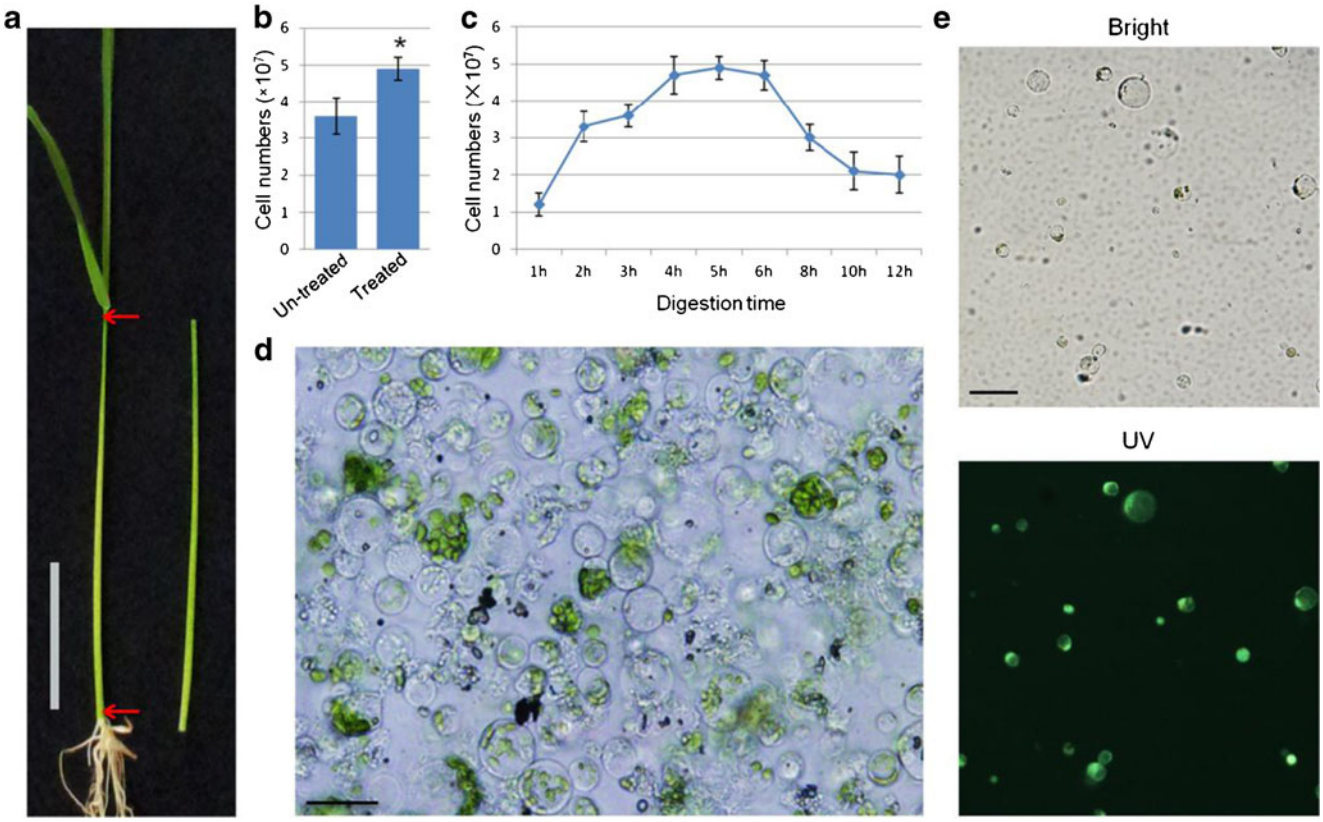

Fig. 1 Isolation of rice leaf sheath protoplasts. **a** The leaf sheath of a 10-day-old green rice seedling was used to isolate protoplasts. The leaf sheath between two *red arrowheads* (*left*) indicated the optimal part for protoplasts isolation (*right*). *Scale bar* 3 cm. **b** Comparison of the yields of protoplasts isolated from leaf sheaths with pre-plasmolysis treatment and non-treatment. The protoplasts yield was improved after treatment. Values are the means ± SE from 3 independent experiments. Asterisk (*) denotes significantly different ($P<0.05$) in comparison with non-treatment. **c** Effect of digestion time on the yield of protoplasts. The amount of protoplasts increased significantly up to 4 h reaching a stable yield level from 4 to 6 h and declined thereafter. Values are the means ± SE from 3 independent experiments. **d** Observation of protoplasts isolated from leaf sheaths. Protoplasts were observed at the microscope at ×200 magnification. *Scale bar* 50 μm. **e** Protoplasts were incubated with FDA for 2 min, and then observed under bright field (*top*) and UV filter (*bottom*). Over 95 % of protoplasts emitted strong fluorescence, indicating a high level of vitality. *Scale bar* 50 μm

of protoplasts were mixed with 10 μl of plasmids (1 μg μl^{-1}) and 110 μl of polyethylene glycol (PEG) solution (40 % PEG 4000, 0.3 M mannitol and 0.1 M $CaCl_2$) in a 2-ml Eppendorf tube. The transformation mixture was incubated for 15 min in darkness at 28 °C. The mixture was then diluted in 1 ml modified W5 and centrifuged at 150*g* for 5 min. The protoplasts were re-suspended in 1 ml WI solution (4 mM MES, pH 5.7, 0.5 M mannitol and 20 mM KCl) and then transferred to multi-well plates. The multi-well plates were incubated for 8–12 h at 28 °C without shaking.

Fluorescence Microscopy

Living cells containing GFP or GFP fusion proteins were imaged under the fluorescence microscope (Olympus, MF30). The transformed protoplasts were incubated with 0.1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI) for 5 min and observed under the fluorescence microscope using the UV filter. Excitation and emission filters Ex480±20/DM505/BA535±25, Ex500±10/DM515/BA535±15, Ex436±10/DM455/BA480±20 and Ex535±25/DM565/BA645±37.5 were used for GFP, YFP, CFP, and chlorophyll auto-fluorescence, respectively.

Data Analysis

All experiments were repeated independently at least three times. The experimental data were analyzed using SAS statistical software (2008). A least significant difference (LSD) procedure was employed to detect staistical differences. A significant level of $P<0.05$ was used for all statistical analyses.

Western Blotting

The protoplasts were harvested by centrifugation at 300*g* for 5 min. The total proteins were extracted by boiling in SDS-PAGE sample buffer (50 mM Tris-HCl, 2 % SDS, 0.1 %

bromophenol blue, 10 % Glycerin, 1 % β-mercaptoethanol) for 5 min. About 1×10^5 cells were subjected to western blot analysis, which was carried out as previously reported (Li et al. 2011) with modifications. The proteins were separated on 12 % minigel and electrophoretically transferred to Nitrocellulose Blotting Membrane (Pall) using the wet transfer apparatus (Bio-Rad). Samples were visualized using an ECL kit (Cell Signaling Technology). All primary antibodies (Abmart) were used at 1:5,000 dilutions. The horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma-Aldrich) was used at 1:10,000 dilution.

Co-IP

The protoplasts were harvested by centrifugation at 300*g* for 5 min. Approximately, 2×10^6 protoplasts were homogenized in IP buffer containing 25 mM Tris-HCl, pH 7.5,150 mM NaCl, 0.5 % Triton X-100, 1 mM EDTA, and 1 % protease inhibitor (Sigma) and incubated for 30 min on ice and then centrifuged at 15,000*g* for 10 min at 4 °C to remove aggregates. The protein extract was then diluted to a concentration of 1–2 μg μl^{-1} in IP buffer. Forty microliters of Protein A/G Agarose Beads (Abmart) were added, and the mixture was incubated for 3 h at 4 °C with gentle shaking (40–50 rpm). The beads were removed by centrifugation at 14,000*g* for 5 min at 4 °C and antibodies were added to the supernatant. After overnight incubation at 4 °C, 40 μl of protein A-Sepharose was added and incubated for further 2–3 h at 4 °C. The beads were collected by centrifugation at 100*g* for 3 min at 4 °C, and then washed five times with ice-cold IP buffer. The proteins were eluted from the beads by boiling in SDS-PAGE sample buffer for 5 min and analyzed by western blotting. The monoclonal mouse anti-GFP and monoclonal mouse anti-FLAG antibodies were obtained from Abmart.

Results

Isolation and Transformation of Rice Leaf Sheath Protoplasts

In this study, the leaf sheaths of 10-day-old ZH11 rice green seedlings were used for protoplast isolation (Fig. 1a). Protoplast isolation was facilitated by a pre-plasmolysis treatment for 30 min in a hyperosmotic mannitol solution (0.6 M mannitol), which resulted in an increased protoplast yield (Fig. 1b). The digestion time was also optimized by counting protoplasts yields at 1, 2, 3, 4, 5, 6, 8, 10, and 12 h post-incubation in enzyme solution. The amount of protoplasts was found to increase significantly up to 4 h reaching a stable yield level from 4 to 6 h and declining thereafter (Fig. 1c; Supplementary Table SII). The above results showed that there was a yield of approximate 5×10^7 cells from 30 seedlings (10-day-old) digested for 4 h in the enzyme solution, which was 5- to 10-fold higher than reported in previous studies (Bart et al. 2006; Chen et al. 2006; Zhang et al. 2011). Healthy protoplasts appeared round and 90.9±5.0 % of the protoplasts contained chloroplasts (Fig. 1d; Supplementary Figure I), and the viability of protoplasts was found to exceed 95 % as determined by FDA staining (Fig. 1e).

The PEG-mediated procedure was used for protoplast transformation. A GFP expression plasmid driven by the p35S was constructed and transformed into rice leaf sheath protoplasts. Transformation efficiencies were assayed at different combinations of protoplast densities (1×10^6, 5×10^6, 1×10^7, 5×10^7 cell ml^{-1}) and plasmid concentrations (0.5, 1.0, 1.5, 2.0 μg μl^{-1}) (Supplementary Table SIII). The transformation efficiency was found to be up to 90 % at protoplast density of 1×10^7 cell ml^{-1} (1×10^6 cell total) and the plasmid concentration of 1 μg μl^{-1} (10 μg total) (Fig. 2a, b; Supplementary Table SIII), which was a significant improvement relative to previously reported data (Bart et al. 2006; Chen et al. 2006; Zhang et al. 2011). The co-transformation efficiency was found to be approximately 80 % (Fig. 2c–f) when p35S-YFP and p35S-CFP were co-transformed into protoplasts.

To test the application of the protoplast isolation and transformation protocol on other rice variety, protoplasts were isolated from an *indica* rice variety AN as described above. There was a yield of approximate 4.0×10^7 cells from 30 seedlings digested for 4 h. The viability of the protoplasts was found to exceed 95 % by FDA staining and the transformation efficiency of p35S-GFP was found to be up to 80.1 % (Supplementary Figure II). The results showed that our protocol could perform well in other genotypes of rice. Therefore, the protocol described by this study represents a high efficiency protoplast transient expression system.

Expression Levels of Exogenous Proteins with Different Molecular Weights

Appropriate expression levels of candidate proteins are crucial for co-IP since the co-IP analysis requires a certain and minimum protein level (Masters 2004; Miernyk and Thelen 2008). However, the expression level of the proteins with high molecular weight was usually low, in that the transformation efficiency of the corresponding plasmids with large size was usually low (Bart et al. 2006). Therefore, the expression levels of the fusion proteins with different molecular weights were assayed in this system to test the effect of plasmid size on expression of exogenous proteins. To improve the transformation efficiency, all transient expression vectors used in this study were constructed based on the 2.7-kb pUC18 vector to minimize the size of the recombinant plasmids. Four rice genes with varying size, i.e. *RbcS* (*ORF1*, 0.5 kb), *ε-cop* (*ORF2*, 0.9 kb), *AmyI-1* (*ORF3*, 1.2kb), and *OsGL1-6* (*ORF4*, 1.9kb), were fused to the *GFP* driven by p35S. The resulting recombinant

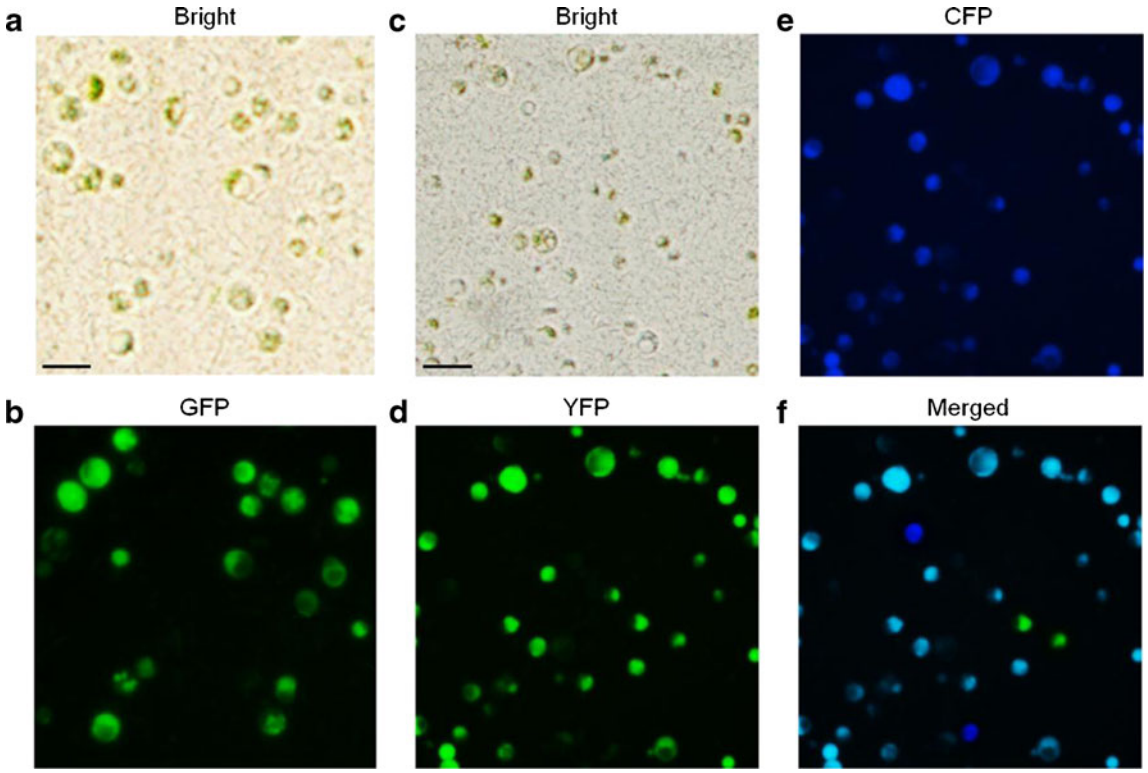

Fig. 2 Transformation of rice leaf sheath protoplasts. **a**–**b** p35S-GFP was transformed into protoplasts and then protoplasts were observed under bright field (**a**) and GFP filter (**b**). About 90 % protoplasts emitted *green* fluorescence. *Scale bar* 50 μm. **c**–**f** p35S-YFP and p35S-CFP were co-transformed into protoplasts. The protoplasts were observed under bright field (**c**), YFP filter (**d**), and CFP filter (**e**). The YFP and CFP images were merged (**f**). *Green* protoplasts in (**f**) were cells expressing YFP only, and *blue* protoplasts were cells expressing CFP only, and all other cells in (**f**) represent those co-expressing YFP and CFP. The co-transformation efficiency was about 80 %. *Scale bar* 50 μm

plasmids are 4.5, 4.9, 5.2, and 5.9 kb in size, and the molecular weights of the corresponding fusion proteins are 44, 59, 74, and 98 kD, respectively (Fig. 3a). The transformation efficiencies were about 70–89 % (Supplementary Table SIV), indicating a high transformation efficiency for large size plasmids. After transformation and 12 h culture, extracts of about 10^5 cells were subjected to western blotting using anti-GFP antibody. We detected expressions of all exogenous proteins (Fig. 3b), demonstrating that the protoplast transient expression system was sufficient for protein assays.

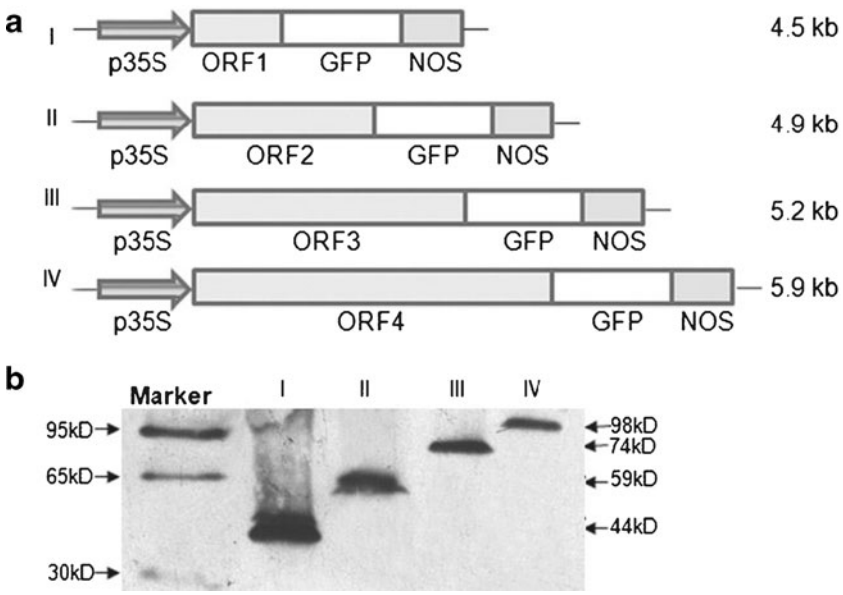

Fig. 3 Western blot analysis of exogenous proteins with different molecular weights. **a** Schematic representation of transient expression constructs in different sizes. The *numbers on the right* represent the size of vectors. **b** Expression vectors with different sizes were transformed into rice leaf sheath protoplasts, followed by western blot analysis against anti-GFP antibody. All exogenous proteins expressed at high levels. *Labels on the right* indicate the molecular weight of exogenous proteins. *Roman numbers* refer to the numbers of expression vectors as given in (**a**)

Protein Subcellular Localization in Rice Leaf Sheath Protoplasts

Since many gene products are associated with particular intracellular compartments in a conditional manner (Lilley and Dupree 2007), it is essential for cell biologists to know the subcellular localization of their proteins of interest to determine the protein interaction network. Therefore, we studied the protein subcellular localization using the green rice sheath protoplasts, wherein the chloroplasts could be clearly observed by their red chlorophyll autofluorescence. The rice chloroplast protein RbcS (Spreitzer and Salvucci 2002) and nuclear protein OsSRT1 (Huang et al. 2007), as well as two rice proteins potentially located to chloroplasts, i.e. RbcA and AtpC, were fused to the N-terminal of GFP (Fig. 4a). The resulting plasmids were transformed into rice leaf sheath protoplasts. After 8 h incubation, the GFP signals were observed and clearly distinguishable (Fig. 4b–e). In p35S-RbcS-GFP-transformed cells, the green RbcS-GFP signal completely overlapped with the red chlorophyll autofluorescence (Fig. 4b). In p35S-AtpC-

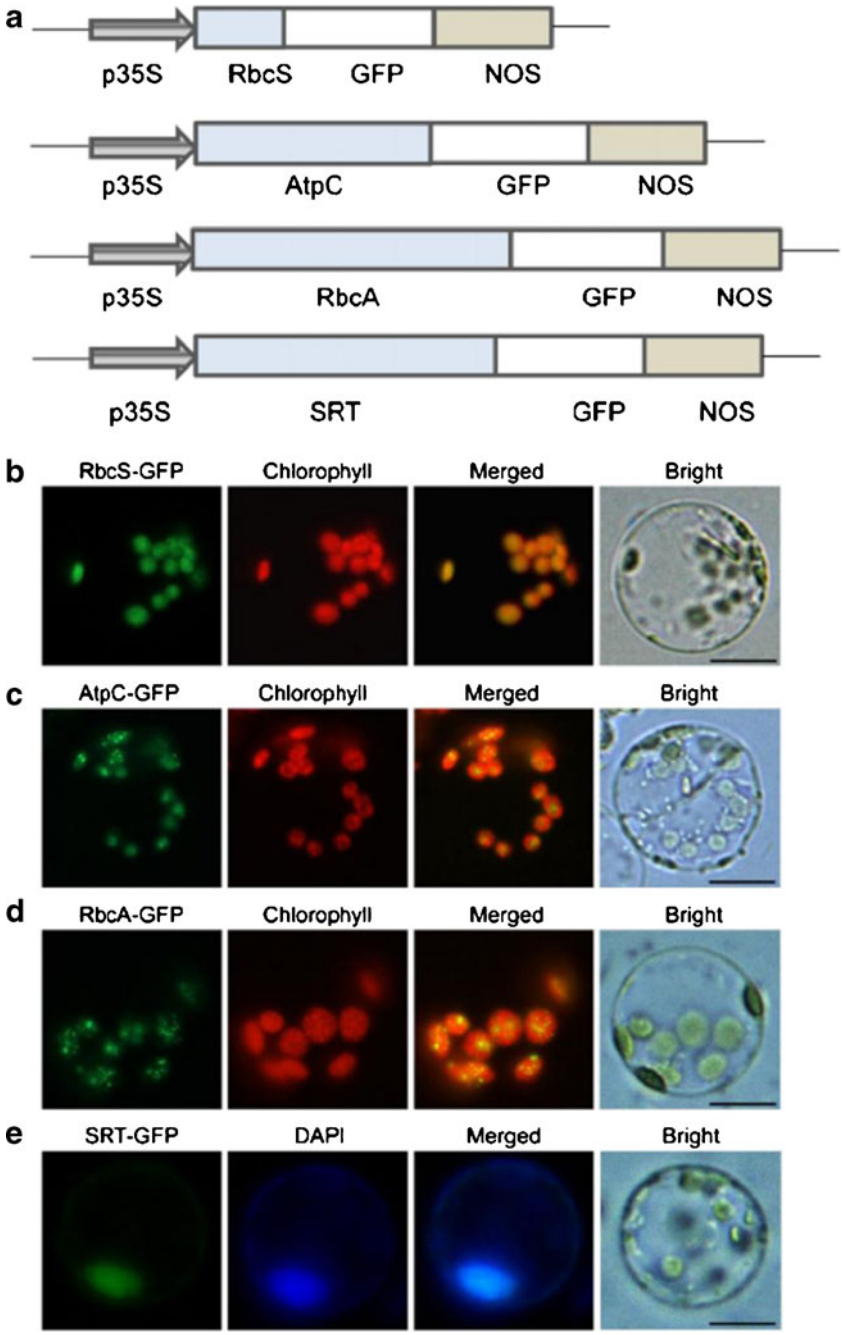

Fig. 4 Fluorescent patterns of GFP fusion proteins in transformed protoplasts. **a** Schematic representation of transient expression constructs used for subcellular localization analysis. **b**–**d** p35S-RbcS-GFP (**b**), p35S-AtpC-GFP (**c**) and p35S-RbcA-GFP (**d**) was transformed into rice leaf sheath protoplasts. The GFP signal (*green*) completely co-localized with chlorophyll autofluorescence (*red*). *Scale bar* 10 μm. **e** p35S-SRT-GFP was transformed into rice leaf sheath protoplasts, and transformed protoplasts were subsequently stained with DAPI for 5 min. The GFP signal (*green*) completely co-localized with the nuclear DAPI signal (*blue*). *Scale bar* 10 μm

GFP- and p35S-RbcA-GFP-transformed cells, the green GFP signals located to the chloroplasts (Fig. 4c, d). The p35S-SRT-GFP-transformed cells were subsequently stained by DAPI, a nuclear fluorescent dye (Pandey et al. 2008). The green SRT-GFP signal completely overlapped with the nuclear DAPI signal (Fig. 4e). These results indicate that fusion proteins could be located to their target organelles accurately in our transient expression system.

Co-IP Experiments with Commercial Tag Antibody Using Rice Leaf Sheath Protoplasts

RuBisCO, a high-abundant chloroplast protein complex, were used to test the validity of our system for co-IP experiments. RuBisCO consists of eight small subunits (RbcS) and eight large subunits (RbcL) (Spreitzer and Salvucci 2002). Approximately 2×10^6 protoplasts were collected after transformation with p35S-RbcS-GFP (Fig. 5a). Protein extracts of transformed protoplasts were incubated with anti-GFP antibody, and then the total extracts and the precipitate were western-blotted against anti-RbcL antibody (Agrisera). An equal amount of untransformed protoplast extracts were used as negative control. Our results showed that RbcL was detected in GFP immunoprecipitates of transformed protoplasts extracts (Fig. 5b, top), indicating that the endogenous RbcL was captured by the anti-GFP antibody. We then investigated a more complicated and lower abundant protein complex in rice, i.e. the coatomer of Coat protein I (COPI) vesicle comprising seven different subunits, α-, β-, β′-, γ-, δ-, ε-, and ζ-COP (Contreras et al. 2000). The ε-COP subunit was fused to GFP (Fig. 5a) and used to transform rice protoplasts. After co-IP using anti-GFP antibody, western blot analysis with antibody against β′-COP showed that the β′-COP was detected in GFP immunoprecipitate of the transformed protoplasts extracts (Fig. 5b, bottom). These data suggest that endogenous proteins interacting with tagged proteins could be successfully captured by the commercially available antibodies against the tag.

Furthermore, a reciprocal co-IP assay was performed using two commercial antibodies, i.e. GFP-tag antibody and FLAG-tag antibody in this system. Plasmids p35S-RbcS-GFP and p35S-RbcS-FLAG were constructed and subsequently used to co-transform rice leaf sheath protoplasts. A RuBisCO complex contains 8 RbcS in vivo (Spreitzer and Salvucci 2002), thus the RbcS-GFP and RbcS-FLAG could interacts in a co-transformed protoplast. The extracts of co-transformed protoplasts, p35S-RbcS-FLAG-transformed protoplasts and untransformed protoplasts were subjected to co-IP assay using anti-GFP antibody, western blot analysis was then performed using anti-FLAG antibody. Our results showed that RbcS-FLAG was detected in GFP immunoprecipitate of co-transformed protoplasts extracts (Fig. 5c), indicating that RbcS-FLAG was captured by anti-GFP antibody. Next, extracts from co-transformed protoplasts, p35S-RbcS-GFP-transformed protoplasts, and untransformed protoplasts were subjected to co-IP assay using anti-FLAG antibody, followed by western blot analysis against anti-GFP antibody. The results showed that RbcS-GFP was detected in FLAG immunoprecipitate of co-transformed protoplasts extracts (Fig. 5d), indicating that RbcS-GFP was captured by anti-FLAG antibody. The control treatment, co-IP of co-expression of two non-interacting proteins, i.e. *RbcS* and *OsSRT1* fused with GFP and FLAG, respectively, demonstrated that anti-GFP and anti-FLAG antibodies did not interact with each other

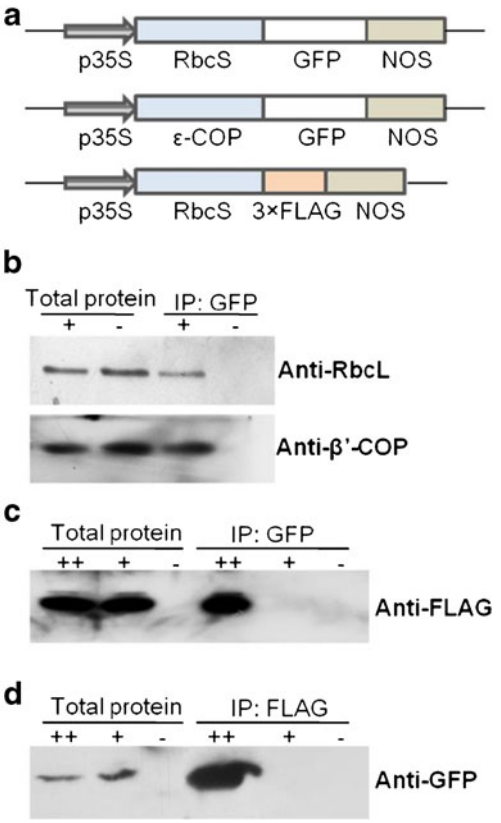

Fig. 5 Co-IP analysis using transformed rice leaf sheath protoplasts. **a** Schematic representation of transient expression constructs used for co-IP assay. **b** *Top* Co-IP of proteins from RbcS-GFP transformed (+) and untransformed (-) protoplasts was performed using anti-GFP antibody, followed by western blot analysis against anti-RbcL antibody. The RbcL was detected in anti-GFP immunoprecipitates (*IP: GFP*) of transformed protoplasts. *Bottom* Co-IP of proteins from ε-COP-GFP transformed (+) and untransformed (-) protoplasts was performed using anti-GFP antibody, followed by western blot analysis against anti-β′-COP antibody. The β′-COP was detected in anti-GFP immunoprecipitates (*IP: GFP*) of transformed protoplasts. **c** Co-IP of proteins from RbcS-FLAG + RbcS-GFP co-transformed (++), RbcS-FLAG transformed (+), and untransformed (-) protoplasts was performed using anti-GFP antibody, followed by western blot analysis against anti-FLAG antibody. RbcS-FLAG was detected in anti-GFP immunoprecipitates (*IP: GFP*) of co-transformed protoplasts. **d** Co-IP of proteins from RbcS-FLAG + RbcS-GFP co-transformed (++), RbcS-GFP transformed (+), and untransformed (−) protoplasts was performed using anti-FLAG antibody, followed by western blot analysis against anti-GFP antibody. RbcS-GFP was detected in anti-immunoprecipitates (*IP: FLAG*) of co-transformed protoplasts

(Supplementary Figure III). Therefore, co-IP of two putative proteins with tag antibody can be performed using the protoplast transient expression system developed by this study, which overcomes the drawbacks of specific antibody preparation and transgenic plant production.

Discussion

Previous studies have shown that a high level of gene expression could be detected in a short period in protoplast transient expression system, making it widely useful for gene functional analysis in plants (Miao and Jiang 2007; Yoo et al. 2007). Here, an improved rice leaf sheath protoplasts transient expression system was developed, which has high protoplast yield and transformation efficiency. Furthermore, this system could be used for co-IP experiments, demonstrating its applicability as a useful tool for investigating protein–protein interactions.

Plant mesophyll protoplasts are a good choice for gene functional studies (Sheen 2001; Yoo et al. 2007). However, unlike the *Arabidopsis* and tobacco leaves, rice leaves are covered with an epicuticular wax layer and contain up to 10 % silicon (Ma et al. 2007; Islam et al. 2009), which in turn hinders the infiltration of enzyme solution into the leaves during protoplast isolation. Consequently, the yield of protoplasts from rice leaves is very low. Our study shows that young leaf sheaths (10-day-old seedlings) with reduced silicon deposition (Ma and Yamaji 2006) is ideal for protoplast isolation. It was also found that pre-plasmolysis treatment (30 min in a hyperosmotic mannitol solution) facilitated protoplast isolation from leaf sheath strips in this study. As a result, the yield and viability of protoplasts improved significantly, which in turn contributed to higher transformation efficiency. Our method also optimized the plasmid concentrations and protoplasts densities in order to achieve higher transformation efficiency with a lower number of cells without compromising the quantity of DNA. Previous studies have shown that transformation efficiency varied significantly with plasmids size and a 12-kb plasmid only obtained 25–30 % transformation efficiency (Bart et al. 2006). For transient gene expression, a big and complicated vector is not needed because the transformed gene was not integrated into plant genome (Davey et al. 2005). Thus, to improve the transformation efficiency, all transient expression vectors used in this study were constucted based on the 2.7-kb pUC18 vector. Consequently, the transformation efficiency of p35S-GFP was found to be up to 90 %, which is significantly higher than previously reported (Bart et al. 2006; Chen et al. 2006; Zhang et al. 2011). In our system, one chloroplast protein, one nuclear protein, and two predicted chloroplast proteins were found to be localized precisely when transiently expressed in rice leaf sheath protoplasts (Fig. 4). In addition, precise localizations of a number of proteins located in nucleus, mitochondria, endoplasmic reticulum, lysosome, etc. have been observed by using this leaf sheath protoplast transient expression system (our unpublished data). Hence, our study provides a reliable technique for the identification of protein subcellular localization in rice.

Traditionally, yeast two-hybrid and GST-pull down systems are the two common choices for detection of protein–protein interactions (Berggard et al. 2007; Miernyk and Thelen 2008). Nevertheless, drawbacks such as a high rate of false-positives and the lack of plant-specific protein modification limit the application of these exogenous systems in plants (Berggard et al. 2007; Miernyk and Thelen 2008). Another widely used method to detect protein–protein interactions is the BiFC based on transient expression system (Chen et al. 2006; Ohad et al. 2007; Citovsky et al. 2008; Zhang et al. 2011). However, this method is imperfect because ectopic expression of split YFP fragments does not necessarily lead to the reconstitution of an active fluorophore, and/or the structure of protein dimers may prevent successful reconstitution of YFP (Citovsky et al. 2008). In contrast, co-IP is a more reliable method than those mentioned above. In several studies, protein–protein interactions could not be detected by co-IP directly due to the difficulty in obtaining suitable antibodies (Miernyk and Thelen 2008). Our studies overcome these drawbacks by using rice leaf sheath protoplasts expressing tagged proteins to perform co-IP experiments. Co-IP of two complicated protein complexes, the high-abundance chloroplast RuBisCO and the low-abundance cytoplasmic coatomer, were investigated in transformed protoplasts using anti-GFP antibody in this study. Endogenous RbcL and β′-COP were detected in GFP immunoprecipitates of p35S-RbcS-GFP- and p35S-ε-COP-GFP-transformed protoplasts, respectively (Fig. 5b). The commercial antibody against the tag was successfully used in this system. As in animal systems (Masters 2004; Ceriani 2007), our system provides a succinct method for studying protein–protein interactions in vivo. The exogenous RbcS-FLAG was detected in GFP immunoprecipitates of p35S-RbcS-FLAG and p35S-RbcS-GFP co-transformed protoplasts, and the exogenous RbcS-GFP was also detected in FLAG immunoprecipitates of p35S-RbcS-FLAG and p35S-RbcS-GFP co-transformed protoplasts (Fig. 5c, d), which demonstrated that co-IP experiments can be performed using protoplasts transformed with tagged versions of two putative interaction partners avoiding the production of specific antibodies. This system can also be applied to co-IP experiments to detect whether a protein could form dimer or polymer complexes using protoplasts expressing candidate proteins in different tagged versions. Our data showed that exogenous proteins ranging in molecular weights from 44 to 98 kD exhibited high expression levels, indicating the possibility that a large proportion of plant proteins could be co-immunoprecipitated in this system. In addition, this system investigates protein–protein interactions by co-IP of proteins

expressed in their native intracellular environment. Therefore, the false-positive rate in this system would be significantly reduced as compared to exogenous systems. This study details a highly reliable and economical tool for the primary determination of interactions of candidate proteins.

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