Application of BioID Technology to Screen Rice GS3 Interacting Proteins (Postprint)

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Abstract

GS3, an atypical subunit in the rice heterotrimeric G-protein system, represents a major quantitative trait locus controlling grain size and functions as a negative regulator in grain size modulation; however, studies on its directly interacting proteins are limited, and the underlying mechanisms remain elusive. BioID (proximity-dependent biotin identification) is a proximity-based protein labeling technology whose operational principle relies on biotin ligase catalyzing the biotinylation of proximal proteins, with biotin binding tightly to streptavidin, thereby enabling enrichment of target proteins using streptavidin-conjugated magnetic beads. This technology offers advantages of high sensitivity, efficiency, and short experimental duration, providing a novel methodology for screening interacting proteins. In this study, employing rice protoplasts as the experimental system, we utilized BioID technology to screen for GS3-interacting proteins in rice. Western blot analysis demonstrated successful expression of the BirAG-GS3 fusion protein in protoplasts and efficient biotinylation of GS3-proximal proteins. Streptavidin magnetic bead enrichment of biotinylated proteins followed by mass spectrometry sequencing yielded proteins that potentially interact with GS3 either directly or indirectly. The identified proteins were subjected to functional enrichment analysis and annotation, and a protein-protein interaction network was constructed. BiFC validation of selected proteins revealed that GS3 may interact with ICL, PPDK, RPN7, and RH15, implicating biological processes including regulation of energy metabolism, storage of seed starch components, the ubiquitin-proteasome system, and apoptotic pathways. These findings establish a foundation for deciphering the protein regulatory network of GS3.

Full Text

Preamble

Screening of Interaction Proteins of GS3 in Rice Using BioID

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Abstract

GS3, the noncanonical subunit of the heterotrimeric G protein system in rice, is a major quantitative trait locus controlling grain size and functions as a negative regulator in this process. However, few studies have investigated its directly interacting proteins, and the underlying mechanisms remain unclear. BioID (proximity-dependent biotin identification) is a proximity-based protein labeling technology that works through a biotin ligase which biotinylates nearby proteins; these biotinylated proteins can then be enriched using streptavidin-conjugated magnetic beads due to the strong affinity between biotin and streptavidin. This technology offers advantages of high sensitivity, efficiency, and short processing time, providing a novel approach for screening interacting proteins. In this study, we employed BioID technology in rice protoplasts to screen for GS3-interacting proteins. Western blot analysis confirmed successful expression of the BirAG-GS3 fusion protein and biotinylation of proteins in proximity to GS3. Streptavidin magnetic beads were used to enrich biotinylated proteins, which were then analyzed by mass spectrometry to identify proteins potentially interacting directly or indirectly with GS3. Functional enrichment and annotation of these proteins were performed, and a protein-protein interaction network was constructed. BiFC validation of selected proteins revealed that GS3 likely interacts with ICL, PPDK, RPN7, and RH15, implicating biological processes including energy metabolism regulation, seed starch storage, the ubiquitin-proteasome system, and apoptotic pathways. These results establish a foundation for elucidating the protein regulatory network of GS3.

Keywords: BioID, GS3, Rice, Grain size, Interaction proteins

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Introduction

In both animals and plants, heterotrimeric G proteins consist of , , and subunits and serve as crucial signaling regulators. In plants, heterotrimeric G protein signaling pathways play indispensable roles in growth, development, and stress responses (Stateczny et al, 2016). Compared to mammalian heterotrimeric G protein signaling networks, plant G protein family members exhibit atypical characteristics in terms of quantity, structure, upstream receptors, downstream effector composition, and even the entire G protein cycling mechanism (Trusov & Botella, 2016). Moreover, no unified heterotrimeric G protein network model has been established in plants. For instance, a seven-transmembrane receptor-like protein AtRGS was identified in Arabidopsis, but no homologous seven-transmembrane receptor protein has been found in rice. Surprisingly, an eleven-transmembrane receptor COLD1 was discovered in rice that works together with heterotrimeric G proteins to regulate Ca2+ channels and confer cold tolerance (Stateczny et al, 2016). Recent perspectives suggest that receptor-like kinases play a primary role in G protein signaling networks (Choudhury & Pandey, 2016).

The noncanonical subunit GS3 of heterotrimeric G proteins plays an important role in regulating rice grain size and shows great potential for rice yield improvement. The noncanonical subunit comprises a conserved -like domain at the N-terminus, a predicted transmembrane domain, and a cysteine-rich domain at the C-terminus (Botella, 2012). The -like domain of GS3 negatively regulates grain size, while its C-terminal tail in turn suppresses the function of the N-terminus, creating a functional "tug-of-war" within the GS3 protein. The stronger the N-terminal function, the more pronounced the inhibition of grain length. When the -like domain of GS3 is mutated or deleted and cannot form a dimer with the subunit to function, grains reach their maximum length; the complete GS3 protein with its tail suppresses the negative regulatory function of the -like domain, weakening the genetic effect and resulting in medium grain length; when only the -like domain is present, the inhibitory effect is strongest and grain length is shortest (Mao et al, 2010).

BioID (proximity-dependent biotin identification) is a proximity-based protein labeling technology that marks proteins physically near a bait protein with biotin as evidence of protein interaction. The core functional component of this technology is the biotin ligase. In *Escherichia coli*, the biotin ligase BirA recognizes substrates with specificity, biotinylating proteins that contain a particular amino acid sequence. Mutation of BirA at position 118 (R118G, BirA*) greatly reduces its substrate specificity, enabling biotinylation of proteins without requiring a specific amino acid sequence (Kwon and Beckett, 2000). BioID was first applied in mammalian cells (Roux et al, 2012). Beyond animal cells, BioID technology has also been used in unicellular organisms such as* Trypanosoma brucei*,* Toxoplasma gondii*, and* Dictyostelium discoideum*, as well as in host-pathogen systems, such as studies of HIV-1 interacting proteins (Varnaite and MacNeill, 2016).

Our laboratory has established a BioID system suitable for rice by investigating various factors affecting BioID application in plants, using rice protoplasts as material (Lin et al, 2017). The operational workflow of BioID technology mainly includes: 1) constructing a bait protein expression vector fused with BirAG and transforming it into plant protoplasts for transient expression of the BirAG-fused bait protein; 2) incubating in the presence of biotin to biotinylate proteins near the bait protein; 3) extracting total protoplast proteins and co-incubating them with streptavidin magnetic beads, allowing biotinylated proteins to bind to the beads; and 4) obtaining enriched biotinylated proteins through washing and elution steps, which can then be identified using western blot and mass spectrometry analysis (Lin et al, 2017). Studies suggest that proteins within approximately 10 nm of the bait protein, including both direct and indirect interactions, can be biotinylated and identified (Varnaite and MacNeill, 2016).

While the function of GS3 in regulating seed size is well established, studies on the underlying mechanism of GS3 regulation remain scarce. This paper employs the BioID method, an approach distinct from conventional yeast two-hybrid screening, to identify GS3-interacting proteins, providing new insights for investigating GS3-related regulatory networks.

Materials and Methods

1.1 Materials

Rice variety "Zhonghua 11" (*Oryza sativa* L.) was preserved in our laboratory. KOD FX polymerase was purchased from Toyobo (Shanghai) Biotechnology Co., Ltd. Restriction enzymes and T4 DNA ligase were purchased from Dalian TaKaRa Biological Co., Ltd. Plasmid extraction kits were from Axygen. *E. coli* competent strain JM109 was preserved in our laboratory. BioID vectors were preserved in our laboratory (Lin et al, 2017). Primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. Streptavidin magnetic beads were provided by Solulink. Anti-BirA rabbit polyclonal antibody was from LifeSpan BioSciences. Secondary antibody was horseradish peroxidase-labeled goat anti-rabbit antibody from Proteintech. RIPA strong protein extraction buffer and horseradish peroxidase-labeled streptavidin were from Beyotime. Sequencing was completed by Beijing Ruibo Xingke Biotechnology Co., Ltd. Protein mass spectrometry sequencing was performed by Shenzhen Weinaifei Biotechnology Co., Ltd.

1.2.1 Construction of Rice GS3 BioID Vector

Total RNA was extracted from rice and reverse-transcribed into cDNA. PCR amplification of the GS3 CDS was performed using primers GS-F: GAAGATC-TATGGCAATGGCGGCGGCGCCCCGGCC and GS-R: GGACTAGTTCA-CAAGCAGGGGGGGCAGCAACGAGGGAC. Underlined sequences indicate introduced BglII and SpeI restriction sites. The reaction conditions were: annealing temperature 60°C, extension for 1 min, 35 cycles. The PCR product

was recovered, digested, and purified.

The BioID vector map is shown in [Figure 1: see original paper]. The vector was double-digested with BamHI and SpeI, denatured, and ligated with the purified GS3 fragment. After transformation into *E. coli*, positive colonies were selected, plasmids were extracted, and sequences were verified.

1.2.2 Rice Protoplast Transformation

Protoplast transformation followed the method of Yang et al (2014). The pUbi::BirAG::GS3 construct was transformed into 400 L of rice protoplasts (concentration approximately 2×10 cells · L ^{1}), with pUbi::BirAG transformed as a positive control. Exogenous biotin was added to the culture medium at a final concentration of 50 mol · L ^{1} and incubated for 24 h. Additionally, another group of pUbi::BirAG::GS3 was transformed without biotin addition as a negative control.

1.2.3 Protein Extraction and Identification

1) After 24 h of protoplast culture, protoplasts were collected by centrifugation at 3000 r · min ^{1} for 3 min, washed with 0.6 M mannitol, and collected again. Total protein was extracted by adding 700 L of RIPA strong extraction buffer, vortexing vigorously, and centrifuging at 13,000 r · min ^{1} for 20 min at 4°C. The supernatant was collected and stored at -20°C.

2) For protein analysis, 15 L of protein sample was separated on 12% SDS-PAGE and transferred to PVDF membrane using wet transfer. After transfer, western blot analysis was performed using anti-BirA rabbit polyclonal antibody as the primary antibody and horseradish peroxidase-labeled goat anti-rabbit antibody as the secondary antibody to detect BirAG protein expression. Similarly, horseradish peroxidase-labeled streptavidin was used to detect biotinylated proteins.

1.2.4 Enrichment of Biotinylated Proteins

1) Magnetic bead preparation: The original magnetic bead stock was vortexed for 1 min to disperse aggregated beads. Twenty microliters of beads were transferred to an EP tube, placed on a magnetic rack for 2 min, and the supernatant was removed. Beads were washed with 1 mL of RIPA strong buffer, vortexed to disperse, and rocked at room temperature for 5 min. This washing step was repeated twice. Beads were then incubated with protein samples overnight at 4°C. After incubation, beads were washed once with 2% SDS and twice with RIPA buffer, then suspended in PBS buffer and sent for mass spectrometry analysis.

2) Protein mass spectrometry sequencing followed the method of Wisniewski et al (2009). Enriched biotinylated proteins on magnetic beads were digested with trypsin to generate peptide solutions for LC-MS/MS analysis

(mass spectrometer model: Triple TOF 5600 LCMS, AB SCIEX). Raw wiff files from mass spectrometry were processed and analyzed using Maxquant software. The database used was the *Oryza sativa* subsp. *japonica* protein database from UniProt. Search parameters were set as follows: cysteine alkylation with iodoacetamide, variable modifications including methionine oxidation and protein N-terminal acetylation, trypsin digestion, secondary spectrum matching tolerance of 40 ppm, peptide false discovery rate controlled at 1% FDR, and protein false discovery rate controlled at 1%.

1.2.5 BiFC Validation of Interacting Proteins

Selected functional genes were cloned and BiFC vectors were constructed. Protoplast transformation was performed and fluorescence was observed using an LSM 800 laser confocal microscope after 12 h. The vectors used were pSAT6-cEYFP-C1-B-(3108) and pSAT6-nEYFP-C1-(E2884). Primers used were as follows (restriction sites underlined and enzymes indicated):

- ICL-F: GAAGATCTATGTCGTCGCCGTTCTCCGTGCCATCT (BglII)
- ICL-R: CGGAATTCCATCCTGGATTTGGCAAGAACATGGCT (EcoRI)
- PPDK1-F: GAAGATCTATGCCGTCGGTTTCGAGGGCCGTGTGC (BglII)
- PPDK1-R: GCTCTAGAGAGGAGCACCTGAGCTGCAGCTAGCCT (XbaI)
- RPN7-F: GAAGATCTATGGACGGCGGCGTAGGCGAGGAAGGG (BglII)
- RPN7-R: CGGAATTCCAGGTCAATGACTCGTGATAGCTTCTG (EcoRI)
- RHD3-F: GAAGATCTATGGACGCCTGTTTTTCAACACAGCTT (BglII)
- RHD3-R: CGGAATTCGTGTGCAATCGGGCTTGAATATTCGGGT (EcoRI)
- RH15-F: CGAGCTCAAAATGGGCGAAGCTGAGGTCAAGGACAAC (ScaI)
- RH15-R: GCAGTACTCGAAGGCATATATGTCGAAGTATCAAT (XbaI)

Results

2.1 Expression of BirAG-GS3 Protein in Rice Protoplasts

The pUbi::BirAG plasmid was transformed into protoplasts with biotin addition as a positive control. Two groups of pUbi::BirAG::GS3 were transformed: one with biotin addition (experimental group) and one without (negative control). After 24 h incubation, total protoplast proteins were extracted and western blot detection was performed using rabbit anti-BirA polyclonal antibody as the

primary antibody. The predicted size of BirAG was 35.29 kDa, while the BirAG-GS3 fusion protein was approximately 59.62 kDa, consistent with experimental results. As shown in [Figure 2: see original paper], the BirAG-GS3 fusion protein was successfully expressed.

Note: After protoplast transformation and incubation in medium with (+) or without (-) biotin, total proteins were analyzed by western blot using anti-BirA antibody to detect BirAG expression. Coomassie Brilliant Blue G-250 staining was used to monitor protein loading amounts.

2.2 Biotinylation of GS3 Proximal Proteins in Rice Protoplasts

Three groups of protein samples were analyzed by western blot using horseradish peroxidase-labeled streptavidin ([Figure 3: see original paper]). The results showed multiple bands in the positive control BirAG group, indicating biotinylation of various rice proteins. The BirAG-GS3 group without biotin addition showed only a single band representing endogenous biotinylated proteins, indicating that BirAG-GS3 was expressed but did not biotinylate surrounding proteins. In contrast, the BirAG-GS3 group with biotin addition showed increased band numbers and unique bands, demonstrating that BirAG-GS3 successfully biotinylated directly or indirectly interacting proteins.

Note: After protoplast transformation and incubation in medium with (+) or without (-) biotin, total proteins were analyzed by western blot using streptavidin-HRP to detect biotinylated proteins in rice. Coomassie Brilliant Blue G-250 staining was used to monitor protein loading amounts.

2.3 Mass Spectrometry Identification of GS3 Proximal Proteins

Biotinylated proteins from each group were enriched by magnetic beads, processed through tryptic digestion, and analyzed by LC-MS/MS. Raw wiff files from mass spectrometry were processed and analyzed using Maxquant software. The identified proteins were visualized in a Venn diagram ([Figure 4: see original paper]). After filtering with the BirAG group and GS3(-) group, we identified 101 proteins specific to the experimental GS3(+) group. These proteins were imported into the website https://www.ebi.ac.uk/QuickGO for gene ontology annotation ([Figure 5: see original paper]). The results showed that GS3 proximal proteins participate in multiple biological functions, including metabolism of various nitrogenous compounds, amino acids, and peptides. In terms of cellular components, GS3 proximal proteins were mainly localized in the cytoplasm and enriched in chloroplasts. For molecular functions, GS3-related proteins primarily exhibited structural molecule activity and regulated functions dependent on small molecules, such as binding various nucleucleotides and anions. These proteins were classified according to their molecular functions (). When imported into the STRING database (http://string-db.org), a protein-protein interaction network for GS3 proximal proteins was constructed ([Figure 6: see original paper]). The analysis revealed numerous experimentally verified or com-

putationally predicted interactions among GS3 proximal proteins, which were enriched in ribosome, porphyrin and chlorophyll metabolism, fatty acid elongation, glyoxylate and dicarboxylate metabolism, and photosynthesis pathways.

Note: Circle nodes represent genes/proteins, rectangles represent KEGG pathways or biological processes. Color gradient from yellow to blue indicates increasing P-values, with yellow representing smaller P-values. Solid lines indicate known interactions, while dotted lines represent predicted interactions.

2.4 Detection of Interactions Between GS3 and Proximal Proteins

To validate the interacting proteins identified by BioID, we performed bimolecular fluorescence complementation (BiFC) assays on filtered proteins. We selected ICL (Isocitrate lyase), PPDK1 (Pyruvate phosphate dikinase 1), RPN7 (26S proteasome non-ATPase regulatory subunit 6), RH15 (DEAD-box ATP-dependent RNA helicase 15), and RHD3 (ROOT HAIR DEFECTIVE 3). The UniProt accessions for these five proteins are noted and framed in . The CDS of these genes were amplified and BiFC expression vectors were constructed. BiFC results showed that ICL, PPDK, RPN7, and RH15 interact with GS3 protein ([Figure 7: see original paper]).

Conclusion and Discussion

Through BioID, we successfully identified 101 candidate proteins. We selected five for preliminary BiFC validation and confirmed interactions with four proteins: ICL, PPDK, RPN7, and RH15, which are involved in multiple plant growth and physiological processes. As a rapid screening method for potential interacting proteins, BioID differs from yeast two-hybrid by detecting proximal proteins in vivo and can identify transient or weak interactions.

ICL and MS (malate synthase) are key rate-limiting enzymes in the glyoxylate cycle. Oil seeds convert fat to sugar through the glyoxylate cycle, which is crucial for seed germination and post-germination growth. Since rice seeds primarily contain starch, the glyoxylate cycle in rice seeds may participate in other physiological responses. Under anaerobic conditions, plant respiration is inhibited, leading to ethanol fermentation for NAD+ production, which generates toxic substances such as acetaldehyde. Under normal growth conditions, ICL and MS are barely detectable but increase rapidly under flooding conditions (Lui et al, 2005), suggesting that GS3 may be involved in stress physiology.

PPDK1 catalyzes the reversible reaction between pyruvate and PEP (phosphoenolpyruvate). Pyruvate provides carbon skeletons for amino acid and fatty acid synthesis. PEP can be carboxylated to form OAA (oxaloacetate) to enter the TCA cycle or gluconeogenesis pathway. Additionally, PEP can react with erythrose-4-phosphate to form shikimate, which participates in lignin and alkaloid synthesis and is an important species in secondary metabolism. In rice, PPDK1 expression is low in mesophyll cells but highly expressed in grains (Kang et al, 2005). Research indicates that PPDK1 functions during rice seed

development rather than after maturation, with its activity primarily regulated by phosphorylation and protein degradation pathways (Chastain et al, 2006). Reduced PPDK1 expression or activity leads to increased grain chalkiness and reduced grain weight (Kang et al, 2005; Wang et al, 2015). Studies in maize show that PPDK1 regulates endosperm quality by controlling the glycolytic pathway (Lappe et al, 2018). We therefore speculate that GS3 participates in regulating sugar metabolism pathways in the endosperm.

DEAD-box RNA helicases (RHs) participate in nearly all biological processes from RNA transcription to decay, powered by ATP hydrolysis, and play important roles in plant growth, development, and stress responses (Linder & Fuller-Pace, 2015). In rice, apoptosis inhibitor 5 (API5) forms a transcription complex with AIP1 (API5-INTERACTING PROTEIN1) and AIP2 (API5-INTERACTING PROTEIN2) to induce expression of cysteine protease 1 (CP1). The "API5-AIP1/2-CP1" module regulates cell apoptosis during rice tapetum degradation (Li et al, 2011). AIP1 and AIP2 are essentially ATP-dependent DEAD-box RNA helicases, namely OsRH56 and OsRH15. In Arabidopsis, DNA/RNA helicases including DEAD-box proteins participate in downstream signaling networks of the heterotrimeric G protein subunit (Khatri et al, 2017). In rice, the G subunit participates in the GA pathway, and its mutant *dl* shows dwarfism, while the OsRH2 and OsRH34 double mutant exhibits a similar phenotype, suggesting that DEAD-box helicases may also be involved in G protein-mediated GA signaling pathways (Huang et al, 2016). We therefore hypothesize that AIP2 may also participate in GS3 downstream network regulation.

The ubiquitin-proteasome system (UPS) selectively degrades proteins to maintain protein homeostasis, thereby participating in plant growth, development, and stress responses. The 26S proteasome consists of one 20S core particle (CP) and two 19S regulatory particles (RP). RPN7 is an essential subunit for 19S particle assembly and forms part of the lid component (Collins and Goldberg, 2017). The 26S proteasome participates in G protein signaling networks. XLG2 directly interacts with FLS2 and BIK1, and together with AGB1 and AGG1/2 functions to reduce proteasome-mediated BIK1 degradation for optimal immune activation (Liang et al, 2016). We therefore speculate that GS3 may also participate in the ubiquitin-proteasome degradation system to transmit signals. In *Saccharomyces cerevisiae*, the ubiquitin ligase SCFUcc1 acts as a metabolic switch regulating the glyoxylate cycle (Nakatsukasa et al, 2015). Combined with the biological functions of PPDK1 and ICL, we hypothesize that GS3 directly participates in regulating glycolysis, TCA, and glyoxylate cycles.

This study successfully captured proximal proteins of GS3 using BioID technology and constructed their interaction network. BiFC experiments demonstrated that among five selected GS3 proximal proteins, four interacted with GS3: ICL, PPDK, RPN7, and RH15. These proteins are involved in energy metabolism regulation, seed starch storage, the ubiquitin-proteasome system, and apoptotic pathways, providing clues for constructing plant heterotrimeric G protein networks and offering new insights for rice yield improvement. Since this experi-

mental system used rice mesophyll protoplasts, the captured proteins may have limitations. We are currently exploring the use of whole transgenic plants as BioID material to more comprehensively screen for target protein interactions across different tissues and cell types.

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Note: Figure translations are in progress. See original paper for figures.