

Article

Dimerization of CRL1 and LBD16 is Essential for Crown Root Development in Rice

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Abstract: The Lateral Organ Boundary Domain (LBD) family proteins, characterized by a highly conserved Lateral Organ Boundaries (LOB) domain at the N-terminus, are plant-specific transcription factors that play essential roles in a wide range of organogenesis processes. Among them, two class I family members, Crown Rootless 1 (CRL1) and Lateral Organ Boundaries Domain 16 (LBD16), have been extensively studied for their crucial functions in crown root development in rice. Although the leucine zipper-like motif in the LOB domain is hypothesized to mediate protein dimerization, the specific mechanisms underlying homo- and heterodimer formation by CRL1 and LBD16, as well as the functional significance of such interactions in regulating crown root morphogenesis, remain largely unknown. In this study, we investigated the structural basis of leucine zipper-like motif-mediated dimerization of CRL1 and LBD16, and its functional consequences. Through pull down, Split-luciferase (LUC) and Co-immunoprecipitation (Co-IP) assays, we demonstrated that rice CRL1 and LBD16 can form homodimers (CRL1-CRL1, LBD16-LBD16) and heterodimers (CRL1-LBD16). Specifically, the conserved leucine residues within their leucine zipper-like motifs are essential for mediating this dimerization. Importantly, our results revealed that CRL1-LBD16 dimerization not only facilitates nuclear localization, but also acts as a molecular switch regulating their functional competence in crown root development. These findings have significantly advanced our understanding of the genetic and molecular mechanisms governing crown root morphogenesis in rice. They may also contribute to the formulation of strategies for the development of rice varieties featuring improved root architecture, enhanced yield potential, and optimized stress resilience.

Keywords: crown root; CRL1; LBD16; dimerization

1. Introduction

Roots, as the principal underground organs of plants, fulfill crucial functions in numerous physiological activities, including water absorption, nutrient uptake, and environmental adaptation [1,2]. Among various root types, crown roots (adventitious roots) are especially important in rice. They initiate growth from the stem base and constitute a major component of the root system, contributing substantially to plant environmental adaptation and crop yield [3,4]. Unraveling the regulatory networks governing crown root development is critical for breeding elite rice varieties with high yield potential and superior adaptability to changing climate conditions to achieve sustainable agricultural production.

The Lateral Organ Boundaries Domain (LBDs) protein family has emerged as a key regulator in plant organogenesis. Characterized by a highly conserved LOB domain at their N-terminus, these plant-specific transcription factors orchestrate diverse plant developmental programs, including lateral root formation, shoot branching, and floral organ development, via intricate regulatory networks [5–13]. The LOB domain, approximately 100 amino acids in length, encompasses a conserved four-Cys motif (CX₂CX₆CX₃C), a Gly-Ala-Ser (GAS) block, and a predicted leucine zipper-like coiled-coil motif (LX₆LX₃LX₆L) [6,14,15]. Recently, our studies uncovered two novel regulatory mechanisms involving two LBD members, CRL1 and LBD16, which are pivotal for the development of crown roots in rice [16,17]. These findings provide new mechanistic insights into the control of root system architecture and underscore the importance of LBD proteins as potential targets for crop improvement strategies.

Protein dimerization assumes a pivotal role in numerous transcription factors, dynamically modulating DNA binding affinity, target specificity, and functional diversity, thereby regulating mRNA transcription, which propels cellular differentiation and biological responses [18–20]. Furthermore, dimerization can stabilize the protein structure and/or augment the interaction with other regulatory molecules and cofactors requisite for transcriptional activation or repression [21–23]. Several LBD proteins have been documented to form homodimers and/or heterodimers with other members of the LBD family. The leucine zipper-like coiled-coil motif within the LOB domain is hypothesized to mediate dimerization in class I LBD proteins, including the homodimerization of LBD16 and LBD18, the heterodimerization of LBD10-LBD27, LBD18-LBD33, and LBD30-LBD6 dimers, as well as the combinatorial interactions of LBD10, LBD22, LBD25, LBD27, and LBD36 [6,24–28]. These interactions directly influence the DNA-binding capabilities and transcriptional activities, thus precisely regulating their biological functions [19]. Despite the significance of dimerization, the specific dimerization interactions among different members of the LBD family are still inadequately characterized, particularly in crop species like rice.

In this study, our research clarifies the crucial role of the leucine zipper-like coiled-coil motif in facilitating the dimerization of CRL1 and LBD16. This dimerization is vital for their appropriate subcellular localization and biological functions during the development of rice crown roots. By comprehending the dimerization interactions between these two proteins, we aim to gain deeper insights into the functional roles of CRL1 and LBD16 in crown root development, which may facilitate the identification of novel genetic targets for improving rice yield and stress tolerance through genetic engineering and breeding strategies.

2. Materials and Methods

2.1. Plant Materials

Rice (*Oryza sativa* ssp. japonica) varieties used in this study are Zhonghua11 (ZH11), which was obtained from the Chinese Academy of Agricultural Sciences (Beijing, China). The *LBD16* knockout mutant (*lbd16*), *Ubi:LBD16:FLAG* (*OE16*) used in this study is described in Geng et al. (2024) [17], and *Ubi:CRL1:FLAG*

(*OECR11*) used in this study is described in Geng et al. (2023) [16], and the *CRL1* knockout mutant (*cr11*) used in this study is described in Inukai et al. (2005) [29].

The *Ubi:CRL1:FLAG/cr11* (*OECR11/cr11*), *Ubi:CRL1:FLAG/lbd16-1* (*OECR11/lbd16-1*), *Ubi:LBD16:FLAG/lbd16-1* (*OELBD16/lbd16-1*), *Ubi:LBD16:FLAG/cr11* (*OELBD16/cr11*), *Ubi:CRL1^Q:FLAG/cr11* (*OECR11^Q/cr11*) and *Ubi:LBD16^Q:FLAG/lbd16-1* (*OELBD16^Q/lbd16-1*) transgenic plants were produced in the *lbd16-1* and *cr11* mutants backgrounds respectively. *LBD16* and *CRL1* full-length cDNAs were amplified and cloned into the *pU2301-3×FLAG* vector under the control of the maize cultivar *B73* ubiquitin-1 (maize cultivar *B73* Ubi-1) promoter [30].

2.2. Plant Phenotype Observation and Growth Conditions

Seeds with the indicated genotypes were surface-sterilized and germinated in media containing 0.3% phytigel supplemented with 2% (w/v) sucrose at 28 °C (in light) and 24 °C (in dark) with a 14 h:10 h, light:dark cycle. Two days after germination, seeds with similar vigorous growth were transferred to normal growth medium for another 5-day growth. The crown root (CR) number was counted.

2.3. RNA Extraction and Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

Total RNA from rice was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by reverse transcription (1 µg RNA per reaction) using Vazyme Mix (R312-01; Vazyme, Nanjing, China). Products were diluted by the addition of 80 µL ddH₂O. Real-time PCR was performed using ChamQ SYBR® qPCR Master Mix (Q311-02; Vazyme, Nanjing, China) on ABI 7500 real-time PCR system (ABI, San Diego, CA, USA). The following thermal profile was used for all reactions: 95 °C for 10 s, and 42 cycles of 95 °C for 5 s and 60 °C for 30 s. RT-qPCR was performed on three replicates of independent pooled samples from different plants of three to five independent transgenic lines. The *ACTIN1* gene (accession number AK101613) served as an internal control and PCR signals were normalized to those of *ACTIN1* transcripts (set as 1). The sequences of the primers used are listed in Table S1.

2.4. Transient Reporter Assays

For co-transfection assays, a mixture of reporter plasmids (3 µg), effector plasmids (3 µg), and internal control plasmids (0.5 µg) was taken and introduced into rice protoplasts. The transfected protoplasts were placed in the dark at room temperature overnight. The protoplasts were collected by centrifugation in 1.5 mL tubes at 100 g for 8 min at room temperature. The activities of luciferase were determined according to the kit instructions (E1910; Promega, Madison, WI, USA). Briefly, 50 µL of lysis buffer (1×) was added to the pelleted protoplasts. After centrifugation at 12,000 rpm for 5 min, the supernatant (40 µL) was transferred to a 96-well enzyme plate (Cat. No. 3917; Corning Costar, Corning, NY, USA) containing 50 µL of Kit A. The fluorescence activities were measured using a Tecan Spark (Tecan, Männedorf, Switzerland). Then 50 µL of Kit B was added and the fluorescence signal was detected again. The relative expression level of the reporter genes was detected as

the ratio of the relative value of firefly LUC to that of Renilla luciferase. Data are means $\pm$ SD of three biological replicates and significant differences were evaluated by one-way ANOVA with Tukey's multiple comparison test.

2.5. In Vitro Pull-down Assays

GST (Glutathione S-transferase), recombinant LBD16-6 $\times$ His, LBD16-GST, CRL1-6 $\times$ His and CRL1-GST proteins were expressed in *E. coli* BL21. Recombinant LBD16-6 $\times$ His or CRL1-6 $\times$ His was incubated with LBD16-GST/CRL1-GST or GST alone immobilized on GST beads (Cat. #45-000-139; GE Healthcare, Chicago, IL, USA) and pulled down from the GST beads. The proteins were subsequently analyzed by immunoblotting. Primers used in this study are listed in **Table S1**.

2.6. Split-luciferase Complementation Assays (Split-LUC)

CRL1, *CRL1^Q*, *LBD16* and *LBD16^Q* cDNA were cloned in-frame upstream or downstream of the sequence encoding the N/C-terminal half of firefly luciferase (nLUC and cLUC). *LBD16-nLUC/CRL1-nLUC/CRL1^Q-nLUC/LBD16^Q-nLUC* and *LBD16-cLUC/CRL1-cLUC/CRL1^Q-cLUC/LBD16^Q-cLUC* were transiently co-infiltrated into *Nicotiana benthamiana* leaves. The infiltrated plants were kept in the dark for 2-day before being infiltrated with 0.15 mg·mL⁻¹ d-luciferin potassium salt as substrate. The leaves were harvested and kept in the dark for 5 min before determining luminescence signals by a CCD camera (Tanaon 5200, Tanon Science & Technology, Shanghai, China). Primers used in this study are listed in **Table S1**.

2.7. Co-immunoprecipitation Assays (Co-IP)

35S:CRL1-GFP/35S:LBD16-GFP/35S:CRL1^Q-GFP/35S:LBD16^Q-GFP or *35S:GFP* were co-transfected into rice protoplasts with *35S:CRL1-FLAG/35S:LBD16-FLAG/35S:CRL1^Q-FLAG/35S:LBD16^Q-FLAG* construct. After 13 h incubation for transgene expression at room temperature, protoplasts were lysed in Co-IP buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM DTT, 1 mM PMSF, DNase 15 U·mL⁻¹) for 30 min, followed by centrifugation at 16,000 g for 5 min to remove cellular debris at 4 °C. The supernatant was transferred to a new tube and incubated with green fluorescent protein (GFP)-conjugated beads (Cat. #2618; Pierce, Rockford, IL, USA) overnight, then precipitated and analyzed by immunoblot with anti-FLAG antibody to detect the protein. Primers used in this study are listed in **Table S1**.

2.8. Western Blotting Analysis

Protein samples were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Chicago, IL, USA). Antibodies against FLAG (Cat. #F3165; Sigma-Aldrich, St. Louis, MO, USA), GFP (Cat. #M20004; Abmart, Shanghai, China) and ACTIN (Cat. No. D191048; BBI Solutions, Cardiff, UK), GST (Cat. #ab19256; Abcam, Cambridge, UK) and His (Abcam, Cambridge, UK; Cat. #ab9108) were used as primary antibodies. After the primary antibody incubation, horseradish peroxidase-conjugated secondary antibodies

goat anti-mouse (Cat. #A21010; Abkine, Wuhan, China) or goat anti-rabbit (Cat. No. A21020; Abkine, Wuhan, China) were used for protein detection by chemiluminescence (Cat. #36208ES76; Yeasen Biotechnology, Shanghai, China). Primers used in this study are listed in **Table S1**.

2.9. Subcellular Localization Assays

For the subcellular localization assay in *N. benthamiana* leaves, plasmids containing *35S:H4-RFP (RFP)*, *35S:LBD16-GFP (LBD16-GFP)*, *35S:CRL1-GFP (CRL1-GFP)*, *35S:LBD16^Q-GFP (LBD16^Q-GFP)* and *35S:CRL1^Q-GFP (CRL1^Q-GFP)* fragments were transformed into *N. benthamiana* leaves by the polyethylene glycol method. Confocal images were visualized using a confocal laser scanning microscope (FV1200; Olympus, Tokyo, Japan). For the subcellular localization assay in rice protoplasts, the above plasmids were mixed and introduced into suspension-cultured rice cells. Transformed rice cells were incubated for 12 h at 24 °C in the dark. Confocal images were visualized using a confocal laser scanning microscope (FV1200; Olympus, Tokyo, Japan). The primers used in this study are listed in **Table S1**.

2.10. Availability of Data and Materials

The data supporting the findings of this study have been provided in the text and supplementary data files and are available upon request. No datasets were generated or analysed during the current study.

3. Results

3.1. Modeling of the Zipper-like Conformation of CRL1 and LBD16

LBD proteins have been categorized into two subtypes based on phylogenetics: Class I LBD proteins possess a conserved CX₂CX₆CX₃C zinc finger motif, while Class II is devoid of a complete leucine zipper domain [31,32]. Among these, Class I LBD members have been posited to promote protein dimerization. To comprehend the structural foundation and the function of the leucine-like zipper coiled-coil motif within CRL1 and LBD16, two well-characterized LBD proteins recognized to regulate crown root development in rice [16,17], we initially constructed the molecular models of their coiled-coil domains using the SWISS-MODEL (web-based service, version: ExPASy 2023 release / accessed via <https://swissmodel.expasy.org>; developed by Biozentrum, University of Basel, Basel, Switzerland). The amino acid sequences from positions 88-106 in CRL1 and 99-124 in LBD16 were inputted into SWISS-MODEL to generate the most probable structural models. Visualization via PyMOL (version 2.0; Schrödinger LLC, New York, NY, USA). disclosed α -helical conformations characteristic of coiled-coil structures, with specific leucine residues predicted to stabilize dimer interfaces. Specifically, Leu-90, Leu-97, Leu-101, and Leu-104 in CRL1, along with Leu-104, Leu-111, Leu-115, and Leu-122 in LBD16, were identified as crucial hydrophobic anchors mediating intermolecular interactions (**Figure 1**). These residues are likely to form a hydrophobic core, facilitating both homodimerization (pairing of the same protein) and heterodimerization (pairing of CRL1 and LBD16) between the two proteins. The findings offer structural insights

into how Class I LBD proteins accomplish dimerization, potentially associating conformational specificity with their combinatorial roles in transcriptional regulation and developmental patterning.

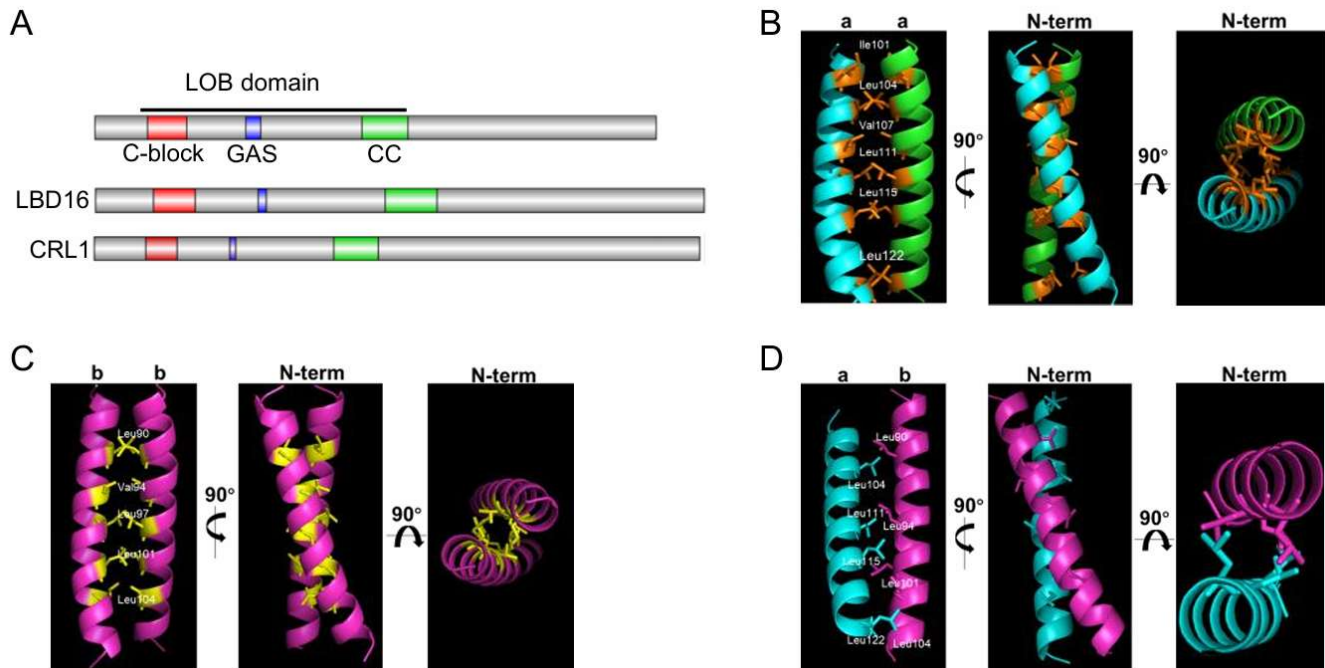


Figure 1. Structure prediction of the zipper-like conformation of the LBD16 and CRL1 dimer using the SWISS-MODEL. **(A)** Protein structure of LBD16 and CRL1, C block, Four-Cys motif with CX₂CX₆CX₃C spacing; GAS block, Gly-Ala-Ser block; CC, predicted Leu-zipper-like coiled-coil motif with LX₆LX₃LX₆L spacing. **(B–D)** Structure prediction of the zipper-like conformation of the LBD16 and CRL1 dimer using the SWISS-MODEL. **(B)** LBD16. a indicates the helices predicted from amino acids 99 to 124 of each monomer. **(C)** CRL1. b indicates the helices predicted from amino acids 88 to 106 of each monomer. **(D)** LBD16 and CRL1. a and b indicate the helices predicted from amino acids 99 to 124 and amino acids 88 to 106 of each monomer.

3.2. Critical Role of the Coiled-coil Motif in the Dimerization of LBD16 or CRL1

To assess the homodimeric interaction of LBD16 or CRL1, glutathione S-transferase (GST) pull-down assays were employed to ascertain the potential homodimeric interactions of CRL1 and LBD16. A significant quantity of CRL1-His/LBD16-His recombinant protein was pulled down upon incubation with CRL1-GST/LBD16-GST, but not with GST alone (**Figure 2A and 2B**), indicating the direct homodimeric binding ability of CRL1 and LBD16 *in vitro*. Additionally, split-luciferase complementation (Split-LUC) assays in *Nicotiana benthamiana* leaf cells verified the formation of homodimers *in planta* (**Figure 2C and 2D**). Moreover, CRL1-FLAG/LBD16-FLAG proteins were effectively co-immunoprecipitated (Co-IP) with CRL1-GFP/LBD16-GFP using anti-GFP antibodies from rice protoplast extracts (**Figure 2E and 2F**), further validating native homodimeric interactions in rice protoplasts.

To determine the role of the predicted leucine-like zipper coiled-coil motif in the dimerization of LBD16 or CRL1, CRL1^Q and LBD16^Q mutants were generated by

substituting critical Leu residues within the coiled-coil motif with Pro residues (Leu to Pro) via site-directed mutagenesis. The impact of these mutations on protein-protein interactions was then evaluated using established assays such as Split-LUC and Co-IP. The results indicated that CRL1^Q and LBD16^Q are unable to form homodimers (Figure 2C,2D,2G and 2H), confirming that the leucine-like zipper coiled-coil motif is indispensable for CRL1/LBD16 self-association. Collectively, these results demonstrate that both CRL1 and LBD16 can form constitutive homodimers in vitro and in vivo, and that their leucine residues in the leucine zipper-like coiled-coil motif are crucial for CRL1/LBD16 dimerization.

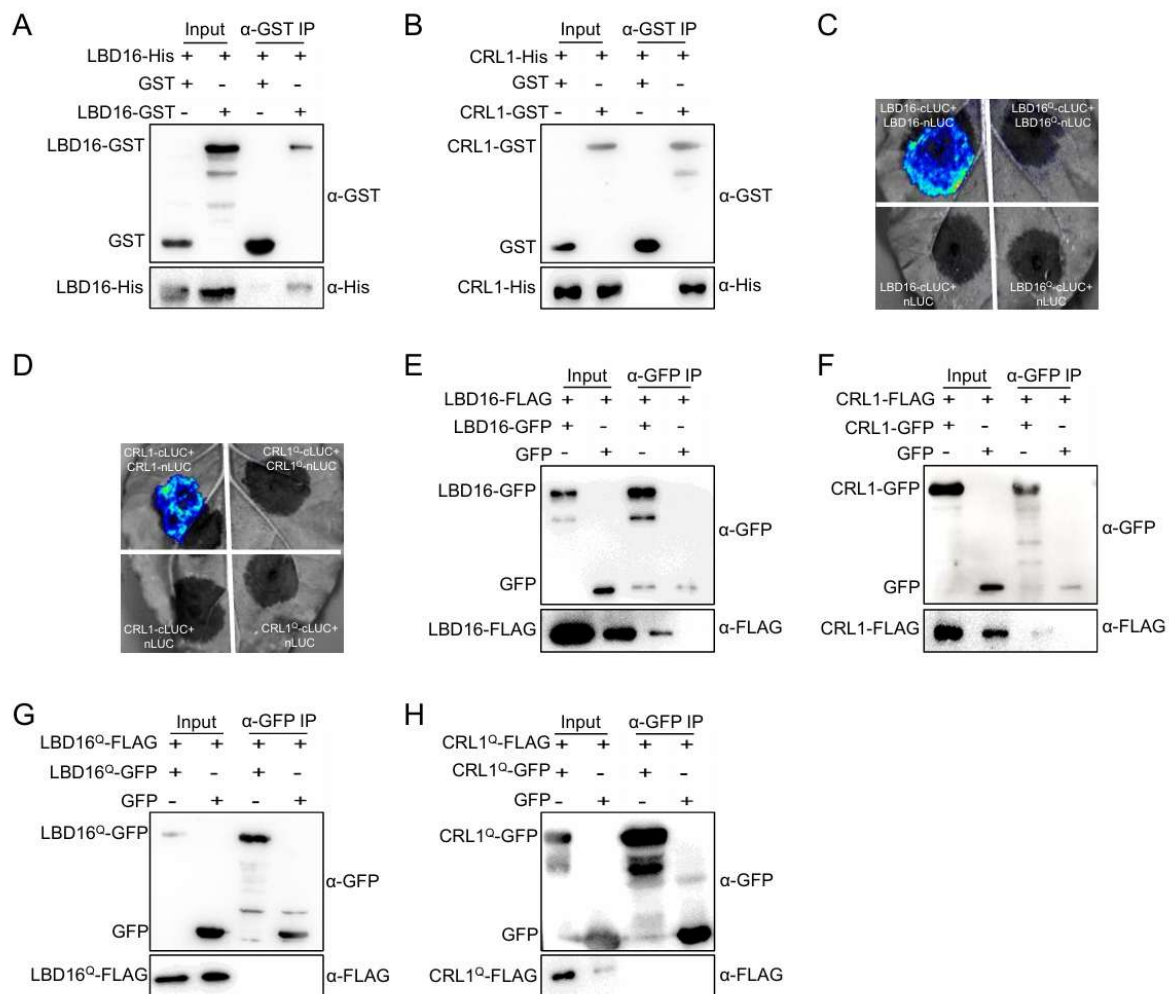


Figure 2. Critical role of the coiled-coil motif in the dimerization of LBD16 and CRL1. (2A and 2B) In vitro GST pull-down assay for LBD16 (A) and CRL1 (B). Recombinant LBD16-6×His and CRL1-6×His protein was incubated with LBD16-6×GST and CRL1-6×GST or GST and was pulled using GST beads conjugated with LBD16-GST and CRL1-GST. The eluted fractions were then analyzed by immunoblots using antibodies against GST and His tags. (C and D) Split-LUC assays for the interaction between LBD16 with LBD16 (C) and CRL1 with CRL1 (D) in *N. benthamiana*. The bottom shows LUC images of *N. benthamiana* leaves coinfiltrated with tumefaciens harboring cLUC, nLUC, LBD16:cLUC, LBD16:nLUC, CRL1:cLUC, CRL1:nLUC and/or LBD16^Q:cLUC, LBD16^Q:nLUC, CRL1^Q:cLUC and CRL1^Q:nLUC. Luminescence signals were quantified after 5 min of dark adaptation of leaves. (E and F) Co-IP assays of LBD16 (E) and CRL1 (F) interaction in 14-day-old rice protoplasts. Protoplasts isolated from 2-week-old WT plants were transfected with *LBD16:GFP* or *GFP* vector and *LBD16:FLAG* plasmid DNA, *CRL1:GFP* or *GFP* vector and

CRL1:FLAG plasmid DNA. Samples prepared after incubation were precipitated with anti-GFP agarose, and immunoblot analysis was performed with monoclonal anti-FLAG or anti-GFP antibody. **(G and H)** Co-IP assay of LBD16^Q **(G)** and CRL1^Q **(H)** interaction in 14-day-old rice protoplasts. Proteins isolated from protoplasts co-expressing LBD16^Q-FLAG with LBD16^Q-GFP or GFP and CRL1^Q-FLAG with CRL1^Q-GFP or GFP were precipitated with anti-FLAG and analyzed by immunoblotting with anti-GFP.

3.3. Physical Interaction between LBD16 and CRL1

In light of their comparable biological functions in crown root development [16,17], an investigation was conducted to explore the potential functional associations between LBD16 and CRL1. Subsequently, GST pull-down assays were carried out to examine whether CRL1 interacts with LBD16. A significant quantity of LBD16-His was pulled down upon incubation with CRL1-GST, but not with GST alone (**Figure 3A**), which indicates a direct physical interaction between LBD16 and CRL1 in vitro. Additionally, Split-LUC assays further verified the heteromerization between LBD16 and CRL1 in *N. benthamiana* leaf cells (**Figure 3B**). To ascertain the interaction between LBD16 and CRL1 in rice cells, full-length LBD16-FLAG was effectively co-immunoprecipitated with CRL1-GFP from rice protoplast extracts using anti-GFP antibodies (**Figure 3C**). This interaction was nullified when the leucine zipper motifs were disrupted in either protein (LBD16^Q-FLAG/CRL1^Q-GFP; **Figure 3D**). Collectively, these results suggest that LBD16 can engage in a physical interaction with CRL1, namely, LBD16 and CRL1 can form a heterodimer. More crucially, the results imply that the conserved leucine residues within these motifs are crucial for complex formation.

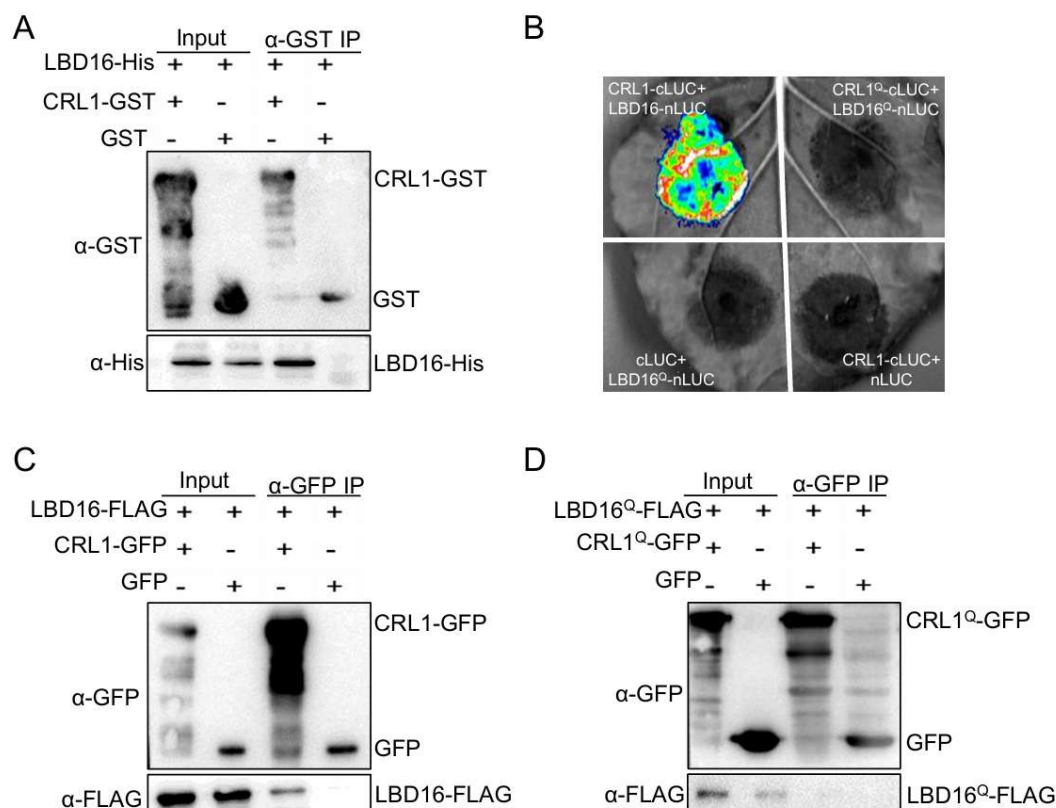


Figure 3. LBD16 physically interacts with CRL1. **(A)** In vitro pull down analysis of LBD16 and CRL1. The LBD16-

His protein purified from *E. coli* was incubated with GST or CRL1-GST, and detected by immunoblotting using anti-His antibody. **(B)** Split-LUC assay for the interaction between LBD16 and CRL1 in *N. benthamiana*. The bottom shows LUC images of *N. benthamiana* leaves coinfiltrated with tumefaciens harboring cLUC, LBD16:nLUC, CRL1:cLUC, LBD16^Q:nLUC, LBD16^Q:cLUC, CRL1^Q:cLUC, CRL1:cLUC and nLUC. Luminescence signals were quantified after 5 min dark adaptation of leaves. **(C and D)** Co-IP assay of LBD16 and CRL1 **(C)** or CRL1^Q and LBD16^Q **(D)** interaction in 14-day-old rice protoplasts. Proteins isolated from protoplasts co-expressing CRL1:GFP with LBD16:FLAG or CRL1^Q:GFP with LBD16^Q:FLAG were precipitated with anti-FLAG and analyzed by immunoblotting with anti-GFP.

3.4. Coiled-coil Motif Site Mutation in the Dimerization of CRL1 and LBD16 Affect Protein Localization

To explore the biological significance of the coiled-coil motif-mediated dimerization between CRL1 and LBD16, the subcellular localization and transactivation capability of LBD16 and CRL1 were investigated. Initially, transient reporter assays in rice protoplasts were carried out using a GAL4-responsive luciferase (LUC) reporter system. The reporter plasmid contained the LUC reporter gene fused to the GAL4 (4X) DNA binding element, and the effector plasmids encoded wild-type LBD16/CRL1 or their leucine-zipper mutants LBD16^Q/CRL1^Q fused to the GAL4 DNA-binding domain under the regulation of the CaMV 35S promoter (**Figure S1A**). Nevertheless, quantification of LUC activity indicated no significant disparities in transactivation potential between the wild-type proteins and their mutants (**Figure S1B**). This implies that the quadruple mutations within the leucine-like zipper motifs may not modify the transactivation capability of CRL1 or LBD16.

To evaluate whether dimerization influences the subcellular localization of the proteins, the subcellular localization of CRL1, LBD16, and their mutant proteins with disrupted leucine-like zipper motifs (CRL1^Q and LBD16^Q) was determined in *N. benthamiana* leaves and rice protoplasts. The findings revealed that CRL1 and LBD16 were solely localized to the nucleus (**Figure 4A and 4B**), whereas the mutants (CRL1^Q and LBD16^Q) displayed dual localization in the nucleus and cytoplasm (**Figure 4A and 4B, Figure S2**). These observations illustrate that the coiled-coil motif-mediated dimerization of CRL1 and LBD16 may be non-essential for transcriptional activation but crucial for the nuclear entry of CRL1 and LBD16. The dimerization acts as an essential prerequisite for protein nuclear localization, and site mutations will intensify protein entry in the cytoplasm. The cytoplasmic mislocalization of the mutant proteins suggests that proper dimerization might be necessary for nuclear import/entry mechanisms and precise spatio-temporal regulation of crown root development.

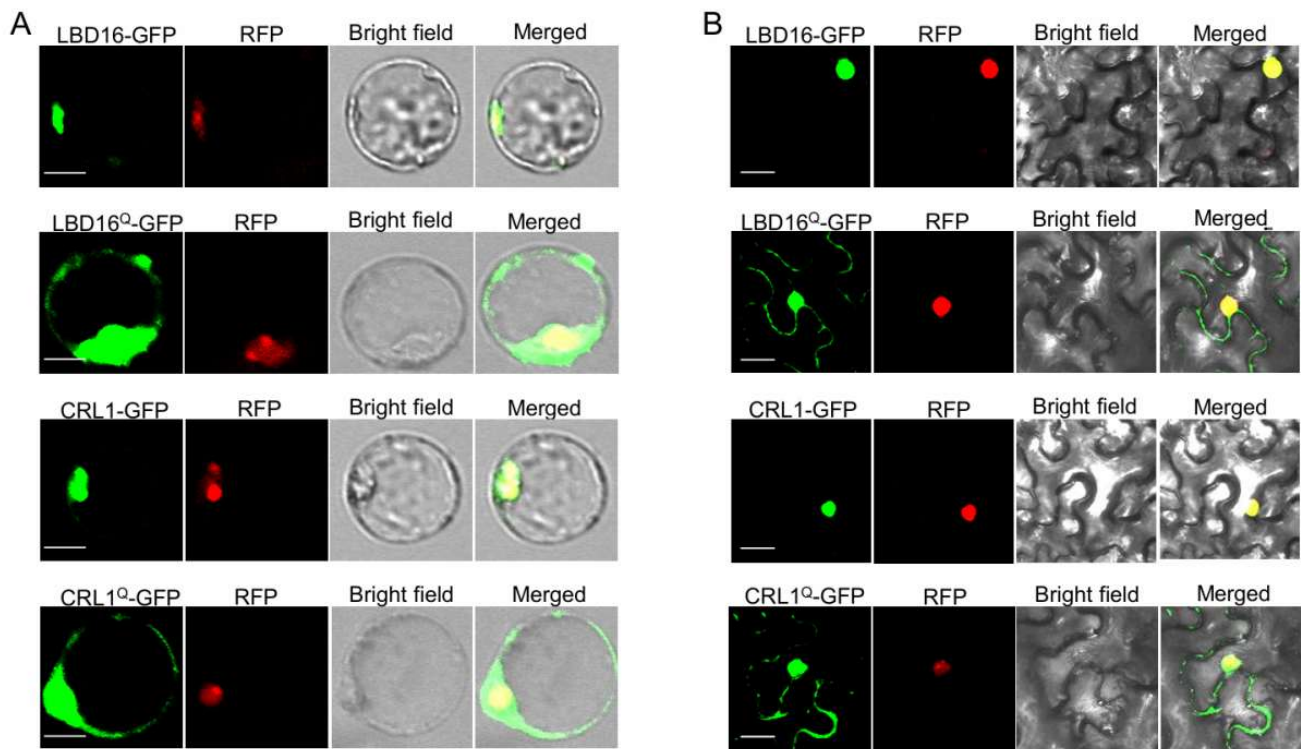


Figure 4. Coiled-Coil motif site mutation in the dimerization of LBD16 and CRL1 affect protein localization. **(A)** Subcellular localization of LBD16-GFP, LBD16^Q-GFP, CRL1-GFP and CRL1^Q-GFP in rice protoplasts. Rice protoplasts were transformed with *LBD16-GFP*, *LBD16^Q-GFP*, *CRL1-GFP* and *CRL1^Q-GFP* or *H4-RFP* plasmid. After 12 hours of co-infiltration in rice protoplasts. **(B)** Subcellular localization of LBD16-GFP, LBD16^Q-GFP, CRL1-GFP and CRL1^Q-GFP in *N. benthamiana* leaves. *N. benthamiana* leaves were transformed with *LBD16-GFP*, *LBD16^Q-GFP*, *CRL1-GFP* and *CRL1^Q-GFP* or *H4-RFP* plasmid. After 2 days of co-infiltration in *N. benthamiana*, the fluorescence signals were checked. GFP, GFP fluorescence; RFP fluorescence; Merged, merged GFP, H4-RFP, and bright field images. H4, a nuclear localization protein, Bar = 20 μ m.

3.5. Homodimerization of LBD16 and CRL1 is Required for the Function in Crown Root Formation

To test whether dimerization is required for crown root formation, we constructed complementation vectors containing the full-length CDS of *CRL1* or *LBD16*, as well as their dimerization-deficient mutants (*CRL1^Q* and *LBD16^Q*), each fused to a FLAG tag and driven by the maize ubiquitin promoter. These constructs were introduced into *cr11* or *lbd16-1* mutants, yielding multiple independent transgenic lines (**Figure S4**). Phenotypic analysis showed that overexpression of *CRL1* and *LBD16* fully restored the crown root number in *cr11* and *lbd16-1* mutants, respectively (**Figure 5** and **Figure S3**). Interestingly, overexpression of *CRL1* in *lbd16-1* partially compensated for the crown root defect (**Figure 5A**), whereas overexpression of *LBD16* failed to rescue the crown root deficiency in *cr11* plants (**Figure 5B**), indicating a non-redundant function of *CRL1*. Notably, overexpression of *LBD16^Q* in *lbd16-1* did not rescue the mutant phenotype (**Figure 5A**). Similarly, overexpression of *CRL1^Q* only partially alleviated the crown root defect in *cr11* mutants (**Figure 5B**), further supporting the functional importance of the coiled-coil motif in LBD16. Together, these results reveal a

cooperative interaction between CRL1 and LBD16, mediated by dimerization, that is essential for proper crown root development.

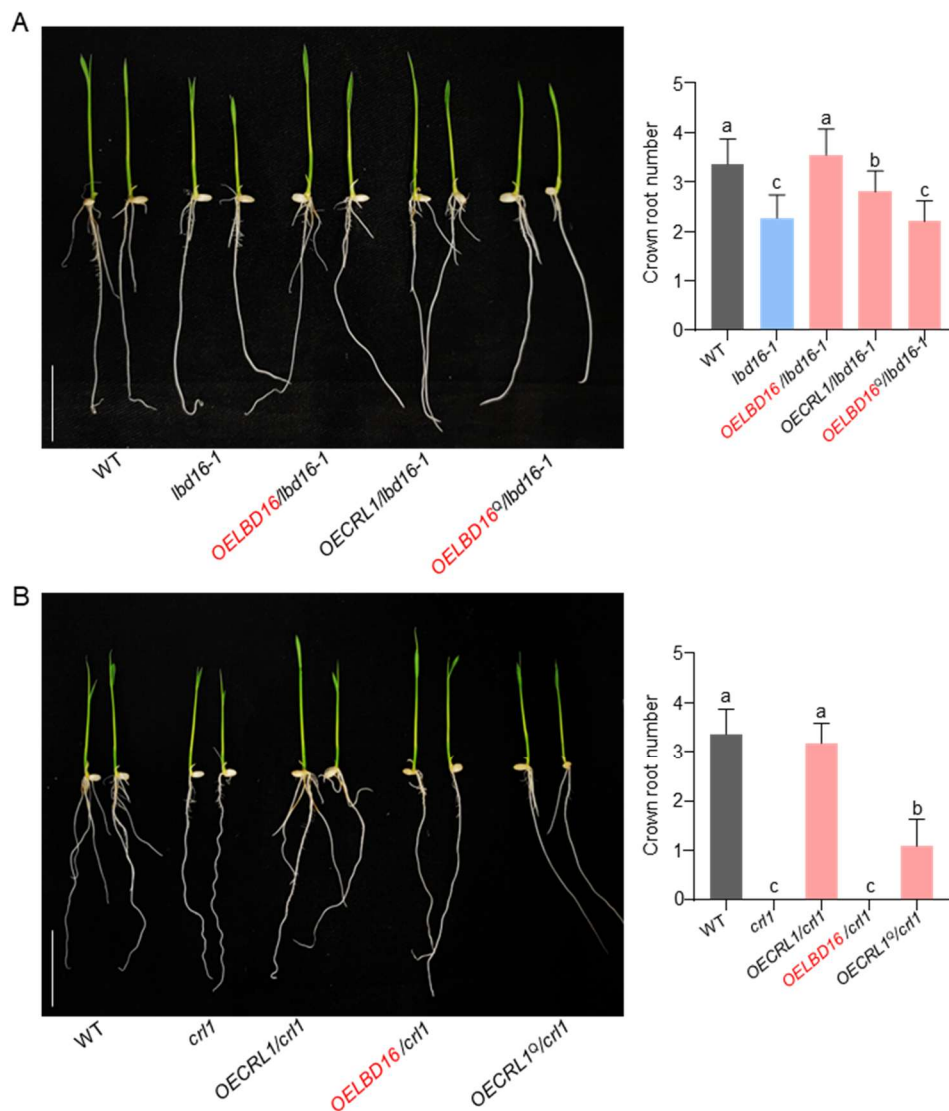


Figure 5. Homodimerization of LBD16 and CRL1 is required for the function in crown root formation. **(A)** Crown root phenotype and statistical data of WT, *lbd16-1*, *OELBD16/lbd16-1*#, *OECRL1/lbd16-1* and *OELBD16^Q/lbd16-1* at the 7-day seedlings after germination. **(B)** Crown root phenotype and statistical data of WT, *crl1*, *OECRL1/crl1*#, *OELBD16/crl1* and *OECRL1^Q/crl1* at the 7-day seedlings after germination. Data are means $\pm$ SD ($n = 11$). The significant difference was calculated using one-way ANOVA with Tukey multiple comparison test. Different letters over the columns indicate significant differences ($P < 0.05$), and the same letters indicate no significant difference. Bar = 5 cm.

4. Discussion

Crown root morphogenesis is a pivotal phase in cereal plant development, exerting profound influences on nutritional acquisition and grain yields [33]. As the primary component of the rice root system, crown roots directly determine the plant's ability to access soil water and nutrients, thereby governing its adaptability to adverse environmental conditions (e.g., drought, nutrient deficiency) and ultimately affecting

crop productivity—an issue of critical importance for global food security amid the growing challenges of climate change and population growth [34]. This complex process involves a sophisticated network of genetic and molecular interactions, with transcription factors as orchestrators. Among these regulatory proteins, two LBD members, CRL1 and LBD16, have been prominently implicated in rice crown root formation [8,17,29,35–37]. Previous studies have established their essential roles in initiating and promoting crown root development, but the precise molecular mechanisms by which these proteins function, particularly the significance of their dimerization patterns modulates their biological activity, remained elusive until now. Here, we present compelling evidence demonstrating that CRL1 and LBD16 can form both homodimers (CRL1-CRL1, LBD16-LBD16) and heterodimers (CRL1-LBD16). Importantly, we identified that the conserved leucine residues within their leucine zipper-like motifs, located within the LOB domain, are crucial for this dimerization process—mutation of these key residues abrogates dimer formation and impairs the biological functions of CRL1 and LBD16. These findings are significant because they provide a molecular basis for understanding the functional implications in rice crown root development have not been previously elucidated. Our results provide a clear molecular basis for understanding how CRL1 and LBD16 interact with each other and potentially with other regulatory proteins to form functional complexes, thereby fine-tuning the transcriptional networks underlying crown root morphogenesis. Of particular significance, our study reveals that dimerization is indispensable for the nuclear localization of CRL1 and LBD16. Nuclear localization is an essential prerequisite for transcription factors to bind DNA and regulate downstream gene expression, and our observation establishes a critical link between protein complex formation (dimerization) and transcriptional control during crown root development. This finding is consistent with the conserved functional logic of many transcription factors, where dimerization serves as a key switch to activate their nuclear localization and subsequent regulatory activity. Collectively, these results not only advance our mechanistic understanding of LBD protein function in rice but also highlight the importance of combinatorial protein-protein interactions in shaping root system architecture, a key agronomic trait for cereal crop productivity.

To place our findings in a broader context, many proteins across diverse biological systems can form homo- and heterodimers (or oligomers) with different partners, thus modulating DNA-binding specificity and affinity, and/or the recruitment of co-factors or downstream binding partners [38]. For instance, the blue-light receptor cryptochrome (CRY) in plants undergoes oligomerization to transduce blue-light signals after irradiation and facilitates blue-light-mediated inhibition of hypocotyl elongation and photoperiodic control of floral initiation [39,40]. The Arabidopsis NPR1 (Nonexpressor of Pathogenesis-Related Genes 1) homodimer is required for its function in inducing defence gene expression and resistance against pathogen challenge [41]. In addition, dimerization always facilitates appropriate nuclear localization, a critical prerequisite for their regulation [42]. Such as, in Arabidopsis, the heterodimerization is required for DYT1(DUO1-LIKE 1) nuclear localization in anthers [42]. STATs (Signal transducer and activator of transcription) dimerize through interactions of their SH2 (Src-homology 2) domain with phosphorylated tyrosine residues and translocate to the nucleus [43,44], involved in a wide range of

other diseases, including autoimmune diseases [45]. In line with these conserved mechanisms, our study demonstrates that CRL1 and LBD16 form dimers that enhance their nuclear localization more effectively, enabling the precise regulation of crown root development (**Figure 2 to Figure 5**). Notably, our findings differ from previous reports on LBD proteins in *Arabidopsis*, where some Class I LBD members primarily function as monomers or homodimers, which suggesting that the dimerization patterns and functional mechanisms of LBD proteins may have diverged during the evolution of monocot and dicot plants, with rice CRL1 and LBD16 adopting a unique combinatorial dimerization strategy to regulate crown root development, a trait specific to cereal crops. This evolutionary divergence highlights the importance of studying LBD protein function in rice, a model cereal crop, to gain insights that are directly relevant to crop improvement.

The practical implications of our study are noteworthy. Crown root development is a key agronomic trait that determines rice yield and stress tolerance. Our identification of the key leucine residues mediating CRL1-LBD16 dimerization, and the critical role of this dimerization in crown root development, provides novel genetic targets for rice breeding. For example, targeted modification of these conserved residues could be used to fine-tune the dimerization efficiency of CRL1 and LBD16, thereby optimizing crown root growth and enhancing rice adaptability to adverse environments (e.g., drought, low-nitrogen conditions). Additionally, our findings lay the foundation for further dissecting the upstream signaling pathways that regulate CRL1-LBD16 dimerization dynamics and understanding how environmental cues (e.g., nutrient availability, drought stress) modulate this dimerization process, which could enable the development of strategies to manipulate crown root development in response to changing environmental conditions.

Despite these advances, our study also has limitations that warrant future investigation. First, while we have demonstrated the importance of CRL1-LBD16 dimerization in nuclear localization and crown root development, the specific downstream target genes regulated by these dimers remain to be identified. Elucidating the transcriptional targets of CRL1-LBD16 dimers will help clarify the complete regulatory network underlying crown root morphogenesis. Second, we have focused on the leucine zipper-like motif in the LOB domain, but other domains or residues of CRL1 and LBD16 may also be involved in dimerization or interaction with other regulatory proteins—future studies should explore these additional interactions to gain a more comprehensive understanding of LBD protein function. Third, the upstream signaling molecules that trigger CRL1-LBD16 dimerization (e.g., kinases, phosphatases) remain unknown; identifying these upstream regulators will provide insights into how external and internal signals are integrated to regulate crown root development. In conclusion, our study provides important insights into the role of CRL1 and LBD16 dimerization in orchestrating crown root development in rice. Our findings not only advance basic research on plant root development but also offer practical genetic targets for rice breeding, with the potential to contribute to global food security. Future investigations will focus on dissecting the intricate upstream signaling pathways that modulate CRL1-LBD16 dimerization dynamics, identifying the downstream effector genes regulated by these dimers, and exploring the potential of manipulating this dimerization process to improve rice yield and stress tolerance.

Supplementary Materials: Figure S1: Coiled-Coil motif site mutation in the dimerization of LBD16 or CRL1 does not affect transcriptional activation ability; Figure S2: Coiled-Coil motif site mutation in the dimerization of LBD16 and CRL1 affect protein localization; Figure S3: Crown root phenotype and statistical data of WT (wild type), *crl1* and *lbd16* at the 7-day seedlings after germination; Figure S4: Identification of positive transgenic lines; Table S1: List of the primers used in this study.

Author contributions: Y.Z. and L.G. conceived and designed the project. M.T. and L.G. performed most of the experiments and analyzed the data. Q.D. contributed to the generation of the experiment materials. Y.Z., L.G. and J.Z. analyzed the data, and wrote the paper with inputs from M.T. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

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