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A versatile nanoluciferase toolkit and optimized in-gel detection method for protein analysis in plants

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Abstract Dissection of gene function requires sophisticated tools to monitor gene expression. Gene tagging with epitope peptides and fluorescent protein tags is a routine method to investigate protein expression using tag-specific antibodies and western blotting with tedious blotting and immunodetection steps. Nanoluciferase (NanoLuc) exhibits extremely bright bioluminescence and is engineered as a sensitive genetic reporter. Due to its small size and high bioluminescent activity, NanoLuc could be engineered to function as a novel protein tag that permits direct detection of tagged protein in the gel matrix (in-gel detection). In this study, we developed Gateway compatible vectors to tag proteins with NanoLuc in plants. We also tailored the in-gel detection conditions which can detect NanoLuc-tagged MPK3 from as low as 200 pg of total protein extracts. Compared to FLAG tag and western blotting-based detection, NanoLuc tag and optimized in-gel detection exhibit increased detection sensitivity but omit the blotting and immunodetection steps. We also demonstrated versatile applications of the NanoLuc-based in-gel detection method for protein expression analysis, probing protein-protein interactions by coimmunoprecipitation, and in vivo protein phosphorylation detection with Phos-tag gel electrophoresis. Finally, NanoLuc was used to tag the gene at its endogenous locus using the wheat dwarf virus replicon and CRISPR/Cas9-mediated gene targeting. Our data suggest that NanoLuc tag and in-gel detection permit fast detection of tagged protein with high sensitivity. The versatile NanoLuc toolkit and convenient in-gel detection method are expected to facilitate in vitro and in vivo protein analysis for plant functional genomics.

Keywords NanoLuc · In-gel detection · Plant · Protein analysis · Knock-in

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Introduction

For plant functional genomics, sophisticated tools are required to analyze gene expression in plants. Gene tagging with epitope peptides and fluorescent proteins is an important approach for investigating protein expression and homeostasis. Peptide tags, such as polyhistidine, FLAG, hemagglutinin (HA), and c-Myc, are routinely used for protein detection and analysis (Terpe 2003). The proteins fused with peptide tags are easily detected by western blotting and can be affinity-purified from plants with tag-specific antibodies. Fluorescent protein tags, such as green fluorescence protein (*GFP*), permit observation of the subcellular location of tagged

protein in plant cells and investigation of protein abundance using western blotting. Moreover, fluorescent protein is engineered to probe protein-protein interactions using bimolecular fluorescence complementation (BiFC) (Walter et al. 2004) and Förster resonance energy transfer (FRET) assays (Gadella Jr. et al. 1999). In western blotting-based protein analysis, the detection specificity and sensitivity of tagged protein are dependent on specific recognition between tags and antibodies. Despite the tedious blotting and immunodetection steps in western blotting, antibodies and tag recognition occasionally require careful validation (Baker 2015). Thus, a convenient and antibody-free method for protein detection is of great value for plant functional genomics.

Luciferases are engineered as popular genetic reporters for functional genomics. Luciferase-based reporters are used to quantitatively analyze promoter activity and protein dynamics in studies of signaling transduction pathways (Cazzonelli and Velten 2006; Xu et al. 2013). Additionally, luciferase is engineered to probe protein-protein interactions using split luciferase complementation assays (Fujikawa and Kato 2007) and bioluminescence resonance energy transfer (BRET) (Pfleger and Eidne 2006). Recently, Nanoluciferase (NanoLuc), which is derived from deep sea shrimp (*Oplophorus gracilirostris*), has been engineered as a useful genetic reporter with extremely bright bioluminescence (England et al. 2016; Hall et al. 2012; Sfarcic et al. 2019). NanoLuc emits light with a peak emission of 460 nm by catalyzing a novel imidazopyrazinone substrate (furimazine). Compared with commonly used firefly luciferase (Fluc) and *Renilla reniformis* luciferase (Rluc), NanoLuc has a small molecular mass but exhibits high physical stability and great tolerance to temperature, pH, and urea (Hall et al. 2012). NanoLuc is used as a sensitive genetic reporter to study signaling transduction in mammalian cells (Hall et al. 2012), *Caenorhabditis elegans* (Sfarcic et al. 2019), and *Arabidopsis thaliana* (Urquiza-Garcia and Millar 2019). NanoLuc is also engineered to probe protein-protein interactions through split NanoLuc assays in *Arabidopsis* (Wang et al. 2020) and NanoLuc BRET assays (Machleidt et al. 2015).

Given its robust luminescent activity and small size (19.1 kDa), NanoLuc-tagged protein should be detectable in a polyacrylamide gel (in-gel detection, Promega). However, such applications of NanoLuc for protein analysis have not been reported in plants to date. Here, we developed Gateway-compatible NanoLuc vectors for tagging proteins with NanoLuc. We optimized the in-gel detection method with enhanced sensitivity and demonstrated its versatile utility for protein analysis using a rice protoplast transient expression system. Our data demonstrate that NanoLuc is a useful tag for coimmunoprecipitation experiments, in vivo protein phosphorylation detection and tagging genes at their endogenous loci using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and wheat dwarf virus (WDV).

Results

NanoLuc is a sensitive bioluminescent reporter in rice protoplasts

We first synthesized a codon-optimized *NanoLuc* gene (see Supplementary Fig. S1 for gene sequences) and compared its luminescence with *Rluc* and codon-optimized *Fluc* (Supplementary Fig. S2). All three luciferase genes were transiently expressed with the rice *Ubiquitin 10* promoter (*UBI10p*) in plant protoplasts (Fig. 1a). Under the optimal conditions, *NanoLuc* exhibited approximately 810- and 280-fold increased luminescent intensities compared with *Fluc* and *Rluc*, respectively (Fig. 1a). The luminescent signals of the three luciferases were linearly correlated with the amount of protein extracts ($R^2 > 0.96$, Fig. 1b). These data are consistent with previous reports that purified recombinant NanoLuc is three orders of magnitude brighter than Fluc and Rluc (Furuhata et al. 2020; Hall et al. 2012). These results indicate that the NanoLuc reporter has extremely high luminescent activity and a broad dynamic range in plant cells.

Construction of NanoLuc tagging vectors

To engineer NanoLuc for protein tagging, Gateway compatible vectors were constructed for monocots (p34NN and p34NC) and dicots (p34NN-35S and p34NC-35S) (Fig. 2a). Notably, p34NN and p34NC use the rice *UBI10* promoter, but p34NN-35S and p34NC-35S use the cauliflower mosaic virus 35S promoter to express NanoLuc-tagged genes. The procedure of in-gel detection of NanoLuc-tagged protein is shown in Fig. 2 b and c. NanoLuc-tagged genes could be expressed in plants via stable transformation, protoplast

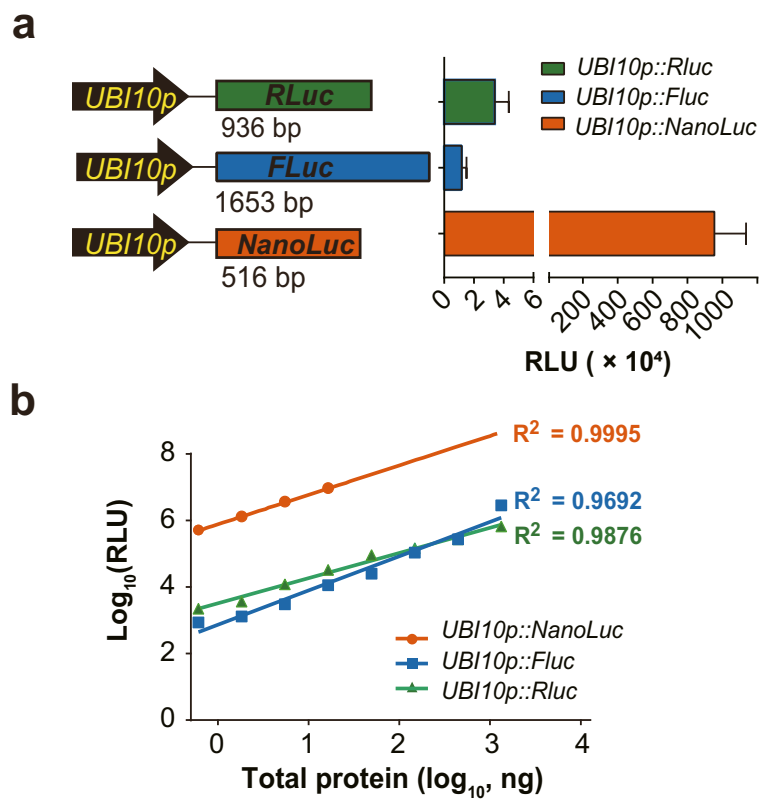

Fig. 1 Comparisons of bioluminescent activities of NanoLuc, Fluc, and Rluc reporters in rice protoplasts. **a** Luminescent activities of NanoLuc, Fluc, and Rluc expressed with the rice *Ubiquitin10* promoter (UBI10p) in rice protoplasts. The structure of reporter constructs and the length of three luciferase genes are shown in the left panel. Error bars indicate standard deviations (n = 3). **b** Dynamic ranges of luminescent signals of NanoLuc, Fluc, and Rluc reporters. Protein extracts from rice protoplasts expressing NanoLuc, Fluc, and Rluc were serially diluted and used for luminescence measurements. The data presented here are the mean of three technical replicates. RLU, relative luminescent unit

transfection, and agroinfiltration. Then, total proteins were extracted and separated by standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following removal of SDS, NanoLuc can be renatured in the gel matrix. After the addition of NanoLuc substrate, the luminescent signal of NanoLuc-tagged proteins could be detected from the gel using a luminescence imager.

Highly sensitive in-gel detection of NanoLuc-tagged proteins using optimized buffers

To demonstrate its utility for in-gel detection, *NanoLuc* tagging was performed by in frame fusion with rice *Mitogen-activated Protein Kinase 3* (*MPK3*, 43.0 kDa, we previously named it *OsMPK5* (Xie et al. 2014)), *Double-stranded RNA Binding Protein 4* (*DRB4*, 34.2 kDa), *Calcium-dependent Protein Kinase 18* (*CPK18*, 57.6 kDa), and *Ethylene Insensitive3-like 1* (*EIL1*, 70.2 kDa). These genes were cloned into the p34NN vector and then transiently expressed in rice protoplasts. However, standard NanoLuc in-gel detection according to Promega's protocol yields weak luminescent signals (Fig. 2d, left panel). As a result, only NanoLuc-MPK3 exhibited clear bands using Promega's in-gel detection method.

We hypothesized that NanoLuc was not fully renatured in the above experiment. Renaturation of proteins in the gel matrix following SDS-PAGE has been used to measure the catalytic activities of protein kinases (Wooten 2002). According to our experience with the in-gel kinase assay, the compositions of buffers are important for protein renaturation. Therefore, we tailored the in-gel detection procedure by replacing the SDS removal, renaturation buffers, and substrate solution according to our in-gel kinase protocol (Fig. 2c) (Xie et al. 2014). In comparison with Promega's standard method, our optimized buffers enhanced the luminescent signals of tagged proteins (MPK3, DRB4, and CPK18) by tenfold (Fig. 2d, right panel). The detection sensitivity of the optimized in-gel detection method was also investigated using 0.2 pg to 200 ng of total proteins from rice protoplasts. The in-gel detection results indicate that the luminescent signal is proportional to the amount of protein loaded (Fig. 2e). More importantly, this optimized in-gel detection method was able to

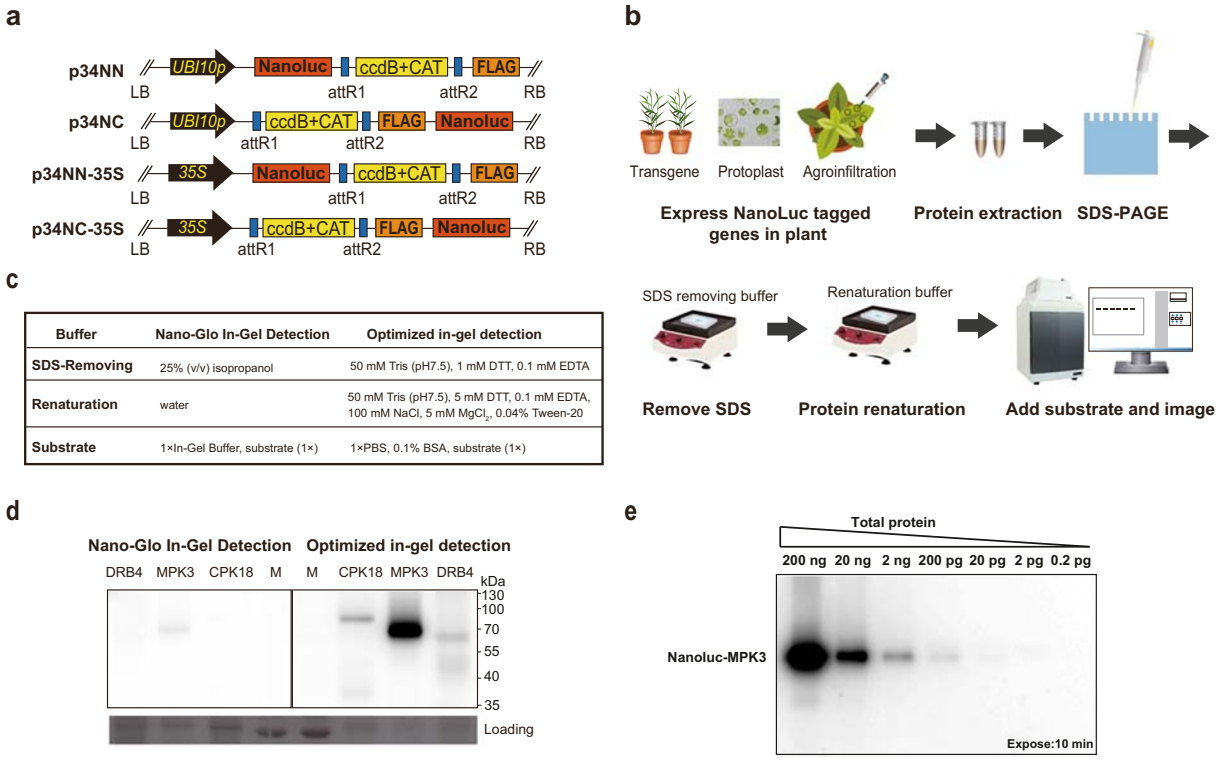

Fig. 2 Optimization of in-gel detection of NanoLuc-tagged protein in plants. **a** Schematic diagram of NanoLuc tag vectors. UBI10p, rice *Ubiquitin10* promoter; 35S, Cauliflower mosaic virus 35S promoter; ccdB+CAT, *ccdB*, and chloramphenicol-resistance genes; LB and RB indicate left and right borders of T-DNA, respectively. **b** Procedure of in-gel detection of NanoLuc-tagged protein. **c** Composition of in-gel detection buffers used in this study. **d** Comparison of in-gel detection results using the buffers from Promega's protocol (Nano-Glo In-Gel Detection) and optimized buffers in this study. Two gels were processed under the same conditions except the difference in SDS-removal and renaturation buffers. The experiments were performed thrice with similar results. **e** Sensitivity of optimized in-gel detection. The photo shows the in-gel detection result of NanoLuc-MPK3 using 0.2 pg to 200 ng of total proteins extracted from rice protoplasts. Loading, Coomassie Blue-stained gel; Expose, the exposure time to capture the photo; M, protein molecular weight markers

detect NanoLuc-MPK3 from as low as 200 pg of total protein (Fig. 2e). These results indicate that the NanoLuc tag with optimized buffers enables antibody-free detection of tagged protein with extremely high sensitivity.

NanoLuc in-gel detection is robust for protein analysis

To test whether NanoLuc exhibits a bias for tag position, we fused NanoLuc to the N- and C-termini of MPK3. After expressing tagged genes in rice protoplasts, two protein fusions exhibited comparable luminescent activities in optimized in-gel detection (Fig. 3a). We also transiently expressed NanoLuc-tagged MPK3 (p34NN-35S and p34NC-35S vectors) in tobacco leaves using agroinfiltration. Again, MPK3 with NanoLuc at the N- and C-termini had comparable luminescent signals in in-gel detection (Supplementary Fig. S3). These results suggest that the tag position does not affect the sensitivity of in-gel detection of NanoLuc-tagged protein.

Next, we compared the in-gel detection with western blotting. To this end, MPK3, DRB4, and EIL1 were fused with N-terminal NanoLuc and C-terminal FLAG. These three dual-tagged genes were expressed in protoplasts and analyzed by in-gel detection and western blotting. The in-gel detection resulted in clear bands with an exposure time of one second (Fig. 3b). In sharp contrast, standard western blotting required fivefold more total protein and 10-min exposure to obtain comparable band intensities even with the use of an ultra-sensitive chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate kit from Thermo Scientific, Fig. 3b). In particular, the low expression of

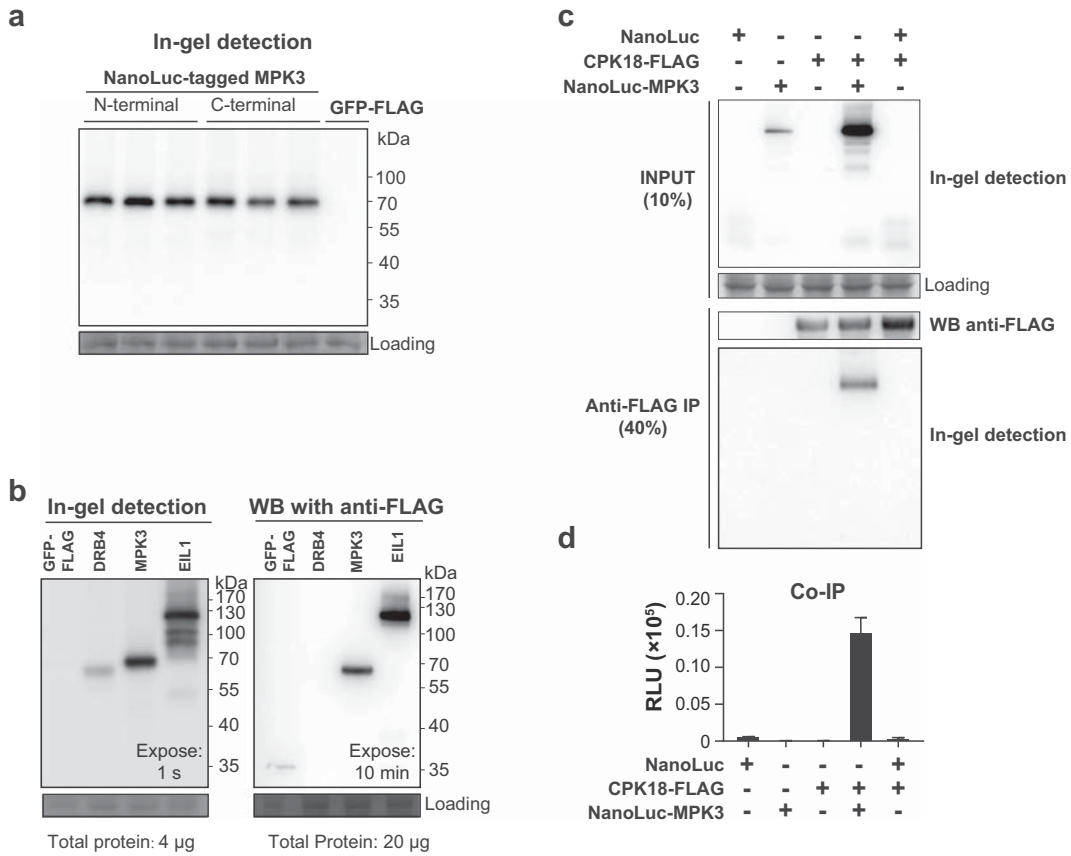

Fig. 3 Assessment of in-gel detection of NanoLuc-tagged proteins. **a** Comparison of the in-gel detection results of MPK3 with NanoLuc at the N- and C-termini. p34NN-MPK3 (N-terminal tagging) and p34NC-MPK3 (C-terminal tagging) constructs were transfected into rice protoplasts for in-gel detection. Three replicates were performed for each construct. Protoplasts expressing FLAG-tagged GFP (GFP-FLAG) were used as controls to monitor nonspecific backgrounds. **b** Comparison of protein detection results of western blotting (WB) and in-gel detection. DRB4, MPK3, and EIL1 were fused with N-terminal NanoLuc and C-terminal FLAG tags and expressed in rice protoplasts. Of note, 20 μg and 4 μg of total protein were used for WB and in-gel detection, respectively. **c, d** Application of the NanoLuc tag in the Co-IP experiment. CPK18-FLAG and NanoLuc-MPK3 were coexpressed in protoplasts for Co-IP. Ten percent of the input sample and 40% of the IP complex were analyzed by WB and in-gel detection, respectively (c). The relative amount of NanoLuc-MPK3 was also quantified by measuring NanoLuc activity using a luminescence reader (d). Error bar, standard deviation (n = 3). Loading, Coomassie Blue stained gel; Expose, the exposure time to capture the image

DRB4 was only detectable with in-gel detection (Fig. 3b). EIL1 is phosphorylated in plants (Yoo et al. 2008); therefore, the main band of the EIL1 fusion protein (~120 kDa) is larger than its predicted size (90 kDa) due to a phosphorylation-dependent electrophoresis shift. In addition, multiple EIL1 bands were observed by in-gel detection, suggesting that EIL1 had multiple phosphorylation forms in rice. In this experiment, we could not exclude the possibility that the sensitivity of western blotting could be improved by fusing more epitope tags (e.g., 3 × FLAG) and optimizing the antibody dilution, blotting, and other conditions. However, our data strongly suggest that NanoLuc-based in-gel detection is a very sensitive method for protein detection and analysis.

We further explored the application of the NanoLuc tag in a coimmunoprecipitation experiment (Co-IP). To this end, CPK18-FLAG and NanoLuc-MPK3 were coexpressed in rice protoplasts. Following standard Co-IP with anti-FLAG beads, NanoLuc-MPK3 was readily detected in the IP complex using in-gel detection (Fig. 3c), confirming the in vivo interaction between CPK18 and MPK3 as shown previously (Xie et al. 2014). In this experiment, NanoLuc-MPK3 in the IP complex was also quantitatively measured using a luminescence reader (Fig. 3d), implying that Co-IP with a

NanoLuc tag potentially enables quantitative analysis of protein-protein interactions.

Analysis of endogenous protein phosphorylation using NanoLuc in-gel detection and Mn2^{+}-Phos-tag SDS-PAGE

Protein kinases play pivotal roles in signal transduction, but detecting the in vivo phosphorylation of protein kinases is challenging. We previously identified MPK3-phosphorylated CPK18 in vitro (Xie et al. 2014), but its phosphorylation has not been characterized in vivo. We attempted to use Mn^{2+}-Phos-tag SDS-PAGE (Kinoshita-Kikuta et al. 2007) and western blotting to analyze the phosphorylation status of CPK18DA (D178A, kinase defect mutant) in protoplasts. To this end, GFP-tagged MKK4DD (MAPK kinase 4 with Thr238-to-Asp and Ser244-to-Asp mutations, constitutively activated), which phosphorylates and activates MPK3, HA-MPK3, and FLAG-CPK18DA, was coexpressed in rice protoplasts. However, Mn^{2+}-Phos-tag SDS-PAGE and western blotting could not detect any phosphorylated form of CPK18DA (Fig. 4a). This finding is likely due to the fact that CPK18DA was not stable in rice cells; thus, the abundance of phosphorylated CPK18DA was too low for detection by regular western blotting (Supplementary Fig. S4). Since the detection signal of CPK18DA was greatly increased using NanoLuc tag and in-gel detection (Supplementary Fig. S4), we then utilized NanoLuc-tagged CPK18 and performed in-gel detection after Mn^{2+}-Phos-tag SDS-PAGE. In-gel detection identified a clear phosphorylation-dependent shift band of NanoLuc-CPK18DA in Mn^{2+}-Phos-tag SDS-PAGE but not in regular SDS-PAGE (Fig. 4 b and c), indicating that MPK3 directly phosphorylates CPK18 in vivo. These results demonstrate that NanoLuc-based in-gel detection is compatible with Mn^{2+}-Phos-tag SDS-PAGE and provides a sensitive and convenient approach to detect phosphorylated proteins with low abundance.

CRISPR/Cas9-mediated tagging of endogenous *MPK3* with NanoLuc

Finally, we investigated the detection of endogenous genes using a NanoLuc tag. To this end, NanoLuc was employed to tag endogenous *MPK3* via targeted knock-in using the CRISPR/Cas9 and WDV replicons (Baltes et al. 2014; Wang et al. 2017). We constructed a pRGEB32-WDV vector (Fig. 5a and Supplementary Fig. S5) based on pRGEB32 (Xie et al. 2015) for plant gene targeting. We then assembled the knock-in construct of pRGEB32-MPK3-NanoLuc, which contains a donor template in the WDV replicon and expresses Cas9 and guide RNA (gRNA) to cleave the 3′-end of the *MPK3* coding region (Fig. 5 a and b). After introduction of the plasmid construct into rice protoplasts, the precise in-frame insertion of *NanoLuc* at the 3′-end of *MPK3* was confirmed by Sanger sequencing (Fig. 5c). NanoLuc-tagged endogenous MPK3 was also detected with in-gel detection (Fig. 5d). Although the efficiency of stable knock-in via CRISPR/Cas9 requires further optimization in plants, tagging endogenous genes with NanoLuc would be useful to study protein function in vivo.

Discussion

In this study, we explored the utility of NanoLuc as a tag for protein analysis. We constructed vectors for NanoLuc tagging and optimized the in-gel detection conditions. Our results show that the NanoLuc tag and optimized in-gel detection provide a fast, convenient, sensitive, and antibody-free method for protein detection.

Recombinant proteins with standardized peptides and fluorescent tags are popular for monitoring gene expression in plant functional genomics. Luciferases, such as Fluc, Rluc, and NanoLuc, are often used as bioluminescent reporters for quantitating promoter activity (Xu et al. 2013) and small RNA-mediated target repression (Cazzonelli and Velten 2006). NanoLuc tag and in-gel detection present a useful alternative for protein detection without antibodies. Antibody-based detection of tagged proteins is extensively used for protein analysis; however, its detection specificity and sensitivity are affected by many factors, especially by the quality of antibody reagents (Baker 2015). The sequences adjacent to the tag also affect the antibody recognition of the peptide tag. For instance, Myc tag antibody recognition is dependent on amino acid residues adjacent to the tag (Schuchner et al. 2020). Compared to classic peptide and fluorescent tags, the NanoLuc tag permits in-gel detection of tagged protein with high sensitivity but omits the tedious blotting and immunodetection steps (Fig. 2). NanoLuc is also flexible to fuse to either the N or C terminus of the protein of interest without loss of detection sensitivity (Fig. 3a and

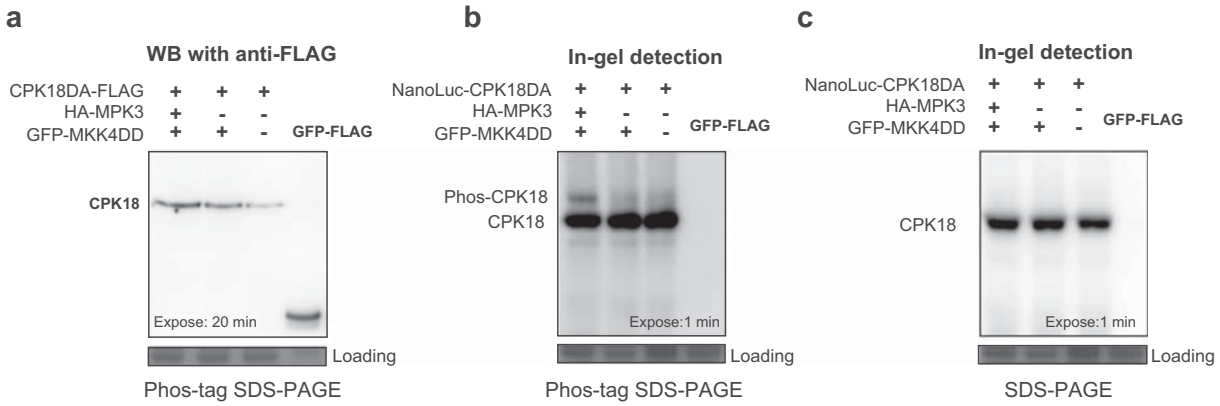

Fig. 4 Analyzing phosphorylation of NanoLuc-tagged protein using Mn^{2+}-Phos-tag SDS-PAGE and in-gel detection. **a** Detection of CPK18DA with western blotting (WB) and anti-FLAG antibody following Mn^{2+}-Phos-tag SDS-PAGE. **b** In-gel detection of NanoLuc-CPK18DA following Mn^{2+}-Phos-tag SDS-PAGE. **c** In-gel detection of NanoLuc-CPK18DA after standard SDS-PAGE. Protoplasts expressing FLAG-tagged GFP (GFP-FLAG) were used to monitor the nonspecific background. Phos-CPK18, phosphorylated CPK18; Loading, Coomassie Blue-stained gel; Expose, the exposure time to capture the image

Supplementary Fig. S3). We tested four NanoLuc-tagged rice proteins with different biochemical characteristics and molecular weights. All fusions were readily detected using optimized in-gel detection, whereas western blotting failed to detect one of them (Fig. 3b). NanoLuc also has a high sensitivity and broad dynamic

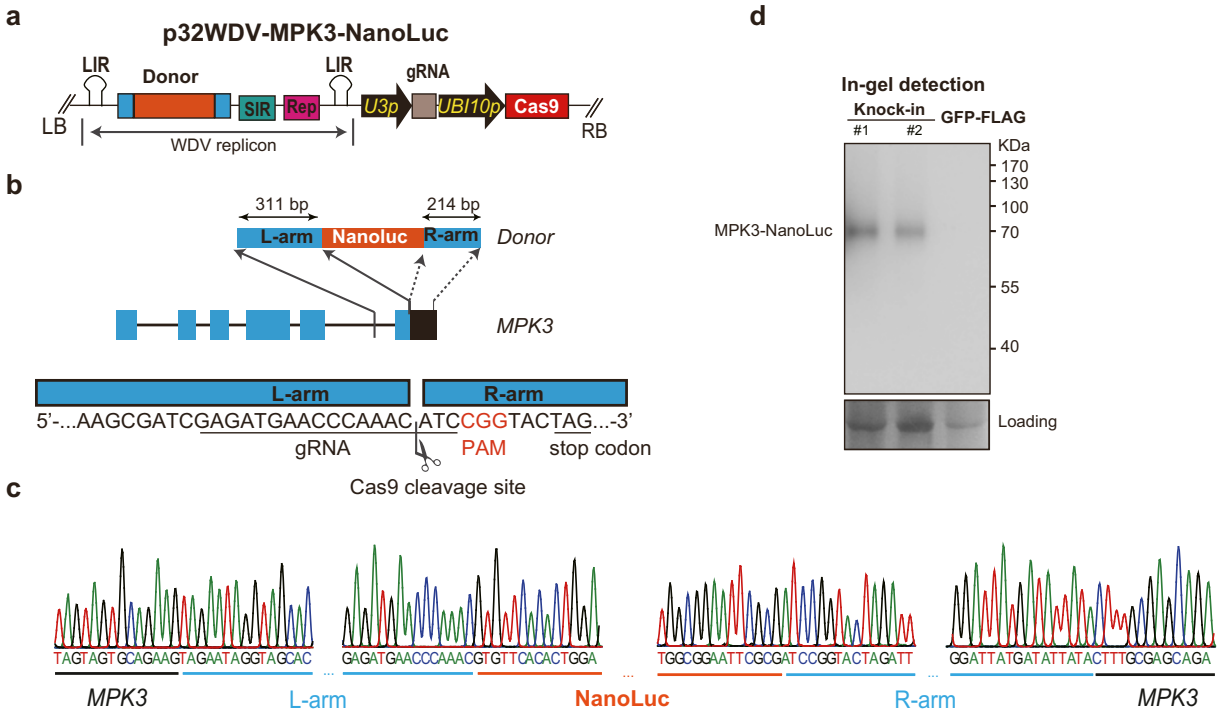

Fig. 5 Tagging *MPK3* with *NanoLuc* at the endogenous locus using CRISPR/Cas9-mediated gene targeting. **a** Design of the knock-in vector expressing Cas9/gRNA and WDV replicon. The WDV replicon is used to amplify the donor template in plant cells. **b** Schematics of the *MPK3* gene, knock-in site (indicated by scissors), gRNA-guide sequence, and donor DNA template. **c** Sanger sequencing confirms precise knock-in of NanoLuc at the desired site in protoplasts. **d** In-gel detection of endogenous MPK3 tagged with NanoLuc. Two replicates (#1 and #2) of knock-in experiments are shown. Protoplast transfection of FLAG-tagged GFP was included to monitor the nonspecific background. LIR and SIR, large- and short-intergenic region; PAM, protospacer-adjacent-motif; L/R-arm, homologous sequences flanking Cas9 cleavage site; Loading, Coomassie Blue-stained gel; U3p, rice snoRNA U3 promoter; UBI10p, rice *Ubiquitin10* promoter; Expose, the exposure time to capture the image

range (Fig. 2e), which is particularly useful for analyzing in vivo protein phosphorylation using Mn^{2+}-Phos-tag SDS-PAGE. For example, a NanoLuc tag based on gel detection detected the phosphorylated and unphosphorylated forms of CPK18DA following Mn^{2+}-Phos-tag SDS-PAGE (Fig. 4). Together, our results suggest that the NanoLuc tag with in-gel detection is a robust and versatile method for protein analysis.

In addition to gene tagging via transgenes, in vivo gene tagging at endogenous loci is a more elegant approach to study gene expression. The rapid development of gene editing technology allows precise knock-in of a protein tag to the desired genomic site (Chen et al. 2019). We tested the feasibility of tagging endogenous *MPK3* with NanoLuc using the WDV replicon and CRISPR/Cas9-mediated gene targeting. In protoplasts, endogenous MPK3 tagged by NanoLuc was readily detected using in-gel detection (Fig. 5). However, the efficiency of CRISPR/Cas9-mediated gene tagging at the endogenous locus in stable transgenic plants requires further validation.

In summary, we developed a NanoLuc toolkit and tailored the in-gel detection method for sensitive and convenient analysis of proteins in vitro and in vivo. We also demonstrated versatile applications of NanoLuc in-gel detection for protein expression analysis, Co-IP, in vivo protein phosphorylation detection, and endogenous gene tagging via CRISPR/Cas9-mediated gene targeting. Compared with epitope tags and western blotting for protein analysis, the NanoLuc tag and our optimized in-gel detection exhibit high detection sensitivity without tedious blotting and immunodetection steps. The versatile NanoLuc toolkit and convenient in-gel detection method are expected to facilitate in vitro and in vivo protein analysis.

Methods

Construction of binary vectors for NanoLuc tagging

Rice codon optimized *NanoLuc* and *Fluc* genes were synthesized (GenScript) and cloned into pUC57 to generate pUC57-Nanoluc and pUC57-Fluc. Sequences of *NanoLuc* and *Fluc* are shown in Supplementary Figs. S1 and S2. The *Rluc* used in this study is from pDuExAn1RG (Fujikawa and Kato 2007).

The NanoLuc tagging vectors were derived from the Gateway destination vector p1300-34, which is used to express genes of interest with the rice *Ubiquitin10* promoter (*UBI10p*) (Ding et al. 2018). For p34NN, the coding sequence of *NanoLuc* was amplified from pUC57-Nanoluc using primers Gib34X-Nano-F and Gib34X-Nano-R. Then, the PCR product and *Xba*I-digested p1300-34 were assembled into p34NN through Gibson cloning. For p34NC, a 9-bp sequence (AAGGGTACC) containing the *Kpn*I site was inserted between the first and second nucleotides of the stop codon (TAG) downstream of the attR2 site in p1300-34 using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent), which resulted in the p1300-34(T-A) plasmid. Subsequently, the *NanoLuc* fragment was amplified from pUC57-Nanoluc using primers Gib-Nanoluc-F and Gib-Nanoluc-Kpn1-R2. Then, the PCR product was inserted in frame at the *Kpn*I site of p1300-34(T-A) through Gibson cloning, which resulted in the p34NC vector. The sequences of primer oligos used to construct these vectors are listed in Supplementary Table S1.

p34NN-35S and p34NC-35S were constructed to express NanoLuc-tagged protein with the cauliflower mosaic virus 35S promoter. Briefly, the 35S promoter was amplified from pRGEB32 (Xie et al. 2015) with primers HindIII-35S-F3 and NruI-35S-R3. The PCR product was inserted into the *Hind*III and *Nru*I sites of p34NN and p34NC to replace the *UBI10* promoter to generate p34NN-35S and p34NC-35S vectors, respectively. The sequences of primer oligos are shown in Supplementary Table S1.

Gene tagging constructs

To compare the bioluminescent activity of *NanoLuc*, *Fluc*, and *Rluc* (Fig. 1a), the *UBI10* promoter was used to express these genes in rice cells. For *NanoLuc*, a 0.5-kb *Nco*I-*Eco*RI fragment from pUC57-Nanoluc was ligated into the Gateway Entry vector pENTR11 (Thermo Fisher Scientific) to generate pENTR11-NanoLuc. The coding sequences of *Fluc* were amplified from pUC57-Fluc using primers FLUC2-F2 and FLUC2-R3 and then cloned into the *Kpn*I and *Eco*RV sites of pENTR11 through Gibson cloning. The *Rluc* gene was subcloned from pDuExAn1RG to the pENTR vector by the Gateway BP reaction. These three luciferase genes were then transferred from pENTR11 to p1300-34 by the Gateway LR reaction to generate *UBI10p::NanoLuc/Fluc/Rluc* constructs.

The plasmids expressing NanoLuc-tagged genes were constructed by Gateway cloning (Thermo Fisher Scientific). Briefly, the full-length cDNA of *DRB4* and *EIL1* were obtained from NARO DNA Bank (https://www.dna.affrc.go.jp/) and then subcloned into pENTR/D-TOPO (Thermo Fisher), respectively (see Supplementary Table S1 for primer sequences). The Gateway Entry clones of *MPK3* (with and without stop codon), *CPK18*, and *CPK18DA* were constructed as previously reported (Xie et al. 2014). Finally, these genes were subcloned from entry plasmids to p34NN by the Gateway LR reaction to generate NanoLuc-tagged *MPK3*, *DRB4*, *CPK18*, and *EIL1* constructs. To fuse Nanoluc at the C-terminus, *MPK3* coding sequences without stop codons in the entry clone were transferred into p34NC through the Gateway LR reaction, and the construct expressing the *MPK3-Nanoluc* fusion was obtained.

The pUGW vectors were used to tag *MPK3* and *CPK18* with HA and FLAG, respectively (Nakagawa et al. 2007). In the Co-IP experiment, the pUGW11-CPK18 plasmid, which expresses CPK18 with C-terminal fused FLAG, was obtained from our previous study (Xie et al. 2014). For in vivo phosphorylation detection, pENTR11-MKK4 was obtained as we reported previously (Xie et al. 2014). The Thr238 and Ser244 residues of MKK4 were substituted with Asp to generate the MKK4DD mutant with constitutively activated kinase activity (see Supplementary Table S1 for primer sequences). Next, MKK4DD in pENTR11 was subcloned into pUGW6 using the Gateway LR reaction to generate a GFP-tagged MKK4DD (GFP-MKK4DD) construct. To generate HA-tagged MPK3 (HA-MPK3), *MPK3* in Entry was cloned into pUGW14 using the Gateway LR reaction. The plasmid vector pUGW11-GFP, which expressed GFP with a C-terminal FLAG (GFP-FLAG), was used as a control for all protoplast experiments in this study.

The GenBank accession numbers of rice genes mentioned in this study are as follows: *CPK18*, AK121471; *DRB4*, AK065829; *MPK3*, AF479883; *MKK4*, AK120525; and *EIL1*, AK103227.

CRISPR/Cas9-mediated knock-in construct for in vivo tagging of *MPK3*

A WDV replicon without the coat protein gene was synthesized (GenScript) and cloned into the pEASY vector (TransGen Biotech). In this synthesized replicon, the coat protein region was replaced by *Kpn*I and *Bam*HI restriction cloning sites. Then, the replicon was amplified from pEASY-WDV using primers 32-Hind III-F and 32-HindIII-R and cloned into the *Hind*III site of p1300-32 (Xie et al. 2015) using Gibson cloning to generate p1300-32-Rep. Then, the Gateway LR reaction was used to clone Cas9 from pENTR11 (Xie and Yang 2013) into p1300-32-Rep, resulting in pRGEB32-WDV (Supplementary Fig. S5).

The knock-in construct (Fig. 5a) was assembled as follows. First, a gRNA targeting *MPK3* was fused with tRNA, and then the tRNA-gRNA cassette was inserted into the dual *Bsa*I sites of pRGEB32-WDV as we described previously (Xie et al. 2015). The intermediate construct pRGEB32-WDV-MPK3 was obtained. Second, the donor DNA template containing NanoLuc and left and right homology arms (L-arm and R-arm) was assembled as follows. The L-arm and R-arm were amplified from rice genomic DNA using primer pairs MPK3-M-F/MPK3-N-R and MPK3-N-F/MPK3-T-R, respectively. The NanoLuc fragment was amplified from pUC57-Nanluc with primers MPK3-NanoLuc-F and MPK3-NanoLuc-R. The PCR products of the L-arm, R-arm, and NanoLuc fragments were assembled together by overlapping extension PCR with primers Gib-MPK3-F and Gib-MPK3-R. Finally, the assembled donor DNA template was inserted into the *Bam*H I and *Kpn*I sites of pRGEB32-WDV-MPK3 using Gibson cloning to generate the final construct pRGEB32-MPK3-NanoLuc.

To tag endogenous *MPK3* with *NanoLuc*, the plasmid pRGEB32-MPK3-NanoLuc was used to transfect rice protoplasts. After incubating protoplasts at room temperature in the dark for 36 h, total protein and genomic DNA were extracted for further analysis. To confirm the precise knock-in of NanoLuc, the *MPK3* genomic fragment across the L- and R-arms was amplified using primers NL-KI-F and NL-KI-R (see Supplementary Table S1 for primer sequences) and sequenced. The endogenous MPK3 tagged by NanoLuc was then examined by the optimized in-gel detection method as we described below.

Plant materials

Rice cultivar Kitaake (*Oryza sativa* spp. *Geng*) was used for protoplast transient expression in this study. *Nicotiana benthamiana* used for agroinfiltration was kindly provided by Dr. Li Yi-bo (Huazhong Agricultural

University, Wuhan, China). The plants were grown in a growth chamber with 14h/10h day/night cycle and the temperature was set to 25~28 °C.

Rice protoplast preparation and transfection

Rice protoplast preparation and transfection were performed as described previously (Xie and Yang 2013). Briefly, 12-day-old young seedlings grown in Murashige and Skoog medium were used for protoplast isolation. In the protein detection experiment, 10–20 μg of plasmid was used to transfect 1×10^6 protoplasts. After incubation in the dark for 16–24 h, protoplasts were collected by centrifugation at 300 *g* for 2 min and frozen at −80 °C.

Agroinfiltration of *Nicotiana benthamiana* leaves

To express NanoLuc-tagged protein in tobacco, *MPK3* in the Entry plasmid was cloned into p34NN-35S and p34NC-35S by the Gateway LR reaction. The constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. For agroinfiltration, the overnight culture of Agrobacteria was diluted in Luria-Bertani broth medium containing kanamycin and rifampicin and continuously cultured until the OD_{600} was 0.6. Bacterial cells were harvested by centrifugation and then suspended in buffer containing 10 mM MES pH 5.6, 10 mM $MgCl_2$, and 200 μM acetosyringone. Bacterial suspensions were infiltrated into the leaves of 4- to 5-week-old *Nicotiana benthamiana* plants. Two days after agroinfiltration, total protein was extracted from infiltrated leaf discs with Plant Protein Extraction Buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, and 1 mM PMSF).

Luminescence assay

For luminescence assays, the protoplasts transfected with the corresponding plasmids were lysed with 1× lysis buffer (25 mM Tris-HCl pH 7.5, 1 mM DTT, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT) and then centrifuged at 12000×*g* for 10 min to remove the cell debris. The concentrations of protein extracts were determined using the Bradford Protein Assay Kit (Tiangen Biotech). To measure NanoLuc bioluminescence, 5 μl of total protein extracts were mixed with 50 μl of Nano-Glo Luciferase Assay Buffer and 0.5 μl of Nano-Glo Luciferase Assay Substrate (Promega) in a 96-well microplate. After 5 min, luminescent signals were measured using a Spark Multimode Microplate Reader (Tecan). To measure Fluc bioluminescence, 5 μl of protein extracts were mixed with 50 μl of Firefly Luciferase Assay Buffer (25 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.125 mM CoA, 0.1 mM ATP) and 150 μg/ml of D-luciferin. For Rluc, 5 μl of protein extracts were mixed with 50-μl H_2O and 0.01 mM coelenterazine. The luminescence signals were measured with a Spark Multimode Microplate Reader (Tecan). Three biological replicates were run for each luciferase.

Western blotting

Total proteins were extracted from rice protoplasts using a modified RIPA buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1% SDS, 1% Triton X-100, 10% glycerol, and 5 μg/ml protease inhibitor cocktail (Sigma-Aldrich)) (Xie et al. 2014). The cell debris was removed by centrifugation at 12000×*g* for 10 min. For western blotting, 20 μg of total protein extracts was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. The tagged proteins were detected using a standard immunodetection procedure using an anti-FLAG antibody (Sigma-Aldrich). In this study, the primary antibody (anti-FLAG) was diluted 1:1000, and the horseradish peroxidase-conjugated secondary antibody (anti-mouse IgG, Sigma-Aldrich) was diluted 1:10000. Finally, the blot was incubated in luminol-based enhanced chemiluminescent substrate from the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher Scientific) and imaged using a Tanon 5200 chemiluminescent imager (Tanon). The band intensities were analyzed using ImageJ (version 1.50, https://imagej.nih.gov/ij). The original, full length western blots images are shown in Supplementary Fig. S6.

Phos-tag-Mn^{2+} SDS-PAGE

Total protein extracts were separated by SDS-PAGE containing 50 μM Phos-tag (FUJIFILM Wako Chemicals) and 100 μM $MnCl_2$. After electrophoresis, the gel was subjected to in-gel detection as described below. For western blotting, the Phos-tag-Mn^{2+} gel was treated and blotted to the membrane as described previously (Xie et al. 2014). The original, full length western

blots, in-gel detection, and stained gel images are shown in Supplementary Fig. S6.

Optimized in-gel detection of NanoLuc-tagged protein

Following SDS-PAGE or Phos-tag-Mn^{2+} SDS-PAGE, the gel was washed at room temperature by gently shaking in SDS-Removing Buffer (50 mM Tris pH 7.5, 1 mM DTT, 0.1 mM EDTA) for approximately 1 h with 3 buffer changes. Then, the gel was incubated in Renaturation Buffer (50 mM Tris pH 7.5, 5 mM DTT, 0.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 0.04% Tween-20) at 4 °C for 1~4 h. Finally, the gel was rinsed with distilled water and then incubated in Substrate Solution (1× PBS, 0.1% BSA, 1× Nano-Glo Luciferase Assay Substrate (Promega)). After 5 min, NanoLuc luminescence in the gel was imaged using a Tanon 5200 chemiluminescent imager (Tanon). After in-gel detection, the gel was stained using Coomassie Brilliant Blue and imaged as a loading control. The band intensities were analyzed using ImageJ (version 1.50, https://imagej.nih.gov/ij). The original, full length stained gel and in-gel detect images are shown in Supplementary Fig. S6.

Coimmunoprecipitation

In the Co-IP experiment, p34NN-MPK3 and pUGW11-CPK18 plasmids were cotransfected with 1×10^7 rice protoplasts. The transfected protoplast was incubated in the dark at room temperature for 16 h. The protoplasts were then collected, and total proteins were extracted using lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF). Co-IP was performed as we described previously (Xie et al. 2014). In brief, 200 μg of total protein was incubated overnight with 20-μl FLAG M2 agarose (Sigma-Aldrich) at 4 °C. Then, the FLAG M2 agarose was collected in a spin column and washed thrice with washing buffer (50 mM Tris pH 7.6, 150 mM NaCl, and 1 mM PMSF). The protein complex was eluted using 0.1 M glycine pH 2.7. Finally, the elution was mixed with an equal volume of 1 M Tris pH 8.0. To analyze proteins in the IP complex, 40% of the elution was subjected to SDS-PAGE for in-gel detection and western blotting. NanoLuc-MPK3 was also measured using a luminescent reader. To this end, 10% of the IP complex was mixed with 200 μl of PBS buffer containing 0.1% BSA and 0.5 μl of Nano-Glo Luciferase Assay Substrate (Promega). The NanoLuc luminescence was measured with a Spark Multimode Microplate Reader (Tecan).

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Authors' contribution KX designed the experiments. HL, CW, MD, and YC performed the experiments. HL, XH and KX analyzed the data. HL, XH, YY and KX wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and material All data generated or analyzed during this study are included in this published article and its supplementary information files. The vectors generated by the current study are available from the corresponding author on request.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

Code availability Not applicable.

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