Novel assays to monitor gene expression and protein-protein interactions in rice using the bioluminescent protein, NanoLuc

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Abstract  Luciferases have been widely utilized as sensitive reporters to monitor gene expression and protein-protein interactions. Compared to firefly luciferase (Fluc), a recently developed luciferase, Nanoluminase (NanoLuc or Nluc), has several superior properties such as a smaller size and stronger luminescence activity. We compared the reporter properties of Nluc and Fluc in rice (Oryza sativa). In both plant-based two-hybrid and split luc complementation (SLC) assays, Nluc activity was detected with higher sensitivity and specificity than that with Fluc. To apply Nluc to research involving the photoperiodic regulation of flowering, we made a knock-in rice plant in which the Nluc coding region was inserted in-frame with the OsMADS15 gene, a target of the rice florigen Hd3a. Strong Nluc activity in response to Hd3a, and in response to change in day length, was detected in rice protoplasts and in a single shoot apical meristem, respectively. Our results indicate that Nluc assay systems will be powerful tools to monitor gene expression and protein-protein interaction in plant research.

Key words: florigen, knock-in, NanoLuc, protein-protein interaction, rice.

Introduction

The study of protein-protein interactions (PPI) is important in understanding protein function and the complicated regulatory networks in all organisms, including plants. To detect PPI effectively, several experimental methods (for example, co-immunoprecipitation (CoIP) assays, two-hybrid assays, bimolecular complementation assays and FRET-FLIM) have been developed and used, depending on the experimental purpose (Bontinck et al. 2018; Ferro and Trabalzini 2013; Xing et al. 2016). Yeast two-hybrid assays (Y2H) have been widely used because they are convenient, quantitative, and extremely powerful in high-throughput screening of interacting proteins. Two-hybrid assays are also performed in plant cells (Ehlert et al. 2006), because plant specific-posttranslational modifications and/or co-factors essential for optimal PPI are expected to be present in these cells. To observe the PPI between protein X and Y in a two-hybrid assay, the DNA-binding domain of the yeast transcription factor GAL4 is fused with X (G4DBD-X) and the transcriptional activation domain of GAL4 (GAL4AD) or herpes simplex virus protein VP16 (VP16) is fused with Y (GAL4AD-Y or VP16-Y). If G4DBD-X interacts with GAL4AD-Y or VP16-Y, the GAL4AD or VP16 activation domain activates the reporter gene expression in a GAL4 binding sequence (GAL4 UAS)-dependent manner. The strength of the interaction can be evaluated by quantifying the reporter activity. However, the two-hybrid assay has three major drawbacks: false-positive results, detection of only a subset of the interactome, and

Abbreviations: Nluc, NanoLuc; Fluc, firefly luciferase; PPI, protein-protein interaction; CoIP, co-immunoprecipitation; FRET-FLIM, Förster/fluorescence resonance energy transfer-Fluorescence Lifetime Imaging Microscopy; Y2H, Yeast two-hybrid assay; GAL4 UAS, GAL4AD GAL4 binding sequence; SLC, Split luc complementation; NanoBiT, NanoLuc Binary Technology; SD, short-day; LD, long-day; SAM, shoot apical meristem; FAC, florigen activation complex; GT, Gene targeting; HR, homologous recombination; KI, knock-in; M15NL, OsMADS15-Nluc; TBSV, Tomato bushy stunt virus; GFP, Green fluorescent protein; FT, Flowering locus T; NLS, nuclear localization signal.

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limited information on the dynamics of a PPI (Stynen et al. 2012). The split luc complementation (SLC) assay is a powerful tool to analyze PPI dynamics (Stynen et al. 2012). In the SLC assay with firefly luciferase (Fluc), two divided fragments of Fluc (FlucN and FlucC) are fused with the proteins of interest, X and Y, respectively. X–Y interaction transiently reconstitutes a functional Fluc, which leads to Fluc luminescence as a reporter of the interaction. For quantitative analysis, Rluc, a luciferase from Renilla reniformis with different substrate specificity, is usually co-introduced with Fluc, as an internal control (Matsuo et al. 2001). Due to the reversible nature of FlucN–FlucC PPI, no need for transcriptional activation after PPI, the absence of nuclear localization constraints, and the intense luminescence, the SLC assay with Fluc has been widely used to detect PPI in plant research (Fujikawa et al. 2014; Gehl et al. 2011; Li et al. 2011). However, it cannot detect very weak PPI due to technical limitations.

Recently, the small luciferase NanoLuc (Nluc) was engineered from a larger luminescent protein isolated from a deep-sea shrimp, Oplopomus gracilirostris (Hall et al. 2012). Nluc has superior biochemical properties, such as its small size (19 kDa, compared to the 59 kDa Fluc protein) and a150-fold brighter signal than that of Fluc (Hall et al. 2012). Furthermore, in order to detect PPI efficiently with Nluc, NanoLuc Binary Technology (NanoBiT) has also been developed (Dixon et al. 2016). Although the use of Nluc in plant research has been reported (Urquiza-Garcia and Millar 2019; Wang et al. 2020), its use is still limited, probably due to the lack of practical protocols and a detailed comparison with conventional Fluc methods. Here, we examined whether Nluc might be useful in PPI analysis in rice by testing a well-characterized interaction in the photoperiodic flowering pathway, the interaction between the Hd3a and OsMADS15 domain (Obsil and Obsilova 2011). The FAC activates floral meristem identity genes such as OsMADS15, a member of the API subfamily in rice, and initiates the reproductive program (Taoka et al. 2011). The interaction among FAC components has been confirmed by Y2H, CoIP, FRET-FLIM, and X-ray crystallographic analysis (Taoka et al. 2011). However, the target DNA sequence of the FAC in rice has not been identified. Therefore, reporter constructs that are faithfully activated by the FAC are lacking, which makes it difficult to analyze the molecular mechanisms of FAC-mediated flowering.

To monitor gene expression, transient expression assays with promoter–reporter constructs have been conventionally used, due to their convenience and the high levels of quantification. However, it is difficult to make a faithful promoter–reporter construct when the transcription of the gene of interest is regulated not only by the 5′ upstream region but also by other genomic components, such as far-upstream or downstream regions, and by higher order chromatin structure (Sabari et al. 2020). Gene targeting (GT) via homologous recombination (HR) is a genetic engineering method that enables precise and flexible genome modification including gene replacement and knock-in (KI) according to the template DNA. Therefore, if the reporter is inserted into the gene of interest by KI, the chimeric gene would be transcribed like the original WT gene, due to the minimal alteration of the original genomic context. Making such KI plants in angiosperms is challenging due to low HR efficiency (Puchta and Fauser 2013; Van Vu et al. 2019), but recently successful protocols have been developed (Miki et al. 2018; Shimatani et al. 2014). In rice flowering research, KI rice plants in which a fluorescent reporter gene, mOrange, was inserted in-frame with OsMADS15, contributed to reveal the spatiotemporal change of OsMADS15 expression during the progression of rice SAM development (Tamaki et al. 2015).

Here, we made rice KI plants in which Nluc was inserted in-frame with OsMADS15, and examined the response of OsMADS15-Nluc (M15NL) to the FAC and to a change of day length. The M15NL gene is effectively activated by the FAC in protoplasts. The activity of M15NL could be easily quantitated in a tiny single SAM. Together with the results from PPI analyses, we show that Nluc is a powerful reporter in PPI and reporter analysis in rice.

Materials and methods

Plant materials and growth conditions
Tobacco plants (Nicotiana benthamiana) were grown at 25°C under long-day conditions (16 h-light/8 h-dark). Rice plants (Oryza sativa cv. Nipponbare) were grown under short-day (10 h-light at 27°C/14 h-dark at 25°C) or long-day (14 h-light at
28°C/10 h-dark at 25°C) conditions.

**Plasmid construction**

General techniques in molecular biology were used for plasmid construction. For PCR reactions, KOD FX Neo (TOYOBO), PrimeSTAR GLX DNA polymerase (TaKaRa bio), or Tks Gflex DNA polymerase (TaKaRa bio) were used according to the manufacturer’s instructions. For seamless assembly of DNA, the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) was used. For Gateway cloning, pENTR-D-TOPO and the Gateway® LR Clonase® II enzyme mix (Thermo Fisher Scientific) were used. The oligonucleotide sequences used for PCR cloning are listed in Supplementary Table S1. Integrity of all the plasmids used was confirmed by PCR and DNA sequencing. The details are described in the Supplementary text.

**Preparation of rice protoplasts**

Rice Oc (Baba et al. 1986) and M15NL-KI suspension cells from callus were cultured in 24 ml of R2S medium (Koyuzuka and Shimamoto 1991) in a 100 ml flask with rotary shaking at 100 rpm at 30°C in the dark. Four days after subculture, two flasks of Oc suspension cells were treated with 10 ml of cellulase solution (40 mg ml⁻¹ Cellulase Onozuka RS (Yakult Pharmaceutical Industry), 10 mg ml⁻¹ Macerozyme R-10 (Yakult Pharmaceutical Industry), 0.5 M sucrose, 10 mM CaCl₂ 2H₂O, 1 mg ml⁻¹ MES-KOH, 1 mg ml⁻¹ bovine serum albumin, pH 5.6) for 4 h with rotary shaking at 50 rpm at 30°C in the dark. After cellulase treatment, the cell suspension was transferred to a 50 ml tube, gently layered with 10 ml of W5 buffer (154 mM NaCl, 125 mM CaCl₂, 2H₂O, 5 mM KCl, 2 mM MES-KOH, pH 5.6), and centrifuged at 123×g for 10 min with a swinging bucket centrifuge, Allegra X-30R Centrifuge (Beckman Coulter). After centrifugation, intact protoplasts, concentrated between the cellulase solution and the W5 layer, were collected, washed three times with W5 buffer, and resuspended in MaMg buffer (0.4 M mannitol, 15 mM MgCl₂, 4 mM MES-KOH, pH 5.7). For the M15NL-KI line, six flasks of suspension cells were treated for 6 h with 20 ml of cellulase solution (40 mg ml⁻¹ Cellulase Onozuka RS (Yakult Pharmaceutical Industry), 10 mg ml⁻¹ Macerozyme R-10, 1 mg ml⁻¹ MES-KOH, pH 5.7). The cell suspensions were mixed by combining equal volumes of individual cultures and infiltrated into the leaves of tobacco at 4 weeks after seeding with a 1 ml syringe.

**Measurement of luciferase activity in tobacco**

Leaves at six days post-infiltration were used for the luciferase analysis. Luminescence of six leaf samples from three plants was analyzed for each analysis with a luminometer, TriStar LB941 (Berthold Technologies). The detection unit of Tristar LB941 is a low-noise photomultiplier, which can detect from 340 to 650 nm. No emission filter set was used, because the autofluorescence from living rice cells is negligible.

For measurement of Nluc activity in leaf tissue lysate, the Nano-Glo® Luciferase Assay System (Promega) was used according to the manufacturer’s instructions. A piece of leaf disc 5 mm×5 mm square (ca. 2.5 mg) was cut out from the infiltrated region and ground in 100 µl of Passive lysis buffer (Promega) with a pestle. To avoid overloading, the lysate was further diluted 10,000-fold with Passive Lysis buffer. Luminescence was measured with TriStar LB941 immediately after mixing 5 µl of the diluted lysate with 5 µl of Nano-Glo Luciferase Assay Reagent (Promega) on a 96-well titer plate.

For measurement of Fluc activity in leaf tissue lysate, the Dual-Luciferase® Reporter Assay System (Promega) was used according to the manufacturer’s instructions. After the cell lysate was prepared in the same way as above, it was further diluted 100-fold to avoid overloading. Luminescence was measured immediately after mixing 2 µl of the diluted lysate with 10 µl of LARII reagent in the kit on a 96-well titer plate.

For measurement of Nluc activity in live cells, the Nano-Glo® Luciferase Assay System and the Nano-Glo® Live Cell Assay System (Promega) were used according to the manufacturer’s instructions. 30 µl of Nano-Glo Luciferase Assay Reagent or Nano-Glo® Live Cell Reagent was infiltrated into the Agro-infiltrated leaf and a piece of leaf disc 5 mm×5 mm square was removed. After 3 min incubation, the leaf disc was
placed into a 96-well plate and luminescence was measured. For measurement of Fluc activity in live cells, 30 µl of 20 mM d-luciferin solution was infiltrated and luminescence was analyzed in the same way as the Nluc analysis in live cells.

**Measurement of luciferase activity in protoplast lysates**

In lysis assays, the activities of Nluc and Fluc in the lysate were measured separately and the ratios between them were calculated. The Nano-Glo® Luciferase Assay System and Dual-Luciferase® Reporter Assay System were used according to the manufacturer’s instructions. Transfected protoplasts which had been stored in the −80°C freezer were lysed in 25 µl of Passive lysis buffer by vigorous vortexing. Luminescence of Nluc was measured with a TriStar LB941 immediately after mixing 10 µl of the lysate with 10 µl of Nano-Glo Luciferase Assay Reagent on a 96-well titer plate. Luminescence of Fluc was measured immediately after mixing 2 µl of the lysate with 10 µl of LARII reagent on a 96-well titer plate.

**Measurement of split luciferase activity in live protoplasts**

In the NanoBiT assay, the reporter Nluc activity was measured in the live cells and the reference Fluc activity was measured in the lysate, separately. The Nano-Glo® Live Cell Assay System was used according to the manufacturer’s instructions. After overnight incubation, transfected protoplasts in W5 buffer were centrifuged at 123 × g for 5 min and resuspended in 50 µl of 0.4 M mannitol solution. 10 µl of the cell suspension was mixed with 2.5 µl of Nano-Glo® Live Cell Reagent on a 96-well plate and luminescence was measured for 6 min with 1 s intervals in the kinetics mode of the TriStar LB941. The maximum relative light unit (RLU) values in the 6 min measurement were estimated as the NanoBiT activity. Reference Fluc activity was measured in the cell lysate with the Dual-Luciferase® Reporter Assay System. Cell lysate was prepared by vigorously mixing 8 µl of protoplast suspension with 2 µl of 5× Passive lysis buffer with a pipette. 2 µl of the cell lysate was mixed with 10 µl of LARII reagent on a 96-well titer plate and Fluc luminescence was measured.

In the SLC assay with Fluc, the Nano-Glo® Luciferase Assay System and D-luciferin was used. After overnight incubation, transfected protoplasts were resuspended in 25 µl of 0.4 M mannitol solution. 10 µl of the cell suspension was mixed with 1 µl of 20 mM D-luciferin solution on a 96-well plate. After 3 min incubation, the luminescence was measured for 30 s with a TriStar LB941. The reference Nluc activity was measured in the cell lysate. The cell lysate was prepared by vigorously mixing 8 µl of protoplast suspension with 2 µl of 5× Passive lysis buffer with a pipette. 10 µl of the cell lysate was mixed with 10 µl of Nano-Glo reagent on a 96-well titer plate and Nluc luminescence was measured immediately.

**Producing KI rice plants**

The Japonica rice (*Oryza sativa* L.) cultivar ‘Nipponbare’ was used for gene targeting. The rice gene targeting strategy using large-scale transformation followed by positive-negative selection was performed according to previous studies (Terada et al. 2002, 2004, 2007) with the following minor modifications. Scutellum-derived embryogenic calli were induced from mature rice seeds on N6D medium. After removing *Agrobacterium*, rice calli were cultured on N6DNU medium for 7–10 days to promote the recovery of calli and ensure *Agrobacterium* elimination. After 4 weeks of positive-negative selection, calli with incomplete targeting were eliminated by expressing *diphtheria toxin A* genes encoded by the vectors (Terada et al. 2002). Surviving callus lines were cultured independently and subjected to PCR screening with the appropriate primers (Supplementary Table S1). Amplified DNA fragments containing L-5F/3F or NL-5F/3F were verified by electrophoresis and DNA sequencing analysis. To induce the regeneration of T₀ plants, the callus lines carrying the modified *OsMADS15* were transferred to MSRE medium and cultured for 4–6 weeks. The T₀ plants obtained were grown and self-pollinated to obtain the T₁ progenies. The homo/heterozygosity of gene targeting was confirmed by PCR to detect Nluc-insertion or intact *OsMADS15* genes. The plant media used in this study are summarized in Supplementary Table S2.

**Measurement of Nluc activity of KI rice plants**

M15NL-KI lines and non-transformed rice (*Oryza sativa* cv. Nipponbare) plants at 27–28 day after sowing (das) were used to detect Nluc activity. The SAM was carefully dissected with a microscope, the Olympus SZX7 (Olympus), according to Saihara and Tsuji (2017). An entire, single SAM was disrupted in a 1.5 ml tube by four cycles of freeze/thaw (1 min in liquid nitrogen/1 min in a water bath at room temperature), and suspended in 30 µl of Passive lysis buffer. 10 µl of the lysate was used to measure the Nluc activity and RLU values/SAM/second were calculated. To obtain the M15NL-KI cell suspension lines, calli were induced from seeds of KI lines, transferred to liquid R2S medium, and maintained in the same way as Oc cells.

**Results and discussion**

**Detection of Nluc activity in tobacco cell lysates**

To evaluate whether Nluc activity can be detected efficiently in plant cells, Nluc was transiently expressed in tobacco using the Agro-infiltration method and the luminescence was measured. For Agro-infiltration, binary vectors expressing luciferase, named pGWB602-Fluc and pGWB602-Nluc (Supplementary Figures S1, S2A), were made using pGWB gateway vectors (Nakamura et al. 2010). *Agrobacterium* transformed with these vectors were co-infiltrated into tobacco with a vector to express a silencing suppressor, p19 (Takeda et al. 2002) as shown in the table in Supplementary Figure S2A. Infiltration with p19 (#1) only was used as background (BG). Six days after infiltration, leaf discs
in the infiltrated areas were dissected and mechanically disrupted in a lysis buffer. The Nluc activity of the lysates was then measured with a luminometer (Supplementary Figure S2B). For comparison, the value of relative light units (RLU) of reporter was calculated as RLU value per second per leaf disk. The average of Rep RLU from pGWB602 Nluc was 1.5×10^5-fold higher than that of BG. This demonstrated that Nluc activity in plant cells can be effectively detected in a cell lysate form and that BG activity is negligible. For comparison, we expressed and measured Fluc in the same way as Nluc (Supplementary Figure S2C). The average RLU value from pGWB602 Fluc was 6.4×10^5-fold higher than that of BG. The fold activity of Nluc to BG was 234.4-fold higher than that of Fluc, which is comparable to previous data showing that the specific activity of Nluc is about 150-fold higher than that of Fluc (Hall et al. 2012). Next, to examine whether Nluc activity can be measured in living plant cells, we infiltrated the substrate solution into the Agroinfiltrated leaves and measured luminescence from the infiltrated leaf pieces (Supplementary Figure S2D, E). We infiltrated the same substrate reagent as used in Supplementary Figure S2B, Nano-Glo, and measured the luminescence (Supplementary Figure S2D). In contrast to the results presented in Supplementary Figure S2B, the fold activity to BG (12.8-fold activity) was much lower. Similarly, infiltration of another Nluc substrate reagent from a commercial live cell assay kit produced weak luminescence (23.2-fold activity to BG, Supplementary Figure S2E). As another control, we infiltrated D-luciferin, a substrate for Fluc, in the same way as shown in Supplementary Figure S2D, E, and detected strong luminescence of Fluc (9.1×10^2-fold activity to BG, Supplementary Figure S2F). These data indicated that Nluc is an efficient luminescent reporter in plant cells, although non-destructive detection in intact plant cells is difficult under current conditions, probably due to the lability or insufficient permeability of the substrate in plant cells.

Detection of Nluc activity in cell lysates of rice protoplasts

Transient expression assays in protoplasts are convenient and powerful in the analysis of PPI and in the study of the activity of promoter or transcription factors. To evaluate whether Nluc can function as an efficient reporter in a transient assay, we made yeast GAL4 two-hybrid-based effector/reporter constructs and compared the reporter activity between Nluc and Fluc (Figure 1A). The effector plasmids were designed to express proteins of interest constitutively as a fusion with G4DBD or VP16 under the control of the CaMV 35S promoter (Supplementary Figure S1, Figure 1A). The Nluc reporter was expressed under the control of GAL4 UAS through the interaction between the test proteins, G4DBD-X and VP16-Y. As an internal reference, a plasmid expressing Fluc was co-transfected. We tested Hd3a-GF14c and GF14c-GF14c interactions in rice protoplasts (Figure 1B) because their interactions have been well-characterized. Hd3a-GF14c interaction is impaired by a R64K/R132K substitution (m2 mutation) in Hd3a (Taoka et al. 2011). GF14c is one of the eight isoforms of rice 14-3-3. The N-terminal dimerization domain is highly conserved among 14-3-3 and its deletion from T14-3c, a tobacco 14-3-3, impairs the homo-dimerization (Gehl et al. 2011). In this assay, Hd3a-GF14c and GF14c-GF14c signals were 5.5- and 62-fold higher than that of the GUS negative control, respectively (Figure 1C). Their interaction-defective mutants, Hd3a m2 and GF14c-dN, showed comparable ratios to those of the GUS control. Next, we swapped the two luciferase genes for reporter and reference: Fluc and Nuc were used as the reporter, and Fluc or Nuc as the reference, respectively. (A) Schematic representation of the assay system. Protein X is expressed as a fusion with G4DBD, whereas protein Y with a nuclear localization signal of SV40 (NLS) and VP16. Nluc or Fluc is used as the reporter, and Fluc or Nuc as the reference, respectively. (B) Combinations of the interactions tested. The Hd3a-GF14c and GF14c-GF14c interaction was examined. GUS was used as a negative control. Hd3a m2; Hd3a R64K/R132K. GF14c-ΔN; 26–257 amino acid region of GF14c. Reporter activity of Nluc (C) and Fluc (D). RLU values from reporter constructs were normalized with that from the reference constructs. The normalized RLU value of #4 was set at 1 and the ratios are represented in relation to this. Fold activation is also represented. For comparison of signal intensities, reporter RLU value of #4 was calculated to be that per second per sample and is shown in the inset. Three independent assays were performed and the averages are shown with their standard error (SE).
Development of PPI assay using NLuc in living rice protoplasts

We performed the SLC assay and evaluated the signal intensity of Nluc by testing the interaction between Hd3a and GF14c (Figure 2). To perform the SLC assay with Nluc, we modified a commercially available split Nluc vector, NanoBiT, to be compatible with experiments in plants. To this end, we made Gateway-compatible expression vectors, in which two split Nluc fragments (LgBiT and SmBiT) are expressed as fusions with proteins of interest under the control of the CaMV 35S promoter (Supplementary Figure S1, Figure 2A, B). First, we screened for the optimal buffer in which to detect Nluc in live cells. After p35S: Nluc vector was transfected and incubated overnight, the transfected protoplasts were resuspended in the five different buffers including W5 and WI buffer, which are usually used for incubation of transfected protoplasts, and Nluc activity was measured in live cells (Supplementary Figure S3A). Among the buffers tested, a 0.4 M mannitol solution showed the highest luminescence (Supplementary Figure S3A). KCl or MES, which are both present in the W5 and WI buffers, may have affected the Nluc bioluminescent reaction. The pH of the media in the NanoBiT assay using animal cells is usually around 7. Therefore, we examined the effect of pH in 0.4 M mannitol with Tris or PBS buffers. However, neither Tris nor PBS improved NanoBiT luminescence (Supplementary Figure S3A). Therefore, we adopted 0.4 M mannitol as a suspension buffer for the NanoBiT assay in rice protoplasts. In the NanoBiT assay manufacturer's technical manual, the testing of all possible combinations of the orientation of the LgBiT and SmBiT fusions are recommended to determine the optimal Nluc activity. Therefore, we tested all eight possible combinations to examine the Hd3a-GF14c interaction (Supplementary Figure S3B). Among them, only three combinations (Hd3a-LgBiT/GF14c-SmBiT, Hd3a-SmBiT/GF14c-LgBiT, and SmBiT-Hd3a/GF14c-LgBiT) showed stronger NanoBiT luminescence (Supplementary Figure S3B). This is consistent with previous reports that the C-terminal fusion of GFP to FT (FT-GFP) has been widely used for functional analysis of florigen and that the 14-3-3 family makes homo- and hetero-dimers through their N-terminal dimerization domain (Obsil and Obsilova 2011). In our experiment, the combination of Hd3a-SmBiT/GF14c-LgBiT was chosen because this tag orientation was not expected to constrain protein function (as discussed below). The NanoBiT signal is stable for at least 2 h in animal cells, according to the manufacturer's instructions. We...
estimated the stability of the NanoBiT signal in rice protoplasts (Supplementary Figure S3C). The change in NanoBiT luminescence between Hd3a and GF14c as shown in Supplementary Figure S3B was measured for 6 min and their RLU values were categorized into three classes according to the intensity: H (10,001–100,000), M (1,001–10,000), and L (1–1,000). The NanoBiT signal rapidly increased just after addition of the NanoBiT substrate, reaching a maximum at 2–5 min, and then started to gradually decrease from 5 min (Supplementary Figure S3C). The average times to reach the maximum signal were 178 s (L), 204 s (M), and 186 s (H), which were not significantly different (p = 0.75 by one-way analysis of variance) (Supplementary Figure S3D). In our NanoBiT analysis, NanoBiT activity was measured until 6 min after addition of substrate. However, it might be more convenient to measure the activity at a defined time point, e.g., at 3 min after substrate addition under our conditions. The rationale behind this is that the relative luminescence from 2 to 5 min was largely constant at a ratio of over 0.9 and that the average time of the L, M, and H classes to reach their maximum was 187.1 s (Supplementary Figure S3D). These parameters, however, should be optimized depending on the experimental conditions.

Under these conditions, we performed NanoBiT assays to test the interaction between Hd3a and GF14c and between GF14c and between GF14c (Figure 2C). The signals between Hd3a-SmBiT and GF14c-LgBiT and between GF14c- SmBiT and GF14c-LgBiT were 59 and 6.6 × 10⁻²-fold higher than that of GUS negative control, respectively (Figure 2C). These activations were about 10-fold higher than that in the two-hybrid assay in Figure 1C. Hd3a-GF14c and GF14c-GF14c interactions are predominantly observed in the cytosol (Purwestri et al. 2009). Whereas two-hybrid assays require the nuclear localization of the test proteins, SLC assays do not. Therefore, this difference could be attributed to the test proteins’ preference of intracellular localization. Although the proteins of interest in the two-hybrid assay are forced to translocate into the nucleus by fusion with a strong nuclear localization signal (NLS), the efficiency of translocation would be dependent on how strongly the proteins are retained in the cytosol. Minimal constraints on intracellular localization and strong sensitivity are advantages of NanoBiT assay. Disadvantages of the NanoBiT assay are firstly that the assay is currently only successful in the protoplasts under our conditions, probably due to insufficient permeability of the substrate. Secondly, the NanoBiT signal was only stable for a few minutes. This would make it difficult to perform high-throughput analysis or kinetic analysis over a longer period. Thirdly, the background activity was slightly higher than in two-hybrid assays. Figure 2C shows that the GUS negative controls worked well with negligible activity detected, whereas the mutant forms of Hd3a and GF14c in the NanoBiT assay showed slightly higher activity than that in the two-hybrid Nluc assay (Figure 1C). This might be because a transient or weak association of the proteins could be detected easily, probably due to the strong luminescence of Nluc. Therefore, careful experimental design and interpretation would be essential for mutation analysis. Fourthly, the NanoBiT samples cannot be analyzed in the cell lysate under our experimental condition. The NanoBiT interaction between Hd3a and GF14c was efficiently detected in live cells, whereas it was impaired in the cell lysate (Supplementary Figure S4B). It is probably because the NanoBiT interaction is not stable in the lysis buffer.

We also compared the NanoBiT assay with the SLC assay using Fluc by testing the Hd3a-GF14c interaction (Figure 2D). To perform the transient expression assay in protoplasts, the essential components for the SLC assay with Fluc were transferred from pDEST-GWNLUC and pDEST-GWCLUC, Agro-infiltration-based split Fluc vectors (Gehl et al. 2011), to the pBI221 plasmid (Supplementary Figure S1). However, a weak enhancement of the Fluc signal (2.1-fold higher than that of the GUS negative control) was observed only in the Hd3a-FlucNt and GF14c-FlucCt combination (Figure 2D). The weak enhancement might be due to steric hindrance from a bulky FlucNt fragment or to weaker luminescence of Fluc than that of Nluc. These results indicated that the NanoBiT assay is superior to the SLC assay with Fluc, in the light of sensitivity to PPI.

In our NanoBiT assay, the reference Fluc activity was separately measured in the cell lysate, because Fluc activity in the cell lysate was detected about 30-fold higher than in the live cell (Supplementary Figure S4A). However, the live cell method for detection of Fluc might be preferable in some cases. The live cell method was also applicable to the two-hybrid Nluc reporter assay (Supplementary Figure S4C), which could be useful.

Detection of Nluc activity in rice KI plants

Although the non-destructive detection of Nluc activity is currently difficult under our conditions, it was easily detected in cell extracts (Supplementary Figure S2). We took advantage of the strong sensitivity of Nluc to analyze the change in florigen activity in planta. To faithfully monitor florigen activity, we developed KI rice plants in which the Nluc coding region was inserted in-frame downstream of the OsMADS15 gene, a target of rice florigen (Figure 3). An established strategy for rice KI-plant development using large-scale Agrobacterium-mediated transformation with a strong positive-negative selection was employed (Terada et al. 2002, 2004). The knock-in vector p15NL was designed to generate an in-frame OsMADS15-Nluc fusion gene (M15NL) at the endogenous OsMADS15 locus by HR-mediated GT...
NanoLuc assay in rice

To obtain rice callus lines carrying \textit{M15NL}, proliferating rice calli derived from mature seeds were inoculated with \textit{Agrobacterium} harboring p15NL. After the transformation process, 288 independent candidate callus lines were selected after 4 weeks of positive-negative selection (Supplementary Table S3). To screen the HR callus lines with a correctly modified \textit{OsMADS15} gene by HR, 5′- and 3′-junction PCR analysis was conducted (Figure 3B). Seven callus lines were found to carry authentic junction fragments generated by HR (Supplementary Table S3). DNA sequencing analysis confirmed that these seven callus lines indeed carry the anticipated \textit{M15NL} gene at its original locus without any mutations (Figure 3C).

First, we examined the activation of the \textit{M15NL} gene by the FAC in the protoplasts derived from the KI rice plants (Figure 4A, B). \textit{M15NL} activity was very low in the absence of the \textit{Hd3a} and \textit{OsFD1} expression vectors (Figure 4B, Supplementary Figure S4D), whereas it was 97-fold higher when both the \textit{Hd3a} and \textit{OsFD1} expression vectors were co-expressed (Figure 4B, lane 2). This indicated that \textit{M15NL} is effectively activated by the FAC. Using this assay system, we also examined the effect of a NanoBiT tag fusion on florigen function. A LgBiT and SmBiT tag fusion to \textit{Hd3a} retained the ability to activate \textit{M15NL}, although the LgBiT tag fusion reduced the activation to 37.3% of the non-fusion control (Figure 4B, lanes 2, 4–6). Consistent with previous reports (Taoka et al. 2011), a \textit{Hd3a} m2 mutant with a NanoBiT tag fusion did not activate \textit{M15NL} (Figure 4B, lanes 7 and 8). These data suggest that the NanoBiT and Nluc tag fusion will not impair the expression and function of fused genes in plants.

To confirm the faithful activation of \textit{M15NL} under changing daylength in planta, we examined \textit{M15NL} activity in a single SAM (Figure 4C–G). \textit{M15NL}\textsuperscript{-KI} plants were grown under inductive SD or non-inductive LD conditions. In our growth conditions, at around 27 das, the SAM expanded longitudinally and developed as an early reproductive meristem with a morphology characteristic of the R1–R2 stage under SD, whereas it did not expand and stayed at a vegetative phase under LD (Figure 4D, E; Tamaki et al. 2015). After a single SAM tissue was micro-surgically dissected, it was mechanically disrupted and the luminescence in the lysate was measured. The \textit{M15NL} signal was 2.1×10\textsuperscript{3} higher from SAM grown under SD than from under LD (Figure 4F, #2, #4). The signal intensity of #4 was comparable to those of non-KI plants (Figure 4F, #1, #3). This indicated that the \textit{M15NL} signal is kept close to a BG level under LD, which is consistent with a previous report (Tamaki et al. 2015). The SD-upregulation of \textit{M15NL} in the SAM was confirmed in three other independent KI lines (Figure 4G). The flowering time and plant growth were not significantly affected by Nluc KI (Figure 4H, I). Prior to the establishment of \textit{M15NL-KI} rice plants, we
also made OsMADS15-Fluc KI rice plants, to monitor florigen activity (Supplementary Figure S5). However, the activation by Hd3a and OsFD1 in protoplasts was too weak (1.3-fold activation) (Supplementary Figure S5D). It might be due to weak luminescence activity of OsMADS15-Fluc fusion protein. Alternatively, it might be because the knock-in of Fluc affected the efficiency of transcription or post-transcriptional control of OsMADS15-Fluc. These data demonstrate that the combination of Nluc and KI is a powerful tool for gene expression analysis in plants, especially when examining it in a tiny tissue such as the SAM.

The experimental scheme of this study is summarized in Supplementary Figure S6. Protoplasts are prepared from rice Oc suspension cells; they can also be prepared from leaves or suspension cells of other plant species (Satoh et al. 2004; Wu et al. 2009; Yoo et al. 2007). Transfection of plasmids was performed with a PEG-mediated method; it would also be feasible in protoplasts, cell suspensions, or whole plants, with electroporation or the particle bombardment method (Matsuo et al. 2001; Muranaka et al. 2013; Satoh et al. 2004). An advantage of the lysis assay is that the tissue samples can be stored at −80°C for later analysis. Recently, a dual Fluc and Nluc detection kit became commercially available, making the assays easier. In these Nluc assays, we used furimazine, a
substrate optimized for Nluc (Hall et al. 2012). Recently, a furimazine analog, coelenterazine \( h \), has been reported to detect Nluc activity in living cells (Wang et al. 2020). Because coelenterazine \( h \) is not an optimal substrate for Nluc (Hall et al. 2012), its permeability into plant cells might be superior to that of furimazine, despite its inferiority as a Nluc substrate in vitro. Whether coelenterazine \( h \) is practically applicable to live cells or tissues of rice remains to be examined. We developed rice M15NL-KI plants to monitor florigen activity. However, once a relevant promoter region has been identified, the use of conventional promoter-Nluc reporter genes in transient assays or in transgenic plants will facilitate the analysis.

In summary, we established a protocol and vector set for PPI and reporter assays with Nluc in rice. The two-hybrid assay is convenient, effective, and sensitive, although the intracellular localization and transcriptional activation activity of the test proteins should be taken into consideration. The NanoBiT assay is tedious but is also sensitive and will be useful for analysis of PPI kinetics. Gene targeting with Nluc created an excellent reporter line to monitor gene activity from a small tissue kinetics. Gene targeting with Nluc created an excellent reporter line to monitor gene activity from a small tissue.

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