Firefly Luciferase Complementation Imaging Assay for Protein-Protein Interactions in Plants

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The development of sensitive and versatile techniques to detect protein-protein interactions in vivo is important for understanding protein functions. The previously described techniques, fluorescence resonance energy transfer and bimolecular fluorescence complementation, which are used widely for protein-protein interaction studies in plants, require extensive instrumentation. To facilitate protein-protein interaction studies in plants, we adopted the luciferase complementation imaging assay. The amino-terminal and carboxyl-terminal halves of the firefly luciferase reconstitute active luciferase enzyme only when fused to two interacting proteins, and that can be visualized with a low-light imaging system. A series of plasmid constructs were made to enable the transient expression of fusion proteins or generation of stable transgenic plants. We tested nine pairs of proteins known to interact in plants, including Pseudomonas syringae bacterial effector proteins and their protein targets in the plant, proteins of the SKP1-Cullin-F-box protein E3 ligase complex, the HSP90 chaperone complex, components of disease resistance protein complex, and transcription factors. In each case, strong luciferase complementation was observed for positive interactions. Mutants that are known to compromise protein-protein interactions showed little or much reduced luciferase activity. Thus, the assay is simple, reliable, and quantitative in detection of protein-protein interactions in plants.
and phototoxicity caused by the external light source for excitation of fluorescence also restrict the application of the reporter-based assays in plants (Dixit et al., 2006).

Alternative reporter-based methods for protein-protein interactions have been developed using protein fragment complementation coupled with enzymatic assays. For example, expression of β-galactosidase fragments fused to interacting proteins reconstitutes the enzymatic activity in Escherichia coli (Rossi et al., 1997). Similarly, 1-β-lactamase has been used to detect protein-protein interactions in mammalian cells (Galarneau et al., 2002). Protein fragment complementation based on the reconstitution of murine dihydrofolate reductase (Remy and Michnick, 1999) was used to detect NPR1-TGA2 interaction in plants (Subramaniam et al., 2001). These assays typically require the addition of fluorescence-generating substrates and thus also suffer from the pitfalls of FRET and BiFC. Recently, an improved firefly luciferase complementation imaging (LCI) assay was developed for protein-protein interactions in animals (Luker et al., 2004). The firefly luciferase (LUC) enzyme is divided into the N- and C-terminal halves that do not spontaneously reassemble and function. LUC activity occurs only when the two fused proteins interact, resulting in reconstituted LUC enzyme, which can be detected by luminometer or a low-light imaging device. The assay measures dynamic changes in protein-protein interactions and can be used for both cell culture and whole animals. Because the luminescence was measured in the dark and is not affected by autofluorescence, LCI is particularly attractive for plant studies. A very recent report successfully used Renilla reniformis LUC complementation assay to detect interactions of two pairs of plant proteins in protoplasts (Fujikawa and Kato, 2007). The utility of the firefly LCI in plant protein-protein interaction studies remains to be tested.

In this study, we developed a series of constructs and comprehensively tested the utility of firefly LUC-based LCI in plants. Tests with nine pairs of proteins that are known to interact with different strength in the plant cell showed that the firefly LUC-based LCI assay is suitable for detecting protein-protein interactions in both protoplasts and intact leaves. The assay is simple, quantitative, highly sensitive, and can be used for transient expression or stable transgenic expression of the interacting proteins. The system provides a new tool for plant protein-protein interaction studies.

RESULTS

Constructs for LCI Assays

The firefly LUC fragments 2-416 (NLuc) and 398-550 (CLuc) were successfully used for protein-protein interaction assays in the mammalian system (Luker et al., 2004). These two fragments roughly correspond to the independently folded N-terminal and C-terminal domains that are linked by a disordered flexible region (Conti et al., 1996). To test the utility of LCI in plants, the NLuc and CLuc fragments were inserted into an expression cassette between the cauliflower mosaic virus 35S promoter and Rubisco small subunit terminator to form 35S::NLuc and 35S::CLuc, respectively (Fig. 1). A Gly/Ser linker between the LUC fragments and the multiple cloning sites was retained in the constructs to allow molecular mobility at the junction of the fusion proteins. Two sets of constructs were made for LCI assays. The first set was made in a pUC19-based plasmid designed for transfection of protoplasts or particle bombardment into plant tissues. The second set was produced in pCAMBIA-based plasmid for generation of stable transgenic plants or Agrobacterium-mediated transient expression. Multiple cloning sites were inserted N terminus to the NLuc fragment and C terminus to the CLuc fragment. We selected nine pairs of proteins that are known to interact with different strength and possess a range of biochemical functions in the plant cell.

Interaction between Bacterial Effectors and Host Proteins

Bacterial pathogens inject effector proteins into the host cells to regulate host susceptibility/resistance to the bacterium (Chisholm et al., 2006; Nomura et al., 2006). We previously showed that the Pseudomonas syringae effector protein AvrB targets Arabidopsis (Arabidopsis thaliana) protein RAR1 to promote virulence (Shang et al., 2006). To test if such an interaction can be detected with the LCI assay, CLuc-AvrB was co-expressed with RAR1-NLuc in protoplasts. For negative

Figure 1. Constructs for LCI assays in plants. A, Schematic diagrams of 35S::NLuc and 35S::CLuc constructs. L, Gly/Ser linker between LUC fragments and multiple cloning sites (MCS). rbs, Transcription terminator derived from the Rubisco small subunit gene. B, Diagram for LUC complementation resulting from NLuc- and CLuc-fusion proteins. [See online article for color version of this figure.]
controls, we included SCaBP8 that functions in salinity tolerance (Quan et al., 2007). As shown in Figure 2A, CLuc-AvrB and RAR1-NLuct coexpression led to strong LUC activity in the protoplasts that can be readily detected with a low-light imaging system after the addition of luciferin, the substrate for firefly LUC. In contrast, RAR1-NLuct coexpressed with SCaBP8 construct showed only background level LUC activity. The rar1-29 mutant carries a single amino acid substitution that specifically disrupts its interaction with SGT1b (Shang et al., 2006). This mutant showed normal interaction with AvrB (Fig. 2A). To determine if the observed LUC activity was caused by different levels of proteins expressed in the protoplasts, we examined respective NLuc and CLuc fusion proteins by western blot. The CLuc-SCaBP8 protein was expressed at a level similar to CLuc-AvrB, and RAR1-NLuct protein was expressed at a similar level in all samples. The results indicate that the strong LUC activity was not caused by higher levels of CLuc-AvrB and RAR1-NLuct proteins expressed in the cell but resulted from a specific interaction between RAR1 and AvrB.

The P. syringae effector AvrPto interacts with the tomato (Solanum lycopersicum) Ser/Thr protein kinase Pto (Tang et al., 1996); the latter subsequently triggers resistance through the association with the N terminus of the resistance protein Prf (NPrf) but not the C terminus of Prf (CPrf; Mucyn et al., 2006). The interaction between AvrPto and Pto, however, has never, to our knowledge, been demonstrated in vivo. We tested if LCI can be used to detect such an interaction. Figure 2B shows that coexpression of Pto-NLuct with CLuc-NPrf, but not CLuc-SCaBP8 or CLuc-CPrf, resulted in strong LUC activity. CLuc-NPrf accumulated to a level approximately 8-fold higher than CLuc-CPrf. However, the reconstituted LUC activity of Pto-NPrf combination was approximately 60-fold greater than the Pto-CPrf combination. The results indicate that the Pto-NPrf interaction resulted in a significant increase in LUC activity, confirming previous co-immunoprecipitation results (Mucyn et al., 2006). Similarly, coexpression of CLuc-AvrPto with Pto-NLuct in Arabidopsis protoplasts resulted in strong complementation of LUC activity. We recently showed that AvrPto<sup>Y89</sup> makes direct contact with Pto, and the AvrPto<sup>Y89D</sup> mutation abolishes the interaction in vitro (Xing et al., 2007). Coexpression of CLuc-AvrPto<sup>Y89</sup> with Pto-NLuct failed to show LUC complementation, although the mutant and wild-type CLuc-AvrPto proteins accumulated to the same level in the plant cell (Fig. 2C), indicating that the interaction detected by LCI is highly specific.

Figure 2. Interactions of P. syringae effectors with host proteins in protoplasts. A, Interaction between AvrB and RAR1. B, Interaction between Pto and the N terminus of Prf. C, Interaction between AvrPto and Pto. The top panels show quantification of LUC activity. Different letters above the bars indicate statistic difference at P < 0.01 (t test). The images in the middle show microtiter plates containing protoplasts expressing the indicated constructs. The pseudocolor bar below shows the range of luminescence intensity in each image. The bottom panels show western blot for proteins isolated from protoplasts. Anti-full-length firefly LUC antibodies or the indicated specific antibodies (anti-RAR1; Shang et al., 2006; anti-CLuc antibodies; Sigma) were used to detect the indicated fusion proteins. The amount of protein loaded in each lane is indicated by Ponceau S staining of Rubisco on a representative protein blot. The data shown are representative of three independent experiments. [See online article for color version of this figure.]
Interaction of the HSP90 Complex Components

The HSP90 protein complex plays an important role in plant innate immunity. HSP90 and its co-chaperones, SGT1 and RAR1, interact with each other, and all three components are required for disease resistance. Arabidopsis contains two SGT1 genes, SGT1a and SGT1b, both functions in stabilizing disease resistance proteins (Shirasu et al., 1999; Azevedo et al., 2002; Takahashi et al., 2003). The RAR1 protein contains a CHORD I (Cys and His-rich) domain, a central Cys-rich domain, and a CHORD II domain. CHORD I is required for interaction with HSP90, whereas the CHORD II domain is required for interaction with SGT1. We showed previously that the rar1-29 allele was compromised in the interaction with SGT1b in the yeast two-hybrid assay (Shang et al., 2006). Figure 3A shows that the LCI assay in protoplasts detected a specific interaction of RAR1 with SGT1b, but not ScarBP8. The rar1-29 mutant protein accumulated to a similar level as the wild-type RAR1 but displayed much weaker interaction with SGT1b. Similarly, we tested the interaction of RAR1 with SGT1a and HSP90. As shown in Figure 3, B and C, the full-length RAR1 was capable of interacting with both SGT1a and HSP90. The CLuc-CHORD I and CLuc-CHORD II domain fusion proteins accumulated to a similar level as the full-length CLuc-RAR1 protein, but showed only a background level LUC activity when coexpressed with SGT1a-NLuc and HSP90-NLuc, respectively. These results are consistent with the respective roles of CHORD I and CHORD II domains in the HSP90 complex.

Protein-Protein Interactions between WRKY Proteins

Transcription factors WRKY18, WRKY40, and WRKY60 play an important role in regulating plant immunity. Interestingly, these transcription factors are able to form homo- or heterodimers, and this interaction is the basis for a complex regulation of downstream gene expression (Xu et al., 2006). We tested the utility of LCI for the interaction between WRKY40 and WRKY18. As shown in Figure 4, coexpression of CLuc-WRKY18 and WRKY40-NLuc in protoplasts strongly complemented the LUC activity compared to the negative control protoplasts coexpressing CLuc-ScarBP8 and WRKY40-NLuc. The Leu zipper motif of these WRKYs is required for the dimerization. Deletion of this motif (CLuc-WRKY18D) significantly reduced the LUC complementation with WRKY40-NLuc, even though 2- to 3-fold more CLuc-WRKY18D protein was expressed in these protoplasts. These results indicate that the LCI assay is also useful for studying interactions among transcription factors.

Interactions between SKP1-Cullin-F-Box Protein E3 Ubiquitin Ligase Complex Components

The SCF (SKP1-Cullin-F-box protein) complex is an E3 ubiquitin ligase regulating 26S proteasome-dependent

![Figure 3](image-url)
degradation of a variety of proteins and is central to plant development and responses to the environment (Callis and Vierstra, 2000). SKP1 directly interacts with the F-box proteins; the latter serve to recruit specific substrate proteins for degradation. F-box proteins form a super family with members such as COI1 that regulates jasmonate signaling and EBF1 and EBF2 that regulate ethylene signaling (Xu et al., 2002; Guo and Ecker, 2003; Potuschak et al., 2003). In Arabidopsis, SKP1 is encoded by ASK1 and ASK2. Both ASK1 and ASK2 interact with COI1 to regulate jasmonate signaling (Xu et al., 2002). We tested if LCI could be used to detect the ASK1-COI1 interaction. Coexpression of CLuc-COI1 and ASK1-NLuc in protoplasts resulted in strong LUC activity (Fig. 5). Cotransfection of an empty CLuc plasmid with ASK1-NLuc showed a much weaker activity that was approximately 14% of protoplasts coexpressing CLuc-COI1 and ASK1-NLuc, suggesting a specific interaction between ASK1 and COI1 in plant cells.

EBF1 and EBF2 directly interact with their substrate protein EIN3 (Guo and Ecker, 2003; Potuschak et al., 2003), a transcription factor, to regulate gene expression. Because this interaction leads to the degradation of EIN3, such an interaction in vivo remains to be demonstrated. To test if LCI was capable of detecting EBF1-EIN3 interaction in the plant cell, we used a truncated EBF1 containing the Leu-rich repeat domain required for substrate binding but lacking the F-box domain. Coexpression of the CLuc-EBF1 with EIN3-NLuc resulted in strong LUC activity in protoplasts, whereas the coexpression of CLuc-EBF1 with SCaBP-NLuc showed only background level activity, indicating that the interaction of an F-box protein with its substrate can be successfully detected by LCI (Supplemental Fig. S1). Although the EBF1-NLuc fusion protein was not detected by western blot, preventing a quantitative assessment of the protein-protein interaction, the results are nevertheless consistent with previous yeast two-hybrid data (Guo and Ecker, 2003; Potuschak et al., 2003).

Comparison of Reconstituted and Full-Length Firefly LUC Activity

We compared protoplasts coexpressing SGT1a-NLuc and CLuc-RAR1 with those transfected with a 35S::LUC (full-length) construct (Supplemental Fig. S2).

Figure 4. Interaction between WRKY40 and WRKY18. The WRKY18D construct lacks the Leu zipper motif. The data shown are representative of three independent experiments. [See online article for color version of this figure.]

Figure 5. Interactions between ASK1 and COI1. The data shown are representative of four independent experiments. [See online article for color version of this figure.]
The latter showed approximately 30-fold stronger luminescence. The simple calculation based on cell number would be such that the reconstituted LUC possesses approximately 3% of the native LUC activity. However, the SGT1a-NLuc accumulated to only approximately 10% of the full-length LUC protein, whereas the CLuc-RAR1 protein was accumulated to a level similar to the full-length LUC. Because the expression of CLuc-RAR1 alone never resulted in significant luminescence in numerous tests (less than five counts), the vast majority of CLuc-RAR1 is unlikely to function in the absence of SGT1a-NLuc (Luker et al., 2004). Therefore, our adjusted estimate of the reconstituted LUC activity is approximately 30% of that of the full-length protein.

Agrobacterium-Mediated Transient Expression for LCI Assays

*Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* provides a convenient system for the rapid analysis of protein functions in plants. We therefore tested if *Agrobacterium*-mediated transient expression could be adopted for the LCI assay. *Agrobacterium* strains carrying CLuc and NLuc constructs were simply mixed, infiltrated into leaves of *N. benthamiana*, and the infiltrated leaves were covered with plastic for 2 d to maintain humidity. Leaves coexpressing different constructs were then examined for LUC activity. CLuc-RAR1 was tested for interactions with SGT1a-NLuc. Figure 6 shows that the expression of SGT1a-NLuc and the empty 35S::CLuc vector or CLuc-RAR1 construct and the empty 35S::NLuc vector did not show LUC complementation, whereas co-infiltration of *Agrobacterium* containing CLuc-RAR1 plus SGT1a-NLuc resulted in strong LUC complementation. The LUC activity was approximately 10-fold greater than the empty vector controls and 7-fold greater than the negative control expressing SGT1-NLuc and CLuc-CHORD I, indicating a specific interaction. Notably, the *Agrobacterium*-based LCI assay was more sensitive and had very low background. We also determined the time course for LUC complementation following the coexpression of SGT1a-NLuc and CLuc-RAR1. Maximum LUC activity was detected 4 to 6 d after infiltration of *Agrobacterium* containing SGT1a-NLuc and CLuc-RAR1, whereas leaves expressing SGT1a-NLuc and the negative control construct CLuc-CHORD I had only negligible LUC activity (Fig. 7, A and B). Western blot showed that maximum protein accumulation occurred between 4 to 6 d after infiltration (Fig. 7C), indicating that the LUC activity is correlated with the SGT1a-NLuc and CLuc-RAR1 protein level in the leaves. The CLuc-CHORD I and CLuc-RAR1 proteins were expressed at a comparable level, indicating that the difference in LUC activity was not caused by different amounts of proteins accumulated in the leaves. Similarly, *Agrobacterium*-based LCI assay detected specific interaction between SGT1b-NLuc and CLuc-RAR1 (Supplemental Fig. S3). Although the accumulation of SGT1b-NLuc in leaves was too low to be detected by western blot, all three negative controls showed only a background level of luminescence that was at least 15-fold less than leaf panels expressing SGT1b-NLuc and CLuc-RAR1.

**DISCUSSION**

In this study, we explored the utility of LCI for protein-protein interaction studies in plants. Using
protoplast- and Agrobacterium-based transient expression, we tested the interactions for nine protein pairs in plants, including components of the SCF E3 ubiquitin ligase complex, HSP90 chaperon complex, bacterial effector-plant resistance protein complex, and transcription factors. The tested proteins possess a variety of biochemical functions, and the strength of interactions varies considerably from protein to protein. We observed expected LUC complementation for all proteins tested. Importantly, we included strict negative controls for protein-protein interactions, including unrelated proteins and/or mutant proteins that are specifically compromised in protein-protein interactions. Whenever possible, the protein level was determined except for one protein pair. These allowed critical assessment of the detected LUC complementation, indicating that LCI is well suited for plant protein-protein interaction studies. In previous studies in animal systems, the interacting proteins have been successfully positioned to both the N-terminal and C-terminal ends of the fusion construct to achieve complementation (Luker et al., 2004; Paulmurugan and Gambhir, 2005), suggesting that the LUC reporter is sufficiently flexible for different construction strategy.

Nonspecific interactions are an inherent problem associated with all protein-protein interaction assays. In our protoplast-based LCI assays, several negative controls showed a certain level of background signal. It is possible that the two halves of firefly LUC are capable of association when present at a high concentration. Nevertheless, the nonspecific LUC activity, as determined by using mutant or truncated proteins that are known to interfere with protein-protein interactions, was significantly lower than the positive interactions, indicating that nonspecific interaction does not impede the proper determination of true interactions. The specificity of interactions was further enhanced when Agrobacterium-mediated transient expression was used for LCI. LUC activity resulting from specific RAR1-SGT1 interactions was 7 to 15 times greater than the negative control (SGT1-NLuc and CHORD I-CLuc) in the Agrobacterium-based LCI assay, indicating that the Agrobacterium-based transient expression is particularly suited for protein-protein interaction studies in plants.

Among the methods measuring protein-protein interactions, the yeast two-hybrid method is most widely used because of the ease of the assay and suitability for large-scale screening. However, the protein-protein interactions are studied in a heterologous system that is prone to false positives. It is not uncommon that the interaction of two proteins occurs in the presence of additional proteins or cellular factors. The lack of these factors in yeast also contributes to false-negatives in yeast two-hybrid assays. Like FRET and BiFC, LCI detects protein-protein in the native physiological environment and is thus relevant to biological problems under investigation. Unlike FRET and BiFC, the current LCI technology does not provide information concerning the subcellular location of the interaction (Fujikawa and Kato, 2007), a caveat that can be addressed by protein colocalization analysis.

Figure 7. Time course of SGT1a-RAR1 interaction in N. benthamiana. A, LUC image of N. benthamiana leaves co-infiltrated with the agrobacterial strains containing SGT1a-NLuc and Cluc-RAR1. B, Quantification of LUC activity in leaves expressing SGT1a-NLuc and Cluc-RAR1. C, Accumulation of fusion proteins in N. benthamiana leaves. Data were collected at the indicated days post infiltration of Agrobacterium. Mock, Leaves infiltrated with water. Arrows indicate infiltrated regions. The data shown are representative of five independent experiments.
The LCI assays described in this study have several advantages over FRET and BiFC assays. First, LCI assays are highly quantitative, which allows linear measurement of luminescence signals over the range of several orders of magnitude. Second, compared to FRET and BiFC, LCI samples the entire tissue or cell population, avoiding bias derived from individual cells. Third, FRET and BiFC assays are complicated by autofluorescence generated by chlorophyll and cell wall. In contrast, LCI measures luminescence at dark and is not affected by the chlorophyll- and cell wall-generated autofluorescence. In addition, LCI can be used to study protein-protein interactions at the organismal level (Luker et al., 2004), and the technique is very useful for studying tissue-specific protein-protein interactions. Finally, LCI does not require the use of a microscope, and data can be collected within 2 min by using a low-light imaging system. This is particularly attractive for studying the dynamics of protein-protein interactions. The assay can also be done with a luminometer, so that a large number of protein pairs can be tested simultaneously. The Agrobacterium-mediated LCI assay requires minimum sample handling and laboratory training. This system enables simultaneous testing of multiple protein pairs with little effort. The ability to simultaneously examine a large number of interacting proteins is a prerequisite of protein interaction construction. Currenty, this is done primarily by using the yeast two-hybrid assays and informatic tools (Piehler, 2005). The availability of LCI as a simple tool for plant protein-protein interaction studies will facilitate the validation of protein interactome data collected from the yeast two-hybrid assays.

**MATERIALS AND METHODS**

**Plants**

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants and *Nicotiana benthamiana* plants were grown in a controlled growth room at 24°C/20°C day/night with 12 h/d light and 70% humidity. Six-week-old Arabidopsis plants were used for protoplast isolation. Seven-week-old *N. benthamiana* plants were used for Agrobacterium-mediated transient expression. Plants were used for protoplast isolation. Seven-week-old *N. benthamiana* plants were used for Agrobacterium-mediated transient expression.

**NLuc and CLuc Constructs**

A plant gene expression cassette containing the cauliflower mosaic virus 35S promoter and rbs terminator was excised from p35S-FAST (Yijia Xia, Danforth Plant Science Center) and ligated to pUC19 at EcoRI and HindIII sites, resulting in 35S-pUC19. CLuc and NLuc were PCR amplified from CLuc-FKBP and FRB-NLuc (Luker et al., 2004) and ligated into 35S-pUC19, resulting in 35S::NLuc and 35S::CLuc plasmids. Derivative NLuc- and CLuc-fusion constructs were made by PCR amplifying the open reading frames of respective genes with primers listed in Supplemental Table S1, digested with KpnI and SalI, or KpnI and PstI, and inserted into 35S::NLuc or 35S::CLuc plasmids. For Agrobacterium-mediated transient expression in *N. benthamiana*, the expression cassette was excised from the 35S::NLuc and 35S::CLuc fusion constructs with EcoRI and HindIII and cloned into pCAMBIA1300 to form pCAMBIA-NLuc and pCAMBIA-CLuc. The constructs were mobilized into *A. tumefaciens* strain GV3101.

**Protoplast Transfection**

Protoplasts were isolated from 6-week-old ecotype Columbia plants according to Sheen (http://genetics.mgh.harvard.edu/sheenweb); $2 \times 10^5$ protoplasts were transfected with indicated constructs and incubated overnight in a 24-well microtiter plate before LUC activity was measured (Li et al., 2005).

**Agrobacterium-Mediated Transient Expression**

*Agrobacterium tumefaciens* (strain GV3101) bacteria containing indicated constructs were grown in Luria-Bertani medium at 28°C overnight, pelleted, and resuspended to 0.3 OD in induction medium according to Bundock et al. (1995). The culture was then grown in induction medium for 8 to 12 h. Bacteria were then washed once with Murashige and Skoog medium containing 10 mM MES, pH 5.6, and resuspended in Murashige and Skoog-MES medium containing 150 μM acetylspermyne to a final concentration of OD_{600} = 0.5. Bacterial suspensions were infiltrated into young but fully expanded leaves of *N. benthamiana* plants using a needleless syringe. After infiltration, plants were immediately covered with plastic bags and placed at 23°C for 48 h before bag removal. Plants were then incubated at 28°C with 16 h light/d before the LUC activity was measured.

**CCD Imaging and LUC Activity Measurement**

One milliliter luciferin was added to protoplasts or sprayed onto leaves, and the materials were kept in dark for 6 min to quench the fluorescence. A low-light cooled CCD imaging apparatus (CHEMIPROHT 1300B/LND, 16 bits; Roper Scientific) was used to capture the LUC image. The camera was cooled to −110°C and relative LUC activity was measured as described (He et al., 2004). An exposure time of 2 min with 3 × 3 binning was used for all images taken. Relative LUC activity is equivalent to luminescence intensity/200 protoplasts or luminescence intensity/0.2 mm² leaf area. Each data point consisted of at least three replicates, and three to five independent experiments were performed for each assay. *t* test was performed to determine statistic significance of differences at *P* < 0.01.

**Western Blot**

Total protein was extracted from equal amounts of protoplasts or leaves, and approximately 100 μg protein was fractionated through SDS-PAGE. Unless indicated otherwise, protein blot was hybridized with the rabbit anti-full-length firefly LUC antibodies (Sigma), which react with both the N-terminal and C-terminal firefly LUC fragments. The protein blot was detected with the ECL kit from Amersham Biosciences. Anti-RAR1 and anti-SGT1 antibodies were raised in-house as described previously (Azevedo et al., 2002).

**Supplemental Data**

The following materials are available in the online version of this article.

Supplemental Figure S1. Interaction between EBF1 and its substrate.

Supplemental Figure S2. Comparison of reconstituted and full-length LUC activities.

Supplemental Figure S3. *Agrobacterium*-based LCI assay for SGT1b-RAR1 interaction.

Supplemental Table S1. Primers used for plasmid construction.

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**LITERATURE CITED**


