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

Huamin Chen\textsuperscript{1,2}, Yan Zou\textsuperscript{2}, Yulei Shang\textsuperscript{2}, Huiqiong Lin\textsuperscript{2}, Yujing Wang\textsuperscript{2}, Run Cai\textsuperscript{1}, Xiaoyan Tang\textsuperscript{3}, and Jian-Min Zhou\textsuperscript{2}*

\textsuperscript{1} School of Agriculture and Biology, Shanghai Jiaotong University

\textsuperscript{2} National Institute of Biological Sciences, Beijing

\textsuperscript{3} Department of Plant Pathology, Kansas State University

* Corresponding author; e-mail zhoujianmin@nibs.ac.cn. fax 86-10-80726687.
Running title: Luciferase complementation for protein-protein interactions

This work was supported by a grant from Chinese Ministry of Science and Technology (2003-AA210090).

Corresponding author: Jian-Min Zhou; e-mail zhoujianmin@nibs.ac.cn, fax 86-10-80726687.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jian-Min Zhou (zhoujianmin@nibs.ac.cn).

RESEARCH CATEGORY: Breakthrough Technologies
ABSTRACT

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 (FRET) and Bimolecular Fluorescence Complementation (BiFC) that are used widely for protein-protein interaction studies in plants require extensive instrumentation. To facilitate protein-protein interactions studies in plants, we adopted the luciferase complementation imaging (LCI) 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 9 pairs of proteins known to interact in plants, including Pseudomonas syringae bacterial effector proteins and their protein targets in the plant, proteins of the SCF 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.
Noncovalent interactions among proteins are vital for all aspects of cellular processes. Thus the identification and characterization of interacting proteins are key to our understanding of protein functions. A plethora of techniques have been developed to detect protein-protein interactions in vitro and in vivo (Piehler, 2005). The most widely used among these techniques is the yeast two-hybrid assay which is ideal for large scale screening for interacting proteins and the construction of protein interactomes (Fields and Song, 1989; Li et al., 2004). However, the yeast two-hybrid assay detects protein-protein interactions under heterologous conditions, and results must be validated by assays under physiological conditions. Examination of protein-protein interactions under physiological conditions is often technically demanding and requires tedious procedures. For example, the co-immunoprecipitation assay requires specific antibodies, lengthy procedures that are influenced by parameters such as schemes for protein extraction, binding and washing, and expertise of individuals performing the experiment. Thus, the results are often variable from laboratory to laboratory. Tandem affinity purification (TAP) represents a more advanced technique primarily designed to identify new proteins in a protein complex in a native state (Puig et al., 2001; Rohila et al., 2006).

The development of reporter-based in vivo protein-protein interaction assays such as Fluorescence Resonance Energy Transfer (FRET; Heim and Tsien, 1996; Ha et al., 1996; Mahajan et al., 1998), the related technology Bioluminescence Resonance Energy Transfer (BRET; Xu et al., 1999; Subramanian et al., 2006), and Bimolecular Fluorescence Complementation (BiFC; Hu et al., 2002) assays has significantly advanced the measurement of protein-protein interactions in vivo. These assays are instrumental for a number of important discoveries in mammalian studies. The
application of FRET and BRET in plant biology, however, has encountered significant difficulties
despite of sporadic successes (Shen et al., 2007). Both assays require sophisticated microscopy
and computation. BiFC is relatively simple compared to FRET and BRET and has been used in a
number of plant protein-protein interaction studies (Walter et al., 2004; Bracha-Drori et al., 2004;
Dong et al., 2006; Quan et al., 2007). FRET and BiFC are technically challenging when a large
number of protein pairs are to be tested. Furthermore, the application of FRET and BiFC assays in
plants is complicated by the autofluorescence generated by cell wall, chloroplast and other cell
structures. Finally, photobleaching 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 E.
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 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 enzyme is divided into the N- and C-terminal halves that do not spontaneously
reassemble and function. Luciferase activity occurs only when the two fused proteins interact, resulting in reconstituted luciferase 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* luciferase complementation assay to detect interactions of two pairs of plant proteins in protoplasts (Fujikawa and Kato, 2007). The utility of the firefly luciferase complementation imaging 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 luciferase-based LCI in plants. Tests with 9 pairs of proteins that are known to interact with different strength in the plant cell showed that the firefly luciferase-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 luciferase 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 CaMV 35S promoter and NOS terminator to form 35S::NLuc and 35S::CLuc, respectively (Figure 1). A Gly/Ser linker between the LUC fragments and the multiple cloning sites (Luker et al., 2004) 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 9 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 (Nomura et al., 2006; Chisholm et al., 2006). We previously showed that the *Pseudomonas syringae* effector protein AvrB targets Arabidopsis 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 controls, we included SCaBP8 that functions in salinity tolerance (Quan et al., 2007). As
shown in Figure 2A, CLuc-AvrB and RAR1-NLuc co-expression led to strong luciferase activity in the protoplasts that can be readily detected with a low light imaging system after the addition of luciferin, the substrate for firefly luciferase. In contrast, RAR1-NLuc co-expressed with SCaBP8 construct showed only background level luciferase 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 (Figure 2A). To determine if the observed luciferase 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-NLuc protein was expressed at a similar level in all samples. The results indicate that the strong luciferase activity was not caused by higher levels of CLuc-AvrB and RAR1-NLuc proteins expressed in the cell but resulted from a specific interaction between RAR1 and AvrB.

The *P. syringae* effector AvrPto interacts with the tomato serine/threonine 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 been demonstrated *in vivo*. We tested if LCI can be used to detect such an interaction. Figure 2B shows that co-expression of Pto-NLuc with CLuc-NPrf, but not CLuc-SCaBP8 or CLuc-CPrf, resulted strong LUC activity. CLuc-NPrf was accumulated to a level ~8 fold higher than CLuc-CPrf. However, the reconstituted luciferase activity of Pto-NPrf combination was ~60 fold greater than the Pto-CPrf combination. The results indicate that the Pto-NPrf interaction resulted in significant increase in luciferase
activity, confirming previous co-immunoprecipitation results (Mucyn et al., 2006). Similarly, co-expression of CLuc-AvrPto with Pto-Nluc in Arabidopsis protoplasts resulted in strong complementation of luciferase activity. We recently showed that AvrPtoY89 makes direct contact with Pto, and the AvrPtoY89D mutation abolishes the interaction in vitro (Xing et al., 2007). Co-expression of CLuc-AvrPtoY89D with Pto-NLuc failed to show luciferase complementation although the mutant and wild-type CLuc-AvrPto proteins accumulated to the same level in the plant cell (Figure 2C), indicating that the interaction detected by LCI is highly specific.

**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 domain (a cysteine and histidine-rich domain), a CC domain (a central cysteine-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 ScaBP8. The rar1-29 mutant protein accumulated to the 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 3B and 3C, 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 luciferase activity when co-expressed 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 down-stream gene expression (Xu et al., 2006). We tested the utility of LCI for the interaction between WRKY40 and WRKY18. As shown in Figure 4, co-expression of CLuc-WRKY18 and WRKY40-NLuc in protoplasts strongly complemented the luciferase activity compared to the negative control protoplasts co-expressing CLuc-SCaBP8 and WRKY40-NLuc. The leucine zipper motif of these WRKYs is required for the dimerization. Deletion of this motif (CLuc-WRKY18D) significantly reduced the luciferase complementation with WRKY40-NLuc, even though 2-3 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 SCF E3 Ubiquitin Ligase Complex Components**

The SCF (SKP1-Cullin-F-box protein) complex is an E3 ubiquitin ligase regulating 26S proteasome-dependent 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. Co-expression of CLuc-COI1 and ASK1-NLuc in protoplasts resulted in strong luciferase activity (Figure 5). Co-transfection of an empty CLuc plasmid with ASK1-NLuc showed a much weaker activity that was approximately 14% of protoplasts co-expressing 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 leucine-rich repeat (LRR) domain required for substrate-binding but lacking the F-box domain. Co-expression of the CLuc-EBF1 with EIN3-NLuc resulted in strong luciferase activity in protoplasts, whereas the co-expression 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 (Figure 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;
Comparison of Reconstituted and Full-Length Firefly Luciferase Activity

We compared protoplasts co-expressing SGT1a-NLuc and CLuc-RAR1 with those transfected with a 35S::LUC (full-length) construct (Figure S2). The latter showed ~30 fold stronger luminescence. The simple calculation based on cell number would be such that the reconstituted luciferase possesses ~3% of the native luciferease activity. However, the SGT1a-NLuc accumulated to only ~10% of the full-length luciferase protein, whereas the CLuc-RAR1 protein was accumulated to a level similar to the full-length luciferase. Because the expression of CLuc-RAR1 alone never resulted in significant luminescence in numerous tests (less than 5 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 luciferase activity is ~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 by plastic for 2 days to maintain humidity. Leaves co-expressing different constructs were then examined for luciferase 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 luciferase complementation, whereas co-infiltration of Agrobacteria containing CLuc-RAR1 plus SGT1a-NLuc resulted in strong luciferase complementation. The luciferase activity was ~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 luciferase complementation following the co-expression of SGT1a-NLuc and CLuc-RAR1. Maximum LUC activity was detected 4-6 days 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 (Figures 7A and 7B). Western blot showed that maximum protein accumulation occurred between 4-6 days post-infiltration (Figure 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 luciferase 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 (Figure S3). Although the accumulation of SGT1b-NLuc in leaves was too low to be detected by western blot, all three negative controls showed only background level of luminescence that was at least 15 fold less than leaf panels expressing SGT1b-NLuc and CLuc-RAR1.

DISCCUSION
In this study we explored the utility of LCI for protein-protein interaction studies in plants. By using protoplast- and *Agrobacterium*-based transient expression, we tested the interactions for 9 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 luciferase 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 luciferase 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 N-terminal and C-terminal ends of the fusion construct to achieve complementation (Luker *et al.*, 2004; Paulmurugan and Gambhir, 2005), suggesting that the luciferase reporter is sufficiently flexible for different construction strategy.

Non-specific interactions are an inherent problem associated with all protein-protein interaction assays. In our protoplast-based LCI assays, several negative controls showed certain level of background signal. It is possible that the two halves of firefly luciferase are capable of association when present at a high concentration. Nevertheless, the non-specific luciferase 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 non-specific
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. Luciferase activity resulted from specific RAR1-SGT1 interactions were 7-15 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 co-localization analysis.

The LCI assays described in this study have several advantages over FRET and BiFC assays. First, LCI assays are highly quantitative which allow 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 microscope, and data can be collected within two minutes by using a low light imaging system. This is particularly attractive for studying the dynamic 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 test of multiple protein pairs with little effort. The ability to simultaneously examine a large number of interacting proteins is a prerequisite of protein interactome construction. Currently, 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* (Col-0) plants and *Nicotiana benthamiana* plants were grown in controlled growth room at 24°C/20°C day/night with 12 h/day 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.

**NLuc and CLuc Constructs**

A plant gene expression cassette containing the CaMV 35S promoter and rbs terminator was excised from p35S-FAST (Yiji Xia, Danforth Plant Science Center, St. Louis) 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 Table S1, digested with KpnI and SalI, or KpnI and PstI, and inserted into 35S::Nluc or 35S::Cluc plasmids.

For Agrobacteriummediate 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.

Upon request, all constructs described in this publication will be made available in a timely manner for non-commercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

**Protoplast Transfection**
Protoplasts were isolated from 6-week-old Col-0 plants according to Sheen

(http://genetics.mgh.harvard.edu/sheenweb/). 2x10^5 protoplasts were transfected with indicated constructs, incubated overnight in a 24 well microtiter plate before luciferase activity was measured.

**Agrobacterium-Mediated Transient Expression**

*Agrobacterium tumefaciens* (strain GV3101) bacteria containing indicated constructs were grown in LB 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 8-12 h. Bacteria were then washed once with MS medium containing 10 mM N-morpholino ethanesulfonic acid (MS-MES; pH5.6), and resuspended in MS-MES medium containing 150 µM acetylsyringone 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/day before the luciferase activity was measured.

**CCD Imaging and Luciferase activity Measurement**

1 mM luciferin was added to protoplasts or sprayed onto leaves, and the materials were kept in dark for 6 minutes to quench the fluorescence. A low light cooled charge-coupled device (CCD) imaging apparatus (CHEMIPROHT 1300B/LND, 16 bits, Roper Scientific, NJ) was used to capture luciferase image. The camera was cooled to -110°C and measurement of relative luciferase
activity as described (He et al., 2004). An exposure time of 2 minutes with 3x3 binning was used for all images taken. Relative LUC activity is equivalent to luminescence intensity/200 protoplasts or luminescence intensity/0.2 mm$^2$ leaf area. Each data point consisted of at least 3 replicates, and 3-5 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 $\mu$g protein was fractionated through SDS Page. Unless indicated otherwise, protein blot was hybridized with the rabbit anti- full-length firefly luciferase antibodies (Sigma, MO), which react with both the N-terminal and C-terminal firefly luciferase fragments. The protein blot was detected with the ECL kit from Amersham Biosciences (NJ). Anti-RAR1 and anti-SGT1 antibodies were raised in-house as previously described (Azevedo et al., 2002).

**ACKNOWLEDGEMENTS**

We thank David Piwnica-Worms for providing the NLuc and CLuc plasmids and Yan Guo for the CLuc-ScaBP construct. J.M.Z. was supported by a grant from Chinese Ministry of Science and Technology (2003-AA210080).
LITERATURE CITED


**Ha T, Enderle Th, Ogletree DF, Chemla DS, Selvin PR Weiss S** (1996) Probing the interaction between two single molecules: Fluorescence resonance energy transfer between a single donor and a single acceptor. Proc Natl Acad Sci USA **93**: 6264-6268.


Figure Legends

Figure 1. Constructs for LCI assays in plants.

A, Schematic diagrams of 35S::NLuc and 35S::CLuc constructs. L denotes the Gly/Ser linker between LUC fragments and multiple cloning sites (MCS). rbs indicates the transcription terminator derived from the Rbisco small subunit gene. B, Diagram for luciferase complementation resulted from an NLuc- and CLuc- fusion proteins.

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 luciferase 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 luciferase 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 a representative of 3 independent experiments.

Figure 3. Interactions among the HSP90 chaperone complex components in protoplasts.

A, Interaction between SGT1b and RAR1. B, Interaction between SGT1a and RAR1. C, Interaction between RAR1 and HSP90. CH I: CHORD I domain; CH II: CHORD II domain; HSP:
HSP90. The data shown are a representative of 3 (A and B) or 5 (C) independent experiments.

**Figure 4.** Interaction between WRKY40 and WRKY18.

The WRKY18D construct lacks the leucine zipper motif. The data shown are a representative of 3 independent experiments.

**Figure 5.** Interactions between ASK1 and COII. The data shown are a representative of 4 independent experiments.

**Figure 6.** Interactions between SGT1 and RAR1 in *N. benthamiana* leaves.

A. Luciferase image of *N. benthamiana* leaves co-infiltrated with the Agrobacterial strains containing SGT1a-NLuc and CLuc-RAR1. Arrows indicate leaf panels that were infiltrated with Agrobacteria containing the indicated constructs. B, Quantification of luciferase activity in leaves expressing SGT1a-NLuc and CLuc-RAR1. The western blot below shows the expression levels of CLuc- and NLuc- fusion proteins. RAR1 derivatives were detected by anti-firefly luciferase antibodies, whereas SGT1a-NLuc was detected by anti-SGT1 antibodies. Ponceau S staining shows equal loading of protein in lanes. Data were collected 4 days after infiltration. The data shown are a representative of 3 independent experiments.

**Figure 7.** Time course of SGT1a-RAR1 interaction in *N. benthamiana*.

A. Luciferase image of *N. benthamiana* leaves co-infiltrated with the Agrobacterial strains containing SGT1a-NLuc and CLuc-RAR1. B, Quantification of luciferase 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 H₂O. Arrows indicate infiltrated region. The data shown are a representative of 5 independent experiments.