Ligand-Dependent Reduction in the Membrane Mobility of FLAGELLIN SENSITIVE2, an Arabidopsis Receptor-Like Kinase

Gul Shad Ali, K. V. S. K. Prasad, Irene Day and A. S. N. Reddy *
Department of Biology and Program in Molecular Plant Biology, Colorado State University, Fort Collins, CO 80523, USA

Arabidopsis FLAGELLIN SENSITIVE2 (FLS2) is a transmembrane leucine-rich repeat receptor-like kinase, which recognizes a conserved 22 amino acid peptide (flg22) of bacterial flagellin and activates downstream defense signaling pathways resulting in enhanced resistance against plant pathogens. The underlying mechanisms for the activation of FLS2 in the cell membrane, however, are not fully understood. Using fluorescence recovery after photobleaching (FRAP), we demonstrate that approximately 75% of the FLS2 in the plasma membrane diffuses laterally with a diffusion coefficient of 0.34 μm²/s⁻¹, indicating that it moves rapidly. Further, we show that FLS2 is less mobile in the presence of flg22, suggesting its ligand-dependent confinement to microdomains or transient interaction with other less mobile membrane proteins. Using an in vivo bimolecular fluorescence complementation (BiFC) system and fluorescence resonance energy transfer (FRET), which reveals in vivo protein–protein interactions, we show that FLS2 does not homodimerize either constitutively or in the presence of flg22. Our data suggest that the reduced mobility of FLS2 after binding flg22 and its existence in monomeric form are important mechanistic features of FLS2 early signaling.

Keywords: BiFC — flg22 — FRAP — FRET — Membrane protein — RLK.

Abbreviations: BiFC, bimolecular fluorescence complementation; BL, BR, brassinosteroid; BR11, BRASSINOSTEROID INSENSITIVE 1; CFP, cyan fluorescent protein; D, diffusion coefficient; FLS2, FLAGELLIN SENSITIVE2; FP, fluorescent protein; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HA, hemagglutinin; LRR, leucine-rich repeat; PAMP, pathogen-associated molecular pattern; PEG, polyethylene glycol; RLK, receptor-like kinase; TLR, Toll-like receptor; YFP, yellow fluorescent protein.

Introduction

Eukaryotic plasma membrane receptor-like kinases (RLKs) play essential roles in mediating intracellular stimuli through recognizing specific ligands and inducing downstream signaling pathways that result in appropriate cellular responses. In the model plant Arabidopsis, >600 RLK genes have been identified, which differ in their potential ligand-binding domains and are accordingly grouped into 15 distinct classes (Shiu and Bleecker 2001, Shiu and Bleecker 2003). Several of these RLKs have been functionally characterized and shown to play a variety of roles in development (CLV1, ERECTA, SERK1), hormone perception [BRASSINOSTEROID INSENSITIVE 1 (BR11)], reproduction (SERK1), symbiosis (NARK), abscission (HAESA) and disease resistance [FLAGELLIN SENSITIVE2 (FLS2) and Xa21] (Becraft 2002, Torii 2004). Among the RLKs, the leucine-rich repeat (LRR)-RLKs constitute the largest group, which according to the number and organization of LRRs are classified into several subfamilies and include many well-studied members such as BR11, CLV1 and FLS2 (Torii 2004).

FLS2, an LRR-RLK, which was originally identified using Arabidopsis mutants that had lost sensitivity to bacterial flagellin and had enhanced susceptibility to pathogenic bacteria (Gomez-Gomez and Boller 2000, Bauer et al. 2001, Zipfel et al. 2004). FLS2 recognizes a highly conserved 22 amino acid peptide (flg22) in the N-terminus of flagellins of plant pathogenic bacteria (Felix et al. 1999). Later it was shown that FLS2 is essential for the flg22-mediated activation of defense mechanisms through the mitogen-activated protein kinase (MAPK) cascade (Asai et al., 2002). Recently, using chemical cross-linking and immunoprecipitation, FLS2 was shown to bind flg22 and determine the specificity of flagellin perception (Chinchilla et al. 2006). Functionally, FLS2 is similar to animal Toll-like receptor 5 (TLR5) that recognizes bacterial flagellin (Hayashi et al. 2001, Mizel et al. 2003) and belongs to a family of ancient and evolutionarily conserved membrane receptors that play important roles in the defense responses of insects and metazoans (Gomez-Gomez and Boller 2002, Akira and Takeda 2004). These receptors are demonstrated to recognize a diverse set of microbial molecules, collectively termed pathogen-associated molecular patterns ‘PAMPs’, which are absent from the host, making these receptors ideal for distinguishing self from non-self (Akira and Takeda 2004). In animals, several TLRs and their respective ligands such as lipopolysaccharides,
single-stranded RNA and flagellin have been identified. Engagement of these receptors by their ligands activates innate and adaptive immune responses, ultimately leading to protection against infections (Barton and Medzhitov 2003, Akira and Takeda 2004). The mammalian TLR5 has been shown to be essential for interaction with flagellin and for the activation of downstream signaling and defense responses (Hayashi et al. 2001, Mizel et al. 2003).

Similar to their animal counterparts, the downstream signaling pathways of FLS2–flg22 interaction have been studied at the genetic and biochemical level (Asai et al. 2002, Gomez-Gomez and Boller 2002). Recently, it was shown that FLS2 localizes to plasma membranes and also to intracellular vesicles (Robatzek et al. 2006). FLS2 was also found to undergo rapid endocytosis in a flg22-dependent manner for degradation. However, several early cellular aspects of its activation mechanism, especially on the plasma membrane, are not known. For example, the plasma membrane distribution state and a change in this state, if any, after stimulation by flg22 are not known. One model of the activation of RLKs involves oligomerization as an essential feature for the ligand-dependent activation of several animal membrane receptors, and evidence for a similar mechanism for plant RLK is also rapidly emerging (Burgess et al. 2003, Russinova et al. 2004, Johnson and Ingram 2005, Wang et al. 2005b). Recently, in two studies it was shown that activation of FLS2 by flg22 requires BAK1 (Chinchilla et al. 2007, Heese et al. 2007), another RLK which acts in brassinosteroid (BR) signaling. These studies have shown that FLS2 rapidly makes a complex with BAK1 in a flg22-dependent manner, suggesting that BAK1 may be a partner of FLS2 in PAMP signaling similarly to it being a partner of BR11 in BL (brassinolide) signaling (Russinova et al. 2004). However, it is not clear if FLS2 exists as a monomer or oligomer and if a change in its oligomerization state is essential for its activation by flg22 binding. Using fluorescence recovery after photobleaching (FRAP) analyses, which reveal in vivo kinetic properties of fluorescently labeled proteins, we show that the mobility of FLS2 is slower than would be expected if it were freely diffusing in the membrane, indicating that FLS2 is part of a pre-assembled complex. Interestingly, the mobility of FLS2 was reduced in the presence of flg22. In addition, we have investigated homodimerization of FLS2 under normal and flg22-treated conditions; we used two different in vivo approaches, a bimolecular fluorescence complementation (BiFC) system (Walter et al., 2004) and fluorescence resonance energy transfer (FRET), that reveal protein–protein interactions. Our results show that FLS2 does not form homodimers in the absence or presence of flg22 on cell surfaces, suggesting that homodimerization is not required for the activation of FLS2 signal transduction pathways.

Results

**Fluorescent protein (FP)-tagged FLS2 is functional**

Schematic diagrams of FLS2 fusion with FPs are shown in Fig. 1A. Before performing the experiments reported below, we established the functionality of FLS2–FP fusion by expressing it transiently in onion epidermal cells and the Arabidopsis mesophyll protoplasts of ecotypes Columbia (Col) and Wassilewskija (Ws-0). As expected, FLS2–yellow fluorescent protein (YFP) localized to plasma membrane in all plant cells tested, providing evidence of its correct subcellular localization. Functional FLS2 is essential for sensing the most conserved 22 amino acid flagellin peptide flg22 and activating the expression of several genes including transcription factors such as WRKY53 (Navarro et al. 2004), WRKY29 and FRK (Asai et al. 2002). Arabidopsis ecotypeWs-0 lacks a functional FLS2 gene and is non-responsive to flg22, thereby providing a genetic complementation system to test the functionality of FLS2–FP fusions (Gomez-Gomez et al. 1999). To test that the FLS2–FP fusion receptors are responsive to flg22, we co-transfected Ws-0 mesophyll protoplasts with an FLS2–FP fusion and an flg22-inducible promoter driving luciferase and treated them with flg22. Fig. 1B shows that expression of FLS2–FPs rendered Ws-0 protoplasts responsive to flg22 by activating WRKY53. Likewise, two other promoters, WRKY29 and FRK, were also activated in an FLS2 fusion protein-dependent manner (data not shown). All fusion proteins with the expected molecular mass were detected on Westerns using antibodies against the fusion tag (Fig. 1C). We also verified the expression and functionality of BiFC vectors (see below) by performing Western blot analyses with total protein from protoplasts transfected with FLS2–YFPN and FLS2–YFPC. Fig. 1C shows that bands corresponding to the expected size of fusion proteins were detected with anti-hemagglutinin (HA) and anti-c-myc antibodies, indicating that full-length fusion proteins are expressed in transfected protoplasts. Next, the functionality of FLS2–YFPN and FLS2–YFPC constructs was verified by their ability to activate the WRKY53 promoter in Ws-0 protoplasts in response to flg22. Fig. 1B (lower panel) shows that expression of both constructs led to a >10-fold increase in WRKY53 activity after flg22 treatment, which occurs only when a functional exogenous FLS2 is expressed in Ws-0 protoplasts. From these data, we conclude that the FLS2 fusion receptors are capable of perceiving and transmitting the flg22 signal and are suitable for investigating protein–protein interactions.

**Subcellular localization of FLS2–YFP in protoplasts**

Consistent with the published reports (Robatzek et al. 2006), FLS2 fused to YFP or cyan fluorescent protein (CFP) localized to plasma membranes in Arabidopsis,
In vivo dynamics of FLS2

FRAP analyses of FLS2–YFP and BRI1–YFP

The mobility of FLS2–YFP on the membrane surfaces of Arabidopsis protoplast was assessed by FRAP analysis, which yields data on biophysical parameters such as mobile/immobile fractions and the diffusion coefficient (D). The mobile fraction provides information about how much of the total available molecules are free to diffuse in the membrane environment and how much is anchored to membranes probably by forming immobile complexes. Similarly Ds and the kinetics of the recovery curves of the mobile fraction reveal information about whether a protein is freely diffusing or is restricted in its mobility by confinement to microdomains in the membrane probably by interacting with other membrane components. Fig. 2 shows a representative pictorial illustration of FRAP carried out on protoplasts expressing FLS2–YFP (Fig. 2A) and BRI1–YFP (Fig. 2B). The recovery of fluorescence in the bleached areas, indicated by arrows, over time indicates that both receptors are mobile in the plasma membrane. Under unstimulated control conditions, the mobile fractions for FLS2–YFP and BRI1–YFP were 74.6 ± 3.5% (n = 25) and 79 ± 2.4% (n = 10), respectively. This indicates that both RLKs have a substantial fraction free to diffuse in the membrane. Next we asked if treatment with flg22 would change the amount of mobile fraction, which in turn would provide us with information on whether ligand binding
Fig. 2 FRAP analyses of FLS2–YFP and BRI1–YFP. (A and B) Representative subsets of pictures taken at different time intervals from a typical FRAP experiment conducted on protoplasts expressing FLS2–YFP (A) or BRI1–YFP (B). The first and second images were taken
In vivo dynamics of FLS2

confines protein to membranes. As is shown in Fig. 2C, flg22 significantly reduced the mobile fraction from 75 to 61.9 ± 3.9%, reflecting a 14% total reduction. This effect was specific to the FLS2/flg22 receptor–ligand combination as flg22 treatment of BR1–YFP-expressing cells did not alter its mobile fraction significantly (Fig. 2D, right graph).

To exclude any general and non-specific effect of flg22 on membrane dynamics, we performed FRAP analysis of BR1 in protoplasts that were transformed with both BR1–YFP and FLS2–CFP. As is shown in Fig. 2E, these protoplasts responded to flg22 by activating WRKY53 promoter. FRAP analysis of the BR1–YFP in these protoplasts revealed no difference between flg22-treated and untreated controls (Fig. 2E). This observation also showed that the reduced mobile fraction of FLS2 after flg22 binding was not due to a non-specific effect of flg22 on the membrane environment.

Next, we quantified the recovery kinetics of mobile fractions of both receptors under flg22-stimulated and control conditions by analyzing the recovery data with an exponential equation as described in Materials and methods. A qualitative examination of the recovery curves of FLS2 clearly shows that flg22 binding slows the mobility of FLS2. We quantified the recovery kinetics by calculating D, which, for FLS2, was reduced from 0.34 μm² s⁻¹ in control protoplasts (n = 25) to 0.22 μm² s⁻¹ in flg22-treated protoplasts (n = 25) (Fig. 2C). Similar to no effect on the mobile fraction of BR1, flg22 had no appreciable effect on the D values of BR1 (Fig. 2D, E).

FLS2 does not homodimerize in either the absence or presence of flg22

To understand the immediate activation mechanisms of FLS2, we investigated homodimerization of FLS2 molecules with FRET and BiFC analyses. CFP- and YFP-tagged BR1 and FLS2 were co-expressed in a pairwise combination in Arabidopsis protoplasts and examined by confocal microscopy. All fusion proteins, as expected, co-localized on the plasma membrane (Fig. 3A–C). Overlay images show that FLS2–YFP and FLS2–CFP perfectly co-localize on the membrane. Similarly BR1–YFP and BR1–CFP, and BR1–CFP and FLS2–YFP also co-localized to plasma membranes. To quantify the level of co-localization, a scatter diagram between the CFP and YFP channel was constructed with LSM 510 software. The close distribution of data points along the diagonal regression line shows that all membrane receptors co-localized on membranes. These analyses, however, are limited to approximately 200 nm resolution and, therefore, cannot determine if these proteins interact.

To investigate the dynamics of proximity between these receptors with greater resolution, we used two different in vivo techniques, namely, BiFC and FRET. The acceptor photobleaching FRET was used to investigate the interaction of FLS2 on the cell surface. First we showed that both FP-tagged receptors were expressed in protoplasts by Western blot analyses with anti-green fluorescent protein (GFP) antibody. Bands of the expected size were detected, showing that full-length fusion proteins are expressed (Fig. 1C). These fusion receptors were also functional in activating several flg22-inducible promoters, including WRKY53 (Fig. 1B). After verifying the expression and functionality of the FP-tagged FLS2, we validated the suitability of our microscope system by performing FRET with another membrane-localized RLK, i.e. BR1, which was recently shown to exhibit FRET on the membrane surfaces of protoplasts (Russinova et al. 2004). Fig. 3D–G shows the typical outcome from an acceptor photobleaching FRET experiment. Bleaching with a 514 nm laser line led to an almost complete loss of YFP fluorescence. As was expected and is shown in Fig. 3F and H, FRET was observed for BR1 on the cell surface, demonstrating that our system is suited for analyzing FRET of FLS2 on cell surfaces. As a further control, we used the FLS2–YFP/BR1–CFP pair as a negative control and FLS2–CFP as the mock FRET control. As shown in Fig. 3H, FRET efficiencies for FLS2 under control and flg22-treated conditions were in the range of 4% (n = 30), which was not significantly different from each other or from negative or mock controls.

To verify the FRET results, we performed BiFC analyses on protoplasts co-transfected with FLS2–YFP⁶ and FLS2–YFP⁷ in control and flg22-treated protoplasts. BiFC relies on the reconstitution of fluorescent YFP when the two fragments of YFP are brought together in close proximity by two interacting proteins that are fused to each YFP fragment separately. BiFC was adopted for plant systems (Walter et al. 2004) and was recently used successfully for probing interaction of membrane-localized proteins in Arabidopsis protoplasts (Xu et al. 2006). In order to use the BiFC system for probing the interaction of FLS2 with itself, we fused full-length FLS2 to the N- and immediately before and after bleaching, respectively. The remaining two images were taken 5 and 100 s after bleaching and reveal gradual recovery of FLS2 and BR1. Bleached areas are indicated by arrows. Bars equal 10 μm. (C–E) Quantitation and kinetic analyses of the FRAP data of FLS2–YFP (C) and BR1–YFP alone (D), and BR1–YFP and FLS2–CFP (E) was accomplished by plotting normalized fluorescence data (average of 10–25 protoplasts) vs. time and fitted with an exponential equation using non-linear curve fitting as described in Materials and Methods. Each data point is the average of 10–25 independent protoplasts. Error bars are the SEM. Control is recovery in the absence of flg22, whereas, flg22 is recovery in the presence of flg22. (F) The WRKY53 reporter activity of protoplasts transformed with BR1–YFP and FLS2–CFP. Activity was determined as in Fig. 1. Average coefficients of diffusion ± SEM are presented next to the legends in each graph.
Fig. 3  Co-localization and FRET analyses of FLS2 on the cell membrane. (A–C) Confocal images of protoplasts co-expressing a YFP- and CFP-tagged receptor in various combinations show co-localization in the plasma membranes. Scatter diagrams to the right of each panel.
C-terminal fragments of the YFP. Unstimulated Ws-0 protoplasts co-expressing FLS2–YFPN and FLS2–YFPC did not reveal any YFP fluorescence, indicating that FLS2 does not undergo constitutive homodimerization (Fig. 4A). Next we investigated if FLS2 would homodimerize in response to flg22. Treatment of protoplasts expressing FLS2–YFPN/FLS2–YFPC with flg22 also did not reveal any fluorescence (data not shown), suggesting that FLS2 does not undergo a ligand-induced homodimerization. To rule out the possibility of the absence of enough flg22 for a sufficiently long time, we tested flg22 at higher concentrations up to 200 μM and monitored the YFP fluorescence for 4–24 h. These treatments also did not reveal any YFP fluorescence (data not shown), suggesting that the absence of FLS2 homodimerization is not due to physiologically lower flg22 levels. Using the same vectors that we have used, the suitability of the BiFC method in assaying protein–protein interactions of a membrane-localized transporter (potassium channel) was recently demonstrated (Xu et al. 2006). Additionally, as a control for BiFC, we co-expressed U1 70K–YFPN and SR–YFPC, which showed reconstitution of YFP fluorescence in accurate subnuclear locations (Fig. 4B). Taken together, our BiFC and FRET data support a scenario, where FLS2 does not undergo homodimerization in either an unstimulated or flg22-dependent manner.

**Discussion**

In this report we studied the in vivo dynamics and homodimerization of FLS2 using FRAP, FRET and BiFC analyses. These microscopy techniques usually use a fusion of a protein of interest with an FP such as GFP. Addition of an extraneous protein may alter the normal functioning of the native protein and, therefore, we first confirmed that all the FLS2 fusion proteins retained their normal activity. All FP-tagged FLS2 proteins localized to cell membranes and were detected at the expected molecular weight positions on Western blots. More importantly, all fusion proteins retained their normal signaling feature demonstrated by activation of several flg22-inducible promoters in an fls2 mutant line. Consistent with the presence of a predicted transmembrane domain in the FLS2 protein, our confocal image analyses in onion cells, tobacco and Arabidopsis show that FLS2 localizes to cell membranes (data not shown). Localization to plasma membrane is consistent with the function of FLS2 which supposedly detects flagellin/flg22 on the membrane (Chinchilla et al. 2006). In addition to localization to cell membranes, in several protoplasts we also observed FLS2 in intracellular vesicles of varying sizes and shapes, indicating that FLS2
may be associated with various subcellular domains at different stages of its life cycle. A similar pattern of localization was also observed for BR1 and BAK1 (Russinova et al. 2004), suggesting that the constitutive appearance of such vesicles is a general feature of the biogenesis/recycling of FLS2 and other plant RLKs. This is consistent with the observations that endocytosis of BR1 and several animal receptor kinases occurred in the absence of ligands as a mechanism of their turnover (Geldner et al. 2007). Recently, using transgenic Arabidopsis plants expressing FLS2-GFP, it was demonstrated that FLS2 is internalized into intracellular vesicles after adding flg22 (Robatzek et al. 2006). In contrast, using FLS2-YFP in protoplasts, we did not observe any significant flg22-dependent internalization of FLS2-YFP in vesicles. The reason for this difference may be that we used protoplasts as opposed to transgenic plants, which indicates that for internalization an intact cell wall is essential. The activation of several flg22-inducible promoters in the absence of FLS2 internalization in our analyses suggests that signal activation and transduction may be independent of internalization of receptors. This is in agreement with several reports, where, for example, signal initiation by TLR4 and TLR2 was shown to be independent of internalization of lipopolysaccharides and lipoteichoic acid (Latz et al. 2002, Triantafillou et al. 2004a). Such a scenario would indicate that the internalization of FLS2 may be a mechanism for recycling of the receptor rather than being required for signaling.

FRAP has been used extensively for studying the dynamics of a variety of proteins (Phair and Misteli 2001, Lippincott-Schwartz and Patterson 2003). In animal cells, the kinetic properties of several membrane-localized receptors have been studied with FRAP (Lippincott-Schwartz and Patterson 2003). Such analyses have revealed that the binding of ligands to their receptors either had no effect on the mobility of receptors or decreased the mobility of receptors, probably by confining them to microdomains that may serve to bring interacting signaling proteins together for activating downstream events (Triantafillou et al. 2004b). In contrast, in plants, studies with membrane receptors using FRAP are very scarce (Bhat et al. 2005). Here, our aim was to initiate investigation into the mechanism of activation of the Arabidopsis RLK, FLS2, on the cell membrane. Our analyses show that FLS2 is a highly dynamic protein in cell membranes with recovery kinetics in the order of seconds. Such a high mobility is probably needed for providing an effective surveillance system for detecting potential pathogens all over the cell surface. Additionally, high mobility may also be necessary for the normal functioning of FLS2 by directing it to the appropriate subdomains on cell membranes. In our analyses, the most interesting observation was the reduced mobility of FLS2, when treated with flg22. This indicates that after engaging its ligand, FLS2 interacts with other components, thereby retarding its mobility. In addition or alternatively, it is also possible that FLS2, after binding flg22, gets confined to microdomains on the cell membrane, which are proposed to be important for signaling of TLRs in animals (Triantafillou et al. 2004b). Using an immuno-precipitation and proteomic approach, Karlova et al. (2006) isolated the Arabidopsis SERK1 protein complex and identified several proteins, which as a whole would add up to a higher order complex that would move with very slow kinetics as compared with individual proteins. Our kinetic data of BR1–YFP in protoplasts suggest a much slower mobility than would be expected for a molecule of the size of BR1–YFP. This can be explained by BR1–YFP being retarded by its interaction with other components in the membrane and/or being present in a higher order complex that simply because of its higher molecular weight exhibits slower mobility. Using blue native gels, the molecular mass for the SERK1 protein complex was estimated to be about 300–500 kDa (Karlova et al. 2006), which is consistent with our FRAP data for BR1–YFP. Due to the high structural similarity of FLS2 and BR1, it is safe to suggest that FLS2 may also reside in a similar higher order molecular complex. Addition of flg22 leads to the confinement of such complexes to lesser mobile complexes probably by interacting with protein components necessary for its activation. In fact, recently it has been shown that FLS2 forms a complex that contains BAK1, suggesting the existence of such a higher order complex (Chinchilla et al. 2007, Heese et al. 2007). It would be interesting to isolate and catalog additional protein components of such a complex, which will add to our understanding of the functioning of FLS2 in particular and RLKs in general.

Several plant and animal membrane receptors are known to undergo homodimerization as part of their activation mechanism (Massague 1998, Li et al. 2002, Nam and Li 2002, Schlessinger 2002, Yu et al. 2002, Russinova et al. 2004, Wang et al. 2005a, Wang et al. 2005b). Here, using two different in vivo approaches, we show that FLS2 does not undergo homodimerization under either unstimulated or flg22-treated conditions. Arabidopsis BR11, but not SERK1, was shown to homodimerize, whereas both these proteins underwent heterodimerization, suggesting that the activation mechanisms of receptors involve differential oligomerization (Russinova et al. 2004). Using BR11 as a membrane-localized positive control, we confirm that BR11 does homodimerize on the cell membrane. Our BIFC and FRET data with FLS2 indicate that similarly to SERK1, FLS2 also does not homodimerize and probably interacts with other membrane receptors. While this manuscript was under review, two studies independently showed that the immunoprecipitation complex...
of FLS2 also contains BAK1 (Chinchilla et al. 2007, Heese et al. 2007). The presence of BAK1 was detected within 2 min of treatment with flg22, suggesting a role for such a complex in early defense responses. However, whether FLS2 interacts directly with BAK1 and whether they phosphorylate each other remains unknown. Our observations using BiFC and FRET started soon after adding flg22 and proceeded for several hours continuously. Therefore, any time-dependent interactions, such as those occurring immediately after adding flg22 or later after a lag period, are ruled out. BiFC analysis is suitable mostly for detecting stable interactions and may not allow detection of transient interactions (Walter et al. 2004) and, therefore, any transient FLS2 interactions may go undetected. Nevertheless, BiFC analyses with FLS2 reported here indicate that FLS2 may not form stable homodimers. FRET analyses, on the other hand, can detect both transient and stable interactions. The absence of FRET between FLS2 molecules suggests that it does not form stable or transient homodimers. BL-dependent heteromerization of BRI1 and BAK1/AtSERK3 is proposed to be essential for the activation of the BR signaling pathway (Russinova et al. 2004, Wang et al. 2005a, Wang et al. 2005). In the absence of any direct interaction of FLS2 with other RLKs, we cannot argue for a similar mode of action for FLS2. However, the general structural similarity of FLS2 to other RLKs indicates that a similar mechanism of activation may also be shared by FLS2. This is also consistent with differential heteromerization of several animal TLRs such as TLR2 with TLR1 or TLR6 in response to different ligands (Ozinski et al. 2000, Hajjar et al. 2001, Sandor et al. 2003).

Materials and Methods

Expression plasmids

BRI1–CFP and BRI1–YFP cloned in pMON999 (Monsanto, St. Louis, MO, USA) vectors driven by the 35S cauliflower mosaic virus promoter were described previously (Russinova et al. 2004). The complete coding sequence of FLS2, except the stop codon, was PCR amplified from an FLS2 expressed sequence tag (EST) clone obtained from Kazusa DNA Research Institute (Kisarazu City, Japan) with Ultra Hotstart PFU (Stratagene) using primers obtained from Kazusa DNA Research Institute (Kisarazu, Japan) in W5 medium (154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES). Approximately 2×10⁶ protoplasts were transfected with 10 μg of plasmid DNA with 40% PEG for 30 min. PEG was removed by diluting the protoplasts with W5 medium followed by centrifugation at 100×g for 2 min. The protoplast pellet was resuspended in W5 medium and incubated in 15 ml polypropylene conical tubes in the dark at 22°C for 18 h. A 50 μl aliquot of the expressing protoplasts was transferred to a glass-bottomed Petri dish with a 30–70 μm thickness coverslip and observed immediately under the microscope. The reporter construct WRKY53::LUC consisting of a 3.2 kb promoter region driving luciferase was co-transfected with either FLS2–YFP, FLS2–YFP², FLS2–YFP, FLS2–CFP, BRI1–YFP or BRI1–CFP effector plasmids and incubated for 12 h. Protoplasts were treated with 5 μM flg22 for 4 h and assayed for luciferase activity using a luciferase assay kit (Promega) according to the manufacturer’s instructions. All transient experiments were done three times with three replicates each time.

Confocal microscopy and fluorescence recovery after photobleaching (FRAP)

FRAP experiments were performed with a Zeiss LSM 510 Meta laser scanning microscope using a 63×, N.A. 1.4 oil immersion apochromate objective essentially as described (Ali and Reddy 2006). A fixed circular area of 5 μm diameter was bleached using the 514 nm line at full intensity at 60% power of a 25 mW laser and 100% transmission with 15 iterations. The first image at the end of bleaching was taken immediately and the rest of the images were taken at 400 ms constant intervals until maximal recovery was reached. For quantitative FRAP analyses, fluorescence intensity of the bleached area and the entire membrane was determined using LSM 510 software. Protoplasts with comparable total fluorescence intensities, which ranged from 2,500 to 3,000 arbitrary units, were used in these experiments. Most of the protoplasts remained stationary without any further manipulation. Those protoplasts that did move were discarded from the analysis. Background-corrected intensities were normalized for photobleaching resulting from bleach pulse and normal scanning according to the following equation (Phair and Misteli 2000): 

\[ I = I_o \times T_o / T, \]

where \( I \) is the normalized intensity of the bleached area, \( T_o \) is the total membrane intensity before bleaching, \( T \) is the total membrane intensity at time interval \( t \) after bleaching, \( I_o \) is the intensity of the bleached area before bleaching, and \( I_o \) is the intensity of the bleached area at time intervals \( t \) after bleaching. The resulting normalized intensity data were fitted to the following exponential models:

\[ I = Y_{\text{max}}[1 - e^{-kt}], \]

where \( Y_{\text{max}} \) is the final level of recovery, \( t \) is the time in seconds and \( k \) is the recovery rate constant. All non-linear curve fitting and the statistical comparisons were performed in GraphPad Prism 4.0 (http://www.graphpad.com). Total maximal

\[ \text{Protoplast transfection and WRKY53 promoter-driven luciferase reporter assay} \]

Transient expression in Arabidopsis mesophyll protoplasts was conducted using a standard polyethylene glycol (PEG) transfection protocol (Abel and Theologis 1994). Briefly, protoplasts were isolated from 4-week-old plants using 2.0% cellulase (Onozuka R-10) and 0.2% macerozyme (R-10, Yakult Hansa Co, Japan) in W5 medium (154 mM NaCl, 125 mM CaCl2, 5 mM KCl and 2 mM MES). Approximately 2×10⁶ protoplasts were transfected with 10 μg of plasmid DNA with 40% PEG for 30 min. PEG was removed by diluting the protoplasts with W5 medium followed by centrifugation at 100×g for 2 min. The protoplast pellet was resuspended in W5 medium and incubated in 15 ml polypropylene conical tubes in the dark at 22°C for 18 h. A 50 μl aliquot of the expressing protoplasts was transferred to a glass-bottomed Petri dish with a 30–70 μm thickness coverslip and observed immediately under the microscope. The reporter construct WRKY53::LUC consisting of a 3.2 kb promoter region driving luciferase was co-transfected with either FLS2–YFP, FLS2–YFP², FLS2–YFP, FLS2–CFP, BRI1–YFP or BRI1–CFP effector plasmids and incubated for 12 h. Protoplasts were treated with 5 μM flg22 for 4 h and assayed for luciferase activity using a luciferase assay kit (Promega) according to the manufacturer’s instructions. All transient experiments were done three times with three replicates each time.
recovery values represent the total mobile population. The immobile fraction was calculated as 1 – mobile fraction, with 1 as 100% recovery. Coefficients of diffusion were calculated according to the following formula: \( D = \frac{0.88(r^2/4 + t/2)}{r} \), where \( r \) is radius of the bleached area and \( t/2 \) is the time when half of the final fluorescence has recovered (Axelford et al. 1976)

**Bimolecular fluorescence complementation (BiFC) assays**

BiFC experiments were performed by co-transfecting FLS2–YFP\( \text{C} \) and FLS2–YFP\( \text{N} \) vectors in the Arabidopsis mesophyll protoplasts as described above (Abel and Theologis 1994, Walter et al. 2004). At 16 h after transfection, protoplasts were treated with flg22 (10 or 200 \( \mu \)M) and observed immediately and at 5 min intervals thereafter for reconstitution of YFP fluorescence for up to 24 h.

**Fluorescence resonance energy transfer (FRET) experiments**

FRET between the FLS2–YFP/FLS2–CFP and other controls was measured with the acceptor (YFP) photobleaching method using the Zeiss LSM 510 Meta confocal microscope according to Karpova et al. (2003) with modifications. The argon laser source operating at 60% of its 25 mW total power was used for exciting YFP at 514 nm (1% transmission) and CFP at 458 nm (5% transmission) with a 458/514 double dichroic filter. The filter set up for the YFP and CFP channels was as follows: YFP, excitation 514 nm, 458/514 dichroic, and emission 560–615 BP filters or 530–594 Chs2 on Meta channel; CFP, excitation 458 nm, 458/514 dichroic, and emission 460–500 nm BP filters or 466–498 ChS1 on Meta channel. Cross-talk between the YFP and CFP channels was avoided by collecting images in the multitracking mode, which allows the collection of images only in one channel at a time. A whole protoplast was bleached with the 514 nm laser (100% transmission; 50 iterations lasting approximately 50 s). Five images before and five after bleaching were recorded in each channel. Average intensity in the bleached area was measured and exported to Excel for further analyses. FRET efficiency was measured by the following formula: \( E = \frac{I_0 - I_f}{I_f} \times 100 \), where \( I_0 \) and \( I_f \) are the average fluorescence intensities of the images immediately before and after bleaching, respectively. Student’s \( t \)-test was used to determine the significance of differences between different treatments.

**Western blot analyses**

Protoplasts from Arabidopsis ecotype Ws-0 transfected with constructs of FLS2 conjugated to YFP, CFP, YFP\( \text{C} \) or YFP\( \text{N} \) were collected and frozen overnight at \(-80 \) °C. They were resuspended in 30 \( \mu \)l of 2\( \times \) sample buffer plus 0.1% Triton X-100, vortexed and sonicated twice, 4 s each. The samples were boiled for 5 min, centrifuged briefly and 30 \( \mu \)l was loaded on a 10% polyacrylamide gel. The gel was blotted and probed with monoclonal GFP antibody (BD Living Colors): anti-[c-myc]-peroxidase (Roche) for FLS2–YFP\( \text{N} \) or anti-HA-peroxidase (Roche) for FLS–YFP\( \text{C} \).

**Acknowledgments**

We thank Dr. Kudla, Institut für Botanik und Botanischer Garten, Molekulare Entwicklungbiologie der Pflanzen, Universität Münster, Schlossplatz 4, 48149 Münster, Germany for pSPY\( \text{NE}-355/p\text{UC-SPY\( \text{NE} \)) and pSPY\( \text{CE}-358/p\text{UCSPY\( \text{CE} \) vectors, and Dr. De Vries, Laboratory of Biochemistry, Wageningen University, 6703 HA Wageningen, The Netherlands for BR1–YFP and BR1–CFP vectors. This work was supported by a grant from the Defense Advanced Research Project Agency.

**References**


