

Single-molecule analysis reveals the phosphorylation of FLS2 regulates its spatiotemporal dynamics and immunity

Reviewed Preprint

v2 • April 3, 2024

Revised by authors

Reviewed Preprint

v1 • October 17, 2023

Yaning Cui, Hongping Qian, Jinhuan Yin, Changwen Xu, Pengyun Luo, Xi Zhang, Meng Yu, Bodan Su, Xiaojuan Li, Jinxing Lin 

State Key Laboratory of Tree Genetics and Breeding, College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China • National Engineering Research Center of Tree Breeding and Ecological Restoration, College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China • College of Life Sciences, Hebei Agricultural University, Baoding, 071001, China • Biotechnology Research Institute, Beijing 100081, China

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Abstract

Summary

Phosphorylation of receptor kinase (RK) is pivotal for signaling in pattern-triggered immunity (PTI). The *Arabidopsis thaliana* FLAGELLIN-SENSITIVE2 (FLS2) is a conserved 22 amino acid sequence in the N-terminal region of flagellin (flg22), initiating plant defense pathways. However, the dynamic FLS2 phosphorylation regulation at the plasma membrane in response to flg22 needs further elucidation. Through single-particle tracking, we demonstrated that the Ser-938 phosphorylation site influences flg22-induced FLS2 spatiotemporal dynamics and dwell time. Förster resonance energy transfer-fluorescence lifetime (FRET-FLIM) imaging microscopy, coupled with protein proximity indexes (PPI), revealed increased co-localization of FLS2/FLS2^{S938D}-GFP with AtRem1.3-mCherry in response to flg22. In contrast, FLS2^{S938A}-GFP shows no significant changes, indicating that Ser-938 phosphorylation influences the efficient FLS2 sorting into AtRem1.3-associated microdomains. Significantly, Ser-938 phosphorylation enhanced flg22-induced internalization and immune responses, thus demonstrating its regulatory role in FLS2 partitioning into functional AtRem1.3-associated microdomains for activating flg22-induced plant immunity.

eLife assessment

This potentially **important** study employs advanced imaging techniques to directly visualize molecular dynamics and of the immune receptor kinase FLS2 in specific microenvironments. The evidence supporting the ligand-induced association with remorin and the requirement of a previously reported phosphosite as presented is **solid**, although support by independent methods would be welcome. The work will be of interest to plant biologists working on cell surface receptors.

<https://doi.org/10.7554/eLife.91072.2.sa2>

Introduction

Receptor kinase (RK) are structurally conserved proteins, featuring an extracellular domain, a single transmembrane region, and a cytoplasmic kinase domain. The cytoplasmic kinase domain consists of a protein kinase catalytic domain (PKC) and a juxtamembrane region, containing crucial phosphorylation sites on specific Ser and/or Thr residues essential for pattern-triggered immunity (PTI), the initial defense layer in plants (Mitra et al., 2015 [↗](#); Gada et al., 2022 [↗](#)). PTI efficiently inhibits pathogen infection by activating defense responses, exemplified by the FLS2 signaling pathway (Li et al., 2016a [↗](#); Zhai et al., 2021 [↗](#); Yu et al., 2023 [↗](#)). Upon recognizing flg22, FLS2 interacts with the co-receptor Brassinosteroid-Insensitive 1-associated Kinase 1 (BAK1), initiating phosphorylation events through the activation of receptor-like cytoplasmic kinases (RLCKs) such as BOTRYTIS-INDUCED KINASE 1 (BIK1) to elicit downstream immune responses (Chinchilla et al., 2006 [↗](#); Li et al., 2016b [↗](#); Majhi et al., 2021 [↗](#)).

Protein phosphorylation, a vital posttranslational modification, plays a well-established and significant role. Research suggests that alterations in protein phosphorylation affect protein dynamics and subcellular trafficking (Palladino et al., 2022 [↗](#); Kontaxi et al., 2023 [↗](#)). In plants, phosphorylation of proteins can coalesce into membrane microdomains, forming platforms for active protein function at the plasma membrane (PM) (Bücherl et al., 2017 [↗](#)). For example, Xue et al. (2018) [↗](#) demonstrated that phosphorylation of the blue light receptor phot1 accelerates PM movement, enhancing interaction with AtRem1.3-mCherry, a sterol-rich lipid marker. This underscores protein phosphorylation's crucial role in PM dynamics and membrane partitioning. Upon flg22 treatment, multiple FLS2 phosphorylation sites activate, with FLS2 Serine-938 phosphorylation playing a pivotal role in defense activation (Cao et al., 2013 [↗](#)). While FLS2 Ser-938 is crucial for plant PTI, its impact on immunity through spatiotemporal dynamics remains unknown.

Membrane microdomains, dynamic structures rich in sterols and sphingolipids, are crucial in regulating plasma membrane (PM) protein behavior (Boutté et al., 2020). Specific proteins like Flotillins and Remorins can uniquely label these microdomains within living cells (Cui et al., 2018 [↗](#); Bücherl et al., 2017 [↗](#)). Various stimuli can induce PM proteins to move into mobile or immobile microdomains, suggesting a connection between microdomains and signal transduction (Kim et al., 2018 [↗](#)). For instance, Xing et al. (2022) [↗](#) demonstrated that sterol depletion significantly impacts the dynamics of flg22-activated FER-GFP, emphasizing microdomains' role in the lateral mobility and dissociation of FER from the PM under flg22 treatment. However, the spatial coordination of FLS2 dynamics and signaling at the PM, along with their relationships with microdomains, remains poorly understood.

To investigate if FLS2 Ser-938 phosphorylation-mediated immune response is linked to its microdomain, we analyzed diffusion and dwell time of FLS2 phospho-dead and phospho-mimic mutants at the PM before and after flg22 treatments. Our results show that flg22-induced dynamic and dwell time changes are abolished in FLS2^{S938A}. Using FLIM-FRET and smPPI techniques, we discovered that FLS2 in different phosphorylation states exhibited distinct membrane microdomain distribution and endocytosis. Additionally, various phenotypic experiments demonstrated that the immune response of phosphorylated FLS2^{S938D} was similar to wild-type FLS2, while weakened in transgenic plants with dephosphorylated FLS2^{S938A}. These findings contribute to the theoretical foundation for studying plant immunity regulation through phosphorylation.

Results and Discussion

The Ser-938 phosphorylation site changed the spatiotemporal dynamics of flg22-induced FLS2 at the plasma membrane

Previous studies highlight the crucial role of membrane protein phosphorylation in fundamental cellular processes, including PM dynamics (Vitrac et al., 2019 [↗](#); Offringa et al., 2013). *In vitro* mass spectrometry (MS) identified multiple phosphorylation sites in FLS2. Genetic analysis further identified Ser-938 as a functionally important site for FLS2 *in vivo* (Cao et al., 2013 [↗](#)). FLS2 Ser-938 mutations impact flg22-induced signaling, while BAK1 binding remains unaffected, thereby suggesting Ser-938 regulates other aspects of FLS2 activity (Cao et al., 2013 [↗](#)). To unravel the immune response regulation mechanisms via FLS2 phosphorylation, we generated transgenic Arabidopsis plants expressing C-terminal GFP-fused FLS2, S938A, or S938D under the FLS2 native promoter in the *fls2* mutant background (Figure S1A). Using VA-TIRFM with single-particle tracking (Figure 1A [↗](#) and 1B [↗](#)), we investigated the diffusion dynamics of FLS2 phospho-dead and phospho-mimic mutants, following previous reports (Geng et al., 2022 [↗](#)). Upon flg22 treatment, FLS2^{S938D}-GFP spots exhibited longer motion trajectories, while FLS2^{S938A}-GFP spots were limited to shorter motion tracks (Figure 1C [↗](#)). The results indicate significant changes in the diffusion coefficients and motion ranges of FLS2/FLS2^{S938D}-GFP after flg22 treatment, whereas FLS2^{S938A}-GFP showed no significant differences (Figure 1D [↗](#) and 1E [↗](#)). Similar outcomes were observed using Uniform Manifold Approximation and Projection (UMAP) technology (Dorrity et al., 2020 [↗](#)) and fluorescence recovery after photobleaching (FRAP) (Greig et al., 2021) (Figure 1F [↗](#), 1G [↗](#) and S1B, F), supporting the essential role of the S938 phosphorylation site in flg22-induced lateral diffusion of FLS2 at the PM.

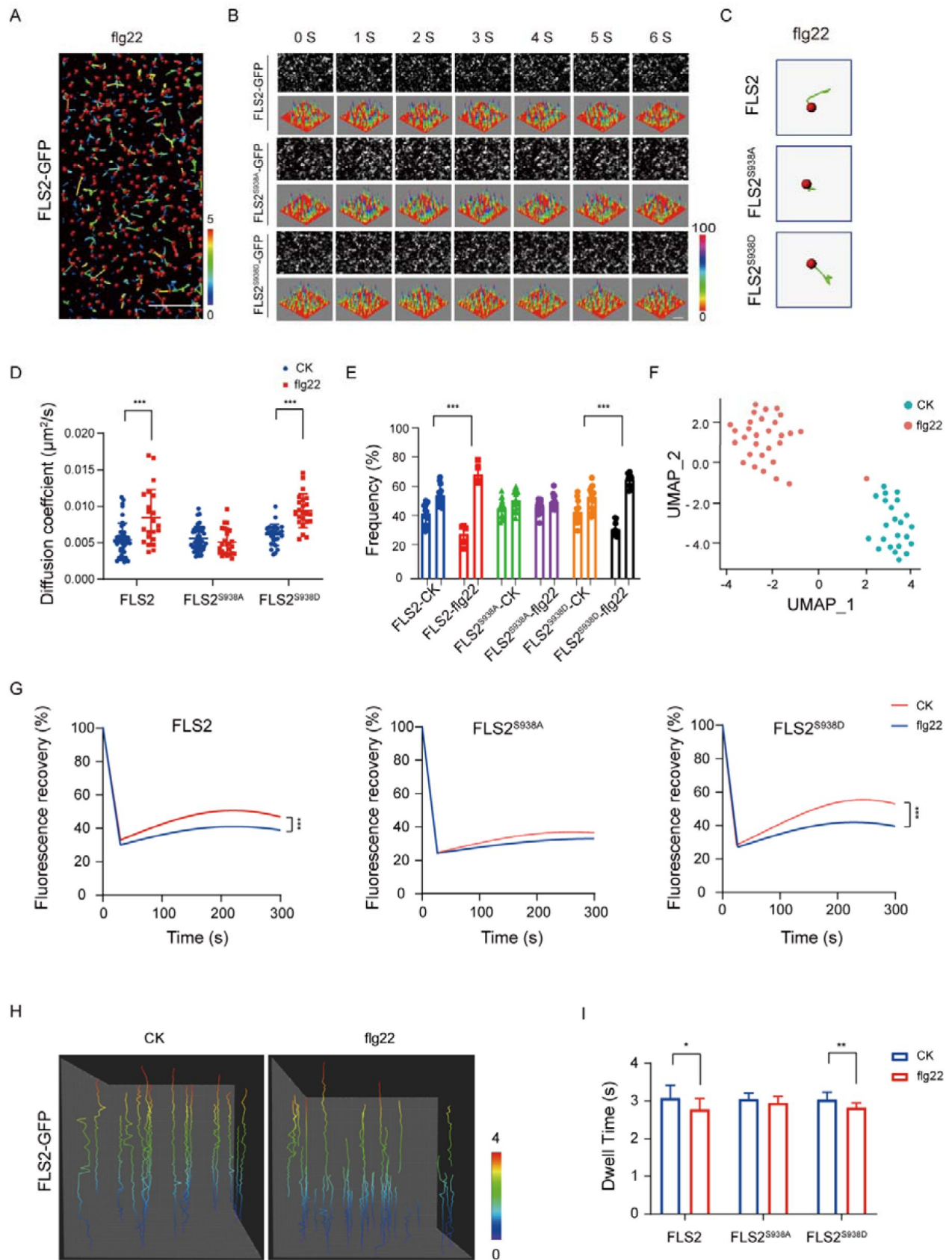


Figure 1.

Effects of Ser-938 phosphorylation on the spatiotemporal dynamics of FLS2 at the plasma membrane.

(A) VA-TIRFM images of a FLS2-expressing hypocotyl cell was analyzed. The 5-day-old transgenic Arabidopsis plant cells were observed under VA-TIRFM. The red balls indicate the positions of the identified points that appeared. Trajectories represent the track length of the identified points. Bar = 10 μ m. (B) Time-lapse images of FLS, FLS2^{S938A}, and FLS2^{S938D}. Bar = 2 μ m. The fluorescence intensity changes among different 3D luminance plots. (C) The trajectories of representative individual FLS2, FLS2^{S938A} and FLS2^{S938D} under 30 min for 10 μ M flg22 processing. (D) Diffusion coefficients of FLS (control, n = 42 spots; flg22, n = 24 spots), FLS2^{S938A} (control, n = 44 spots; flg22, n = 25 spots) and FLS2^{S938D} (control, n = 27 spots; flg22, n = 23 spots) under different environments. Statistical significance was assessed using the Student's *t*-test ($***p < 0.001$). Error bars represent the SD. (E) Frequency of long- and short-range motions for FLS, FLS2^{S938A}, and FLS2^{S938D} under different environments. Statistical significance assessed with the Student's *t*-test ($***p < 0.001$). Error bars represent the SD. (F) UMAP visualization of FLS2^{S938D} samples under different conditions (control, n = 32 spots; flg22, n = 21 spots). Dots represent the individual images, and are colored according to the reaction conditions. (G) Fluorescence recovery curves of the photobleached areas with or without the flg22 treatment. Three biological replicates were performed. (H) The single-molecule trajectories of FLS2 analyzed by Imaris could be faithfully tracked for 4 s under control and flg22 treatments. (I) Dwell times were analyzed for FLS2 (control, n = 18 spots; flg22, n = 16 spots), FLS2^{S938A} (control, n = 19 spots; flg22, n = 24 spots), and FLS2^{S938D} (control, n = 11 spots; flg22, n = 14 spots) under the control and flg22 treatments. Statistical significance assessed with Student's *t*-test ($*p < 0.05$; $**p < 0.01$). Error bars represent the SD.

Using VA-TIRFM, we analyzed FLS2 particle dwell time across various phosphorylation states. The results revealed that flg22 treatment significantly reduced the fluorescence trajectory of FLS2 molecules compared to the control (Figure 1H and S1C), indicating that Ser-938 phosphorylation influences flg22-induced dwell times of FLS2 at the PM. Subsequently, real-time dynamic analysis through the Kymograph technique provided spatiotemporal information via frame-by-frame tracking (Zhou et al., 2020). Compared to FLS2-GFP and FLS2^{S938D}-GFP, FLS2^{S938A}-GFP showed nearly linear fluctuations in fluorescence intensity under flg22 conditions, and the duration of fluorescence retention was essentially unchanged (Figure S1D). To further confirm this, we quantified FLS2 dwell times using exponential function fitting (Figure 1I and S1E). After flg22 treatment, FLS2^{S938D}-GFP dwell times significantly decreased, resembling those of FLS2-GFP. Although the FLS2^{S938A}-GFP plants, dwell times appeared to slightly decrease with flg22 treatment, the change was not significant. This aligns with previous findings that NRT1.1 phosphorylation affects dynamics and dwell time (Zhang et al., 2019). Additionally, numerous FLS2 exhibited short-lived dwell times, indicating abortive endocytic events associated with the endocytic pathway and signal transduction (Bertot et al., 2018). Therefore, these results underscore the impact of Ser-938 phosphorylation on the spatiotemporal dynamics of flg22-induced FLS2.

Ser-938 phosphorylation enhances recruitment of FLS2/BAK1 heterodimerization into AtRem1.3-associated microdomains

Proteins rarely act independently; they typically form multimers to enhance downstream signaling (Li et al., 2022). Previous studies have shown that inactive FLS2 mainly exists as monomers. Flg22 treatment induces FLS2 and BAK1 to heterodimerize at the PM, signifying flg22 as a ligand promoting this heterodimerization (Orosa et al., 2018). Therefore, we further investigated if Ser-938 phosphorylation affects FLS2/BAK1 heterodimerization. Tessler segmentation, FRET-FLIM, and smPPI analyses revealed no impact of Ser-938 phosphorylation on FLS2/BAK1 heterodimerization (Figure 2A-C and S2). This aligns with the previous finding that

flg22 acts as a molecular glue for FLS2 and BAK1 ectodomains (Sun et al., 2013 [DOI](#)), confirming the independence of FLS2/BAK1 heterodimerization from phosphorylation, with these events occurring sequentially.

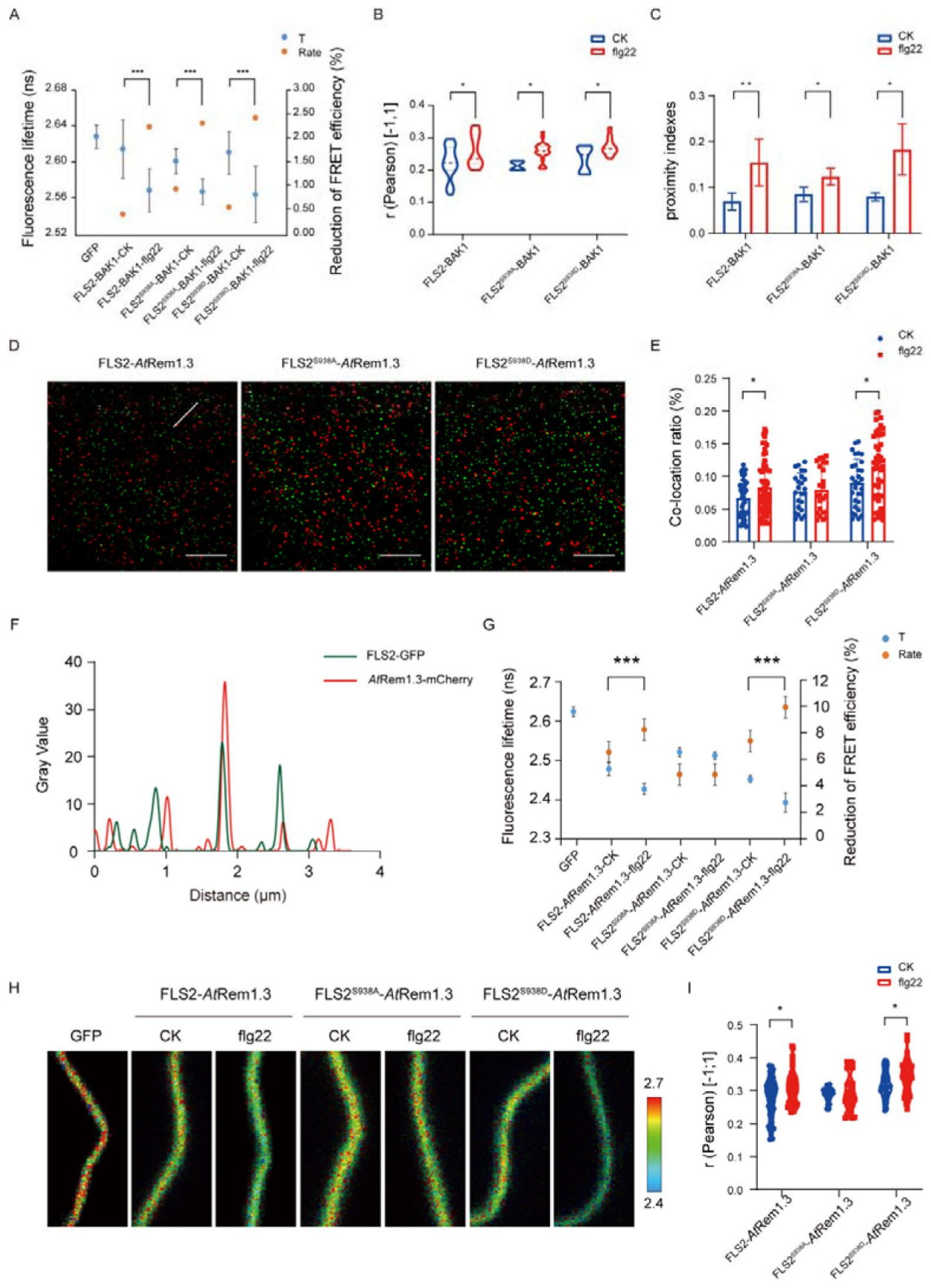


Figure 2.

Different Ser-938 phosphorylation states of FLS2 affect its partitioning into AtRem1.3-associated microdomains.

(A) FLIM-FRET was used to detect the co-expression of FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP and BAK1-mCherry in the *N. benthamiana* epidermal cells stimulated by 1/2 MS or flg22 (10 μ M) for 30 min. Average fluorescence lifetime (τ) and the FRET efficiency were analyzed for FLS2, FLS2^{S938A} or FLS2^{S938D} and BAK1. Statistical significance was assessed with Student's *t*-test ($***p < 0.001$). Error bars represent the SD. (B) Pearson correlation coefficient values of co-localization between FLS2 (control, *n* = 24 images; flg22 = 15 images), FLS2^{S938A} (control, *n* = 18 images; flg22 = 22 images), or FLS2^{S938D} (control, *n* = 15 images; flg22 = 20 images) and BAK1 upon stimulation with control or flg22. Statistical significance was assessed with the Student's *t*-test ($*p < 0.05$). Error bars represent the SD. (C) Mean protein proximity indexes showing FLS2 (control, *n* = 8 images; flg22 = 5 images), FLS2^{S938A} (control, *n* = 4 images; flg22 = 3 images) or FLS2^{S938D} (control, *n* = 4 images; flg22 = 3 images) and BAK1 degree of proximity under different conditions. Statistical significance was assessed with the Student's *t*-test ($*p < 0.05$; $**p < 0.01$). Error bars represent the SD. (D) TIRF-SIM images of the Arabidopsis leaf epidermal cells co-expressing FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP and AtRem1.3-mCherry. The white line represents the fluorescence signals. Bar = 5 μ m. (E) The histogram shows the co-localization ratio of FLS2-GFP (control, *n* = 52 images; flg22 = 61 images), FLS2^{S938A}-GFP (control, *n* = 30 images; flg22 = 36 images) or FLS2^{S938D}-GFP (control, *n* = 34 images; flg22 = 54 images) and AtRem1.3-mCherry. Statistical significance assessed with the Student's *t*-test ($*p < 0.05$). Error bars represent the SD. (F) FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP and AtRem1.3-mCherry fluorescence signals as shown in D. (G) The fluorescence mean lifetime (τ) and the corrected fluorescence resonance efficiency (Rate) of FLS2, FLS2^{S938D} or FLS2^{S938A} with co-expressed AtRem1.3. Statistical significance was assessed using the Student's *t*-test ($***p < 0.001$). Error bars represent the SD. (H) Intensity and lifetime maps of the Arabidopsis leaf epidermal cells co-expressing FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP and AtRem1.3-mCherry as measured by FLIM-FRET. (I) Quantification of co-localization between FLS2, FLS2^{S938A} or FLS2^{S938D} and AtRem1.3 with and without stimulation with ligands. Pearson correlation coefficient values (*r*) were obtained between FLS2 (control, *n* = 66 images; flg22 = 53 images) /FLS2^{S938A} (control, *n* = 42 images; flg22 = 54 images) /FLS2^{S938D}-GFP (control, *n* = 29 images; flg22 = 38 images) and AtRem1.3-mCherry. Statistical significance assessed with the Student's *t*-test ($*p < 0.05$). Error bars represent the SD.

Membrane microdomains serve as pivotal platforms for protein regulation, impacting cellular signaling dynamics (Martinière et al., 2021). Studies reveal that specific plasma membrane (PM) proteins, responsive to developmental cues and environmental stimuli, exhibit dynamic movements within and outside these microdomains (Lee et al., 2019). For instance, treatment with the secreted peptides RAPID ALKALINIZATION FACTOR (RALF1 and RALF23) enhances the presence of FERONIA (FER) in membrane microdomains (Gronnier et al., 2022). Conversely, flg22-activated BSK1 translocates from membrane microdomains to non-membrane microdomains (Su et al., 2021). In a previous investigation, we demonstrated that flg22 induces FLS2 translocation from AtFlot1-negative to AtFlot1-positive nanodomains in the plasma membrane, implying a connection between FLS2 phosphorylation and membrane nanodomain distribution (Cui et al., 2018). To validate this, we assessed the association of FLS2/FLS2^{S938D}/FLS2^{S938A} with membrane microdomains, using AtRem1.3-associated microdomains as representatives (Huang et al., 2019). Employing SPT, FRET-FLIM, and Pearson correlation, we found increased correlation coefficients between FLS2-GFP/FLS2^{S938D}-GFP and AtRem1.3-mCherry during flg22 treatment compared to the control (Figure 2D–2F and Movies 1–3). Significantly, flg22 treatment did not increase colocalization levels of FLS2^{S938A}-GFP and AtRem1.3, indicating the necessity of phosphorylation (Figure 2G–2I). These findings suggest that the phosphorylation state of FLS2 at Ser-938 influences its aggregation into AtRem1.3-associated microdomains, providing an efficient mechanism for triggering the immune response.

Ser-938 phosphorylation maintains FLS2 protein homeostasis via flg22-induced endocytosis

The PM protein endocytosis is crucial for regulating intercellular signal transduction in response to environmental stimuli. Notably, Thr 867 mutation, a potential phosphorylation site on FLS2, results in impaired flg22-induced endocytosis, underscoring the significance of phosphorylation in FLS2 endocytosis (Robatzek et al., 2006 [\[1\]](#)). As mentioned earlier, FLS2 phosphomimetic mutants showed a reduced dwell time (Figures 1 [\[2\]](#) H and I), implying a probable connection between FLS2 dwell time and endocytosis. Results found that FLS2/FLS2^{S938D}/FLS2^{S938A}-GFP accumulated in BFA compartments labeled with FM4-64, indicating that various Ser-938 phosphorylation states of FLS2 undergo BFA-dependent constitutive endocytosis (Figure 3A [\[3\]](#), S3A).

Next, we investigated the impact of Ser-938 on flg22-induced FLS2 internalization. Results revealed increased endocytic vesicles for FLS2 during flg22 treatment, aligning with previous studies (Leslie et al., 2017; Loiseau et al., 2017). Employing confocal laser-scanning microscopy (CLSM) during 10 μ M flg22 treatment, we tracked FLS2 endocytosis and quantified vesicle numbers over time (Figure 3B [\[4\]](#)). It is evident that both FLS2 and FLS2^{S938D} vesicles appeared 15 min after-flg22 treatment, significantly increasing thereafter (Figure 3C [\[5\]](#)). Notably, only a few vesicles were detected in FLS2^{S938A}-GFP, indicating Ser-938 phosphorylation's impact on flg22-induced FLS2 endocytosis. Additionally, fluorescence correlation spectroscopy (FCS) (Chen et al., 2009 [\[6\]](#)) monitored molecular density changes at the PM before and after flg22 treatment (Figure S3F). Figure 3D [\[7\]](#) shows that both FLS2-GFP and FLS2^{S938D}-GFP densities significantly decreased after flg22 treatment, while FLS2^{S938A}-GFP exhibited minimal changes, indicating Ser-938 phosphorylation affects FLS2 internalization. Western blotting confirmed that Ser-938 phosphorylation influences FLS2 degradation after flg22 treatment (Figure 3E [\[8\]](#)), consistent with single-molecule analysis findings. Therefore, our results strongly support the notion that Ser-938 phosphorylation expedited FLS2 internalization, potentially regulating its immune response capacity.

Ser-938 Phosphorylation affects FLS2-mediated responses

PTI plays a pivotal role in host defense against pathogenic infections (Lorrai et al., 2021; Ma et al., 2022). Previous studies demonstrated that FLS2 perception of flg22 initiates a complex signaling network with multiple parallel branches, including calcium burst, mitogen-activated protein kinases (MAPKs) activation, callose deposition, and seedling growth inhibition (Baral et al., 2015 [\[9\]](#); Marcec et al., 2021; Huang et al., 2023 [\[10\]](#)). Our focus was to investigate the significance of Ser-938 phosphorylation in flg22-induced plant immunity. Figure 4A-F [\[11\]](#) illustrates diverse immune responses in FLS2 and FLS2^{S938D} plants following flg22 treatment. These responses encompass calcium burst activation, MAPKs cascade reaction, callose deposition, hypocotyl growth inhibition, and activation of immune-responsive genes. In contrast, FLS2^{S938A} (Figure S4A-D) exhibited limited immune responses, underscoring the importance of Ser-938 phosphorylation for FLS2-mediated PTI responses.

In summary, our study confirmed that FLS2 phosphorylation regulated PAMP-triggered plant immunity by influencing spatiotemporal dynamics at the PM (Figure 4 G [\[12\]](#) and H [\[13\]](#)). Following flg22 treatment, activated FLS2 undergoes hetero-oligomerization and phosphorylation sequentially. Crucially, phosphorylation at the Ser-938 site promotes FLS2 recruitment into AtRem1.3-associated microdomains and endocytosis. These results provided new insights into the phosphorylation-regulated dynamics of plant immunity, thereby providing a reference for future studies on signal transduction in intracellular complex nanostructures.

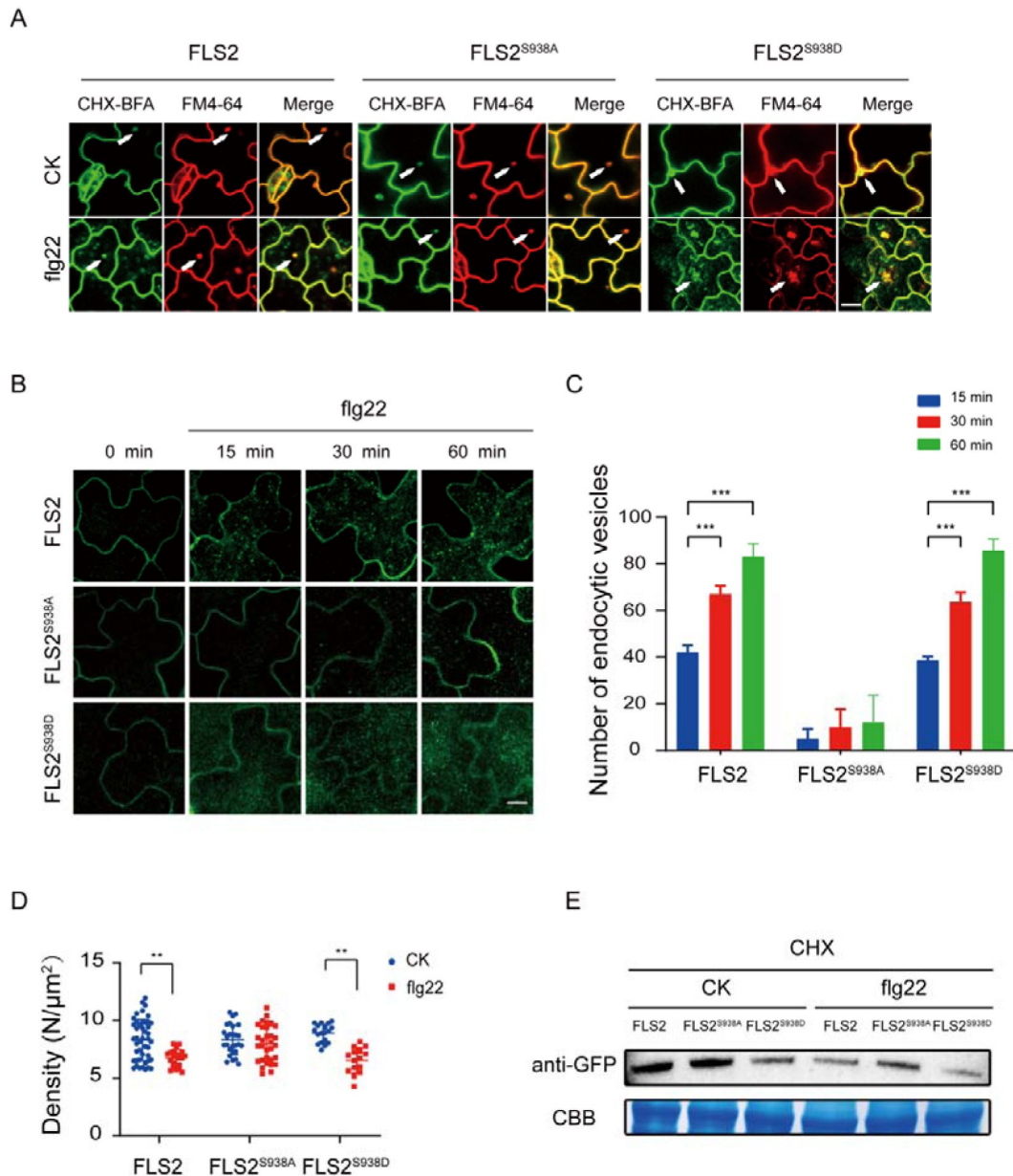


Figure 3.

The Ser-938 phosphorylation site affects flg22-induced endocytosis.

(A) Confocal images of FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP in *Arabidopsis thaliana* leaf epidermal cells. The 5-day-old transgenic seedlings were pre-treated with 50 μ M CHX for 30 min, then treated with 50 μ M BFA for 60 min or CHX + BFA + 5 μ M FM4-64 for 30 min, followed by 10 μ M flg22 treatment for a total of 30 min. White arrows indicate BFA bodies. Bar = 3 μ m. (B) Images of FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP in *Arabidopsis thaliana* leaf epidermal cells treated with 10 μ M flg22 for 15, 30, and 60 min. Bar = 3 μ m. (C) Analysis of FLS2, FLS2^{S938A}, and FLS2^{S938D} endocytic vesicle numbers in cells treated with 10 μ M flg22 treatment over time. Statistical significance assessed using the Student's *t*-test ($***p < 0.001$). Error bars represent the SD. (D) The signal density of FLS2, FLS2^{S938A} and FLS2^{S938D} in cells after control or 10 μ M flg22 treatments for 30 min as measured by FCS. Statistical significance was assessed using Student's *t*-test ($**p < 0.01$). Error bars represent the SD. (E) Immunoblot analysis of FLS2 protein in 10-day-old transgenic *Arabidopsis* plant upon stimulation with or without 10 μ M flg22. CBB, a loading control dyed with Coomassie Brilliant Blue.

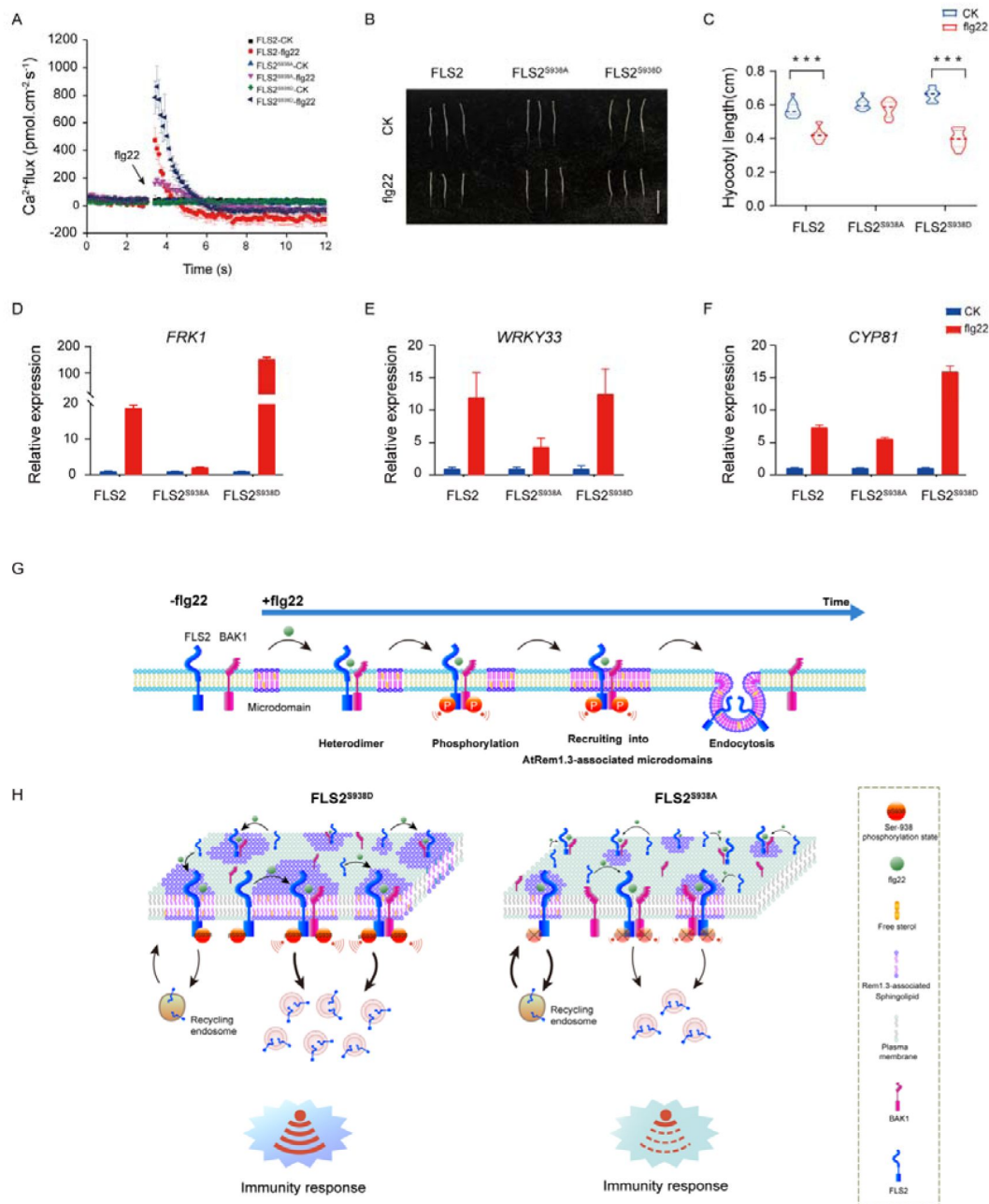


Figure 4.

Ser-938 phosphorylation is essential for various flg22-induced PTI responses.

(A) The flg22-induced transient Ca^{2+} flux in 20-day-old transgenic leaf cells. The Ca^{2+} flux was continuously recorded for 12 min in the test medium. Each point represents the average value for about 12 individual plants $\pm$ SEM (B) Phenotypes of 5-day old etiolated seedlings grown in the presence of 1/2 MS (control) or 10 μM flg22 solid medium. Bar=0.5cm. (C) Hypocotyl length of FLS2 (control, n = 13 seedlings; flg22=13 seedlings), FLS2^{S938A} (control, n = 13 seedlings; flg22=13 seedlings) and FLS2^{S938D} (control, n = 13 seedlings; flg22=13 seedlings) transgenic plants. Statistical significance was assessed with the Student's *t*-test (****p* < 0.001). Error bars represent the SD. (D-F) mRNA levels of the PTI marker genes *FRK1*/*WRKY33*/*CYP81* were significantly different between the FLS2, FLS2^{S938A}, and FLS2^{S938D} 10-day-old transgenic Arabidopsis plant after treatment with 10 μM flg22 for 30 min. Total RNA was extracted from 10-day-old seedlings and analyzed using qRT-PCR, with transcript levels being normalized to *UBQ5*. Error bars represent SD. (G) The working model for the spatiotemporal dynamic regulation of FLS2 phosphorylation at the plasma membrane upon flg22 stimulation. (H) Dynamic model of FLS2 with different Ser-938 phosphorylation states upon stimulation with flg22.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32030010, 91954202, 32370740, 32000483, 32170689), the Fundamental Research Funds for the Central Universities (NO.QNTD202301), National Key Research and Development Program of China (2022YFF0712500, 2022YFD2200603), Beijing Nova Program (20230484251), 5-5 Engineering Research & Innovation Team Project of Beijing Forestry University (BLRC2023C06) and the program of Introducing Talents of Discipline to Universities (111 project, B13007).

Author contributions

J.L., Y.C. and X.L. designed the research; Y.C., H.Q., C.X. and J.Y. performed the research; C.X., P.L. and M.Y. contributed new reagents/analytic tools; X.Z., H.Q. and B.S. analyzed the data; and Y.C., H.Q. and J.L. wrote the paper.

Declaration of interests

The authors declare no competing interests.

STAR Methods

Plant Materials and Construction

Mutants and transgenic lines used in all experiments were in the *Arabidopsis thaliana* Colombia-0 (Col-0) background. To generate the transgenic plants, specific constructs were PCR-amplified and cloned into the vector pCambia2300. Plasmids were introduced into the mutant plants by *Agrobacterium*-mediated transformation. Dual-color lines expressing FLS2^{S938A}-GFP and FLS2^{S938D}-GFP with REM1.3-mCherry were generated by hybridization.

Drug Treatments

All chemicals were obtained from Sigma-Aldrich, and dissolved in 100% DMSO to yield stock solutions at the following concentrations: BFA (50 mM in DMSO, 50 μ M in working solution), CHX (50 mM in DMSO, 50 μ M in working solution), and FM4-64 (5 mM in DMSO, 5 μ M in working solution). The flg22 of flagellin peptides was synthesized by Shanghai GL Biochem Company, and was used in a concentration of 10 μ M in double-distilled H₂O. *Arabidopsis* seedlings were treated in 1/2 MS growth liquid medium with added hormone or drug.

Quantitative Reverse-transcription PCR

Total RNA was extracted using the Plant Kit (Tiangen), and then reverse transcribed into cDNA with FastQuant RT Kit (Tiangen). Next, qPCR was performed using TiangenSurperRealPreMix Plus (SYBR Green). The primers used were as follows: WRKY33 (At At2g38470) 5'-GAAACAAATGGTGGGAATGG-3' and 5'-TGTCGTGTGATGCTCTCTCC-3'; CYP81 (At At2g23190) 5'-AAATGGAGAGAGCAACACAATG-3' and 5'-ATCGCCCATTCGAATGTTAC-3'; FRK1 (AtAt2g19190) 5'-TATATGGACACCGGTATAGTG-3' and 5'-ATAAACTTTGCGTTAGGGTCG-3'.

Aniline Blue Staining

To detect callose deposition, aniline blue staining was performed as described. *Arabidopsis thaliana* leaves were completely de-colored by the destaining solution (3 mL ethyl alcohol and 1 mL glacial acetic acid), rinsed in water and 50% ethanol, and then stained in 150 mM KH_2PO_4 (pH 9.5) plus 0.01% aniline blue for 2 h. Samples were mounted in 25% glycerol, and then observed under a microscope that was equipped with a Leica DM2500 UV lamp.

Confocal Laser Scanning Microscopy and Image Analysis

Confocal microscopy was done with a TCS SP5 Confocal Microscope fitted with a 63X water-immersion objective. GFP and FM4-64 were assayed using 488-nm and 514-nm wavelengths (multitrack mode). The fluorescence emissions were respectively detected with spectral detector set LP 560–640 (FM4-64) and BP 520–555 (GFP). Image analysis was performed with Leica TCS SP5 software and quantified using the ImageJ software bundle (NIH).

VA-TIRFM and Single-Particle Fluorescence Image Analysis

The dynamics of FLS2 phospho-dead and phospho-mimic mutants were recorded using VA-TIRFM. This was done using an inverted microscope (IX-71, Olympus) equipped with a total internal reflective fluorescence illuminator (model no. IX2-RFAEVA-2; Olympus) and a 1003 oil-immersion objective (numerical aperture = 1.45). To track GFP-labeled proteins at the PM, living leaf epidermal cells of 6-day-old seedlings were observed under VA-TIRFM. To visualize GFP or mCherry fluorescent proteins, appropriate corresponding laser excitation (473-nm or 561-nm) was used and emission fluorescence was obtained with filters (BA510IF for GFP; HQ525/50 for mCherry). A digital EMCCD camera (Andor Technology, ANDOR iXon DV8897D-CS0-VP, Belfast, UK) was used to acquire the fluorescent signals, which were stored directly on computers and then analyzed with Image J software. Images of single particles were acquired with 100-ms exposure time.

TIRF-SIM imaging and the colocalization analysis

The SIM images were taken by a $60 \times \text{NA } 1.49$ objective on a structured illumination microscopy (SIM) platform (DeltaVision OMX SR) with a sCMOS camera (Camera pixel size, $6.5 \mu\text{m}$). The light source for TIRF-SIM included diode laser at 488 nm and 568 nm with pixel sizes (μm) of 0.0794 and 0.0794 (Barbieri et al., 2021 [DOI](#)).

For the dual-color imaging, FLS2/FLS2^{S938A}/FLS2^{S938D}-GFP (488 nm/30.0%) and AtRem1.3-mCherry (561 nm/30.0%) were excited sequentially. The exposure time of the camera was set at 50 ms throughout single-particle imaging. The time interval for time-lapse imaging was 100 ms, the total time was 2s, and the total time points were 21s. The Imaris intensity correlation analysis plugin was used to calculate the co-localization ratio.

Ca^{2+} Flux Measurements in Arabidopsis Leaves

Net Ca^{2+} fluxes in Arabidopsis leaf cells were measured using the non-invasive micro-test technique (NMT), as described previously (Zhong et al., 2023 [DOI](#)). First, a small incision was made in the leaves of fourteen-day-old seedling. Then it was fixed at the bottom of 35 mm petri dish, and incubated in the test buffer (pH = 6.0, $0.1 \text{ mmol L}^{-1} \text{ KCl/CaCl}_2/\text{MgCl}_2$, $0.2 \text{ mmol L}^{-1} \text{ Na}_2\text{SO}_4$, $0.3 \text{ mmol L}^{-1} \text{ MES}$, $0.5 \text{ mmol L}^{-1} \text{ NaCl}$) Ca^{2+} concentration in the leaf cells was measured at 0.2 Hz near and $30 \mu\text{m}$ away from the cells. Each plant was measured once, and then use 1/2 MS (CK) or $10 \mu\text{M}$ flg22 treated the leaf and measured Ca^{2+} flux again. The Ca^{2+} flux was calculated as described (Jiao et al., 2022 [DOI](#)).

Analysis of Root Growth

Arabidopsis seedlings were treated with different conditions, and imaging was performed by scanning the root systems at 500 dpi (Canon EOS 600D). ImageJ was used to analyze the root growth parameters. Three biological replicates were performed.

Fcs

FCS was performed in point-scanning mode on a Leica TCS SP5 FCS microscope equipped with a 488-nm argon laser, an Avalanche photodiode, and an in-house coupled correlator. After acquiring images on the PM of a cell in transmitted light mode, the diffusion of protein molecules into and out of the focal volume transformed the local concentration of fluorophores, leading to spontaneous fluctuation in the fluorescence intensity. Finally, the protein density was calculated on the basis of the protocol described previously.

Fret flim

FRET-FLIM analysis was performed using an inverted Olympus FV1200 microscope equipped with a Picoquant picoHarp300 controller. The excitation at 488 nm was implemented by a picosecond pulsed diode laser at a reduplication rate of 40 MHz, by way of a water immersion objective (603, numerical aperture [NA] 1.2). The emitted light passed through a 520/35 nm bandpass filter and was detected by an MPD SPAD detector. Data were collected and performed using the SymphoTime 64 software (PicoQuant).

Western Blot

Total proteins were extracted from 10-day-old seedlings of the acidic phosphomimic mutants FLS2^{S938A}-GFP and FLS2^{S938D}-GFP and transgenic FLS2-GFP lines under different conditions. Proteins were extracted using buffer E [includes 1.5125 g Tris-HCl (pH 8.8), 1.2409 g Na₂S₂O₅, 11.1 ml glycerine, 1 g SDS, and 5 mM DTT]. The proteins in PM fractions were obtained using the Invent Minute kit. Proteins were separated by 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blotted with anti-GFP antibody (Sigma-Aldrich) at a 1:4000 dilution.

Supplemental data

Supplemental Figure S1. Different Ser-938 phosphorylation states lead to specific spatiotemporal dynamics of FLS2.

Supplemental Figure S2. Different Ser-938 phosphorylation states do not affect the hetero-oligomerization of FLS2/BAK1.

Supplemental Figure S3. Effects of Ser-938 phosphorylation on the endocytosis of FLS2.

Supplemental Figure S4. Ser-938 phosphorylation affects flg22-induced PTI responses.

Supplemental Movies 1-3. TIRF-SIM movies of FLS2 (WT, A, D) and AtRem1.3-mCherry.

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Editors

Reviewing Editor

Jian-Min Zhou

Chinese Academy of Sciences, Beijing, China

Senior Editor

Jürgen Kleine-Vehn

University of Freiburg, Freiburg, Germany

Reviewer #1 (Public Review):

Summary:

Organization of cell surface receptors in membrane nanodomains is important for signaling, but how this is regulated is poorly understood. In this study the authors employ TIRFM single-molecule tracking combined with multiple analyses to show that ligand exposure increases diffusion of the immune receptor FLS2 in the plasma membrane and its co-localization with remorin REM1.3 in a manner dependent on the phosphosite S938. They additionally show that ligand increases dwell time of FLS2, and this is linked to FLS2 endocytosis, also in a manner dependent on S938 phosphorylation. The study uncovers a regulatory mechanism of FLS2 localization in the nanodomain crucial for signaling.

Strengths:

TIRFM single-molecule tracking, FRAP, FRET and endocytosis experiments were nicely done. A role of S938 phosphorylation is convincing.

Weaknesses:

In the previous submission, reviewers pointed out multiple issues, which the reviewers believed the authors can address in the revision. The revised version does improve to some extent but still contains many issues in terms of data analysis and writing.

<https://doi.org/10.7554/eLife.91072.2.sa1>

Reviewer #2 (Public Review):

Summary:

The research conducted by Yaning Cui and colleagues delves into understanding FLS2-mediated immunity. This is achieved by comparing the spatiotemporal dynamics of a FLS2-S938A mutant and FLS2-WT, especially in relation to their association with the remorin protein. To delineate the differences between the FLS2-S938A mutant and FLS2-WT, they utilized a plethora of advanced fluorescent imaging techniques. By analyzing surface dynamics and interactions involving the receptor signal co-receptor BAK1 and remorin proteins, the authors propose a model of how FLS2 and BAK1 are assembled and positioned within a remorin-specific nano-environment during FLS2 ligand-induced immune responses.

Strengths:

These techniques offer direct visualizations of molecular dynamics and interactions, helping us understand their spatial relationships and interactions during innate immune responses.

Advanced cell biology imaging techniques are crucial for obtaining high-resolution insights into the intracellular dynamics of biomolecules. The demonstrated imaging systems are excellent examples to be used in studying plant immunity by integrating other functional assays.

Weaknesses:

It's essential to acknowledge that every fluorescence-based method, just like biochemical assays, comes with its unique limitations. These often pertain to spatial and temporal resolutions, as well as the sensitivity of the cameras employed in each setup. Meticulous interpretation is pivotal to guarantee an accurate depiction and to steer clear of potential misunderstandings when employing specific imaging systems to analyze molecular attributes. Moreover, a discerning interpretation and accurate image analysis can offer invaluable guidance for future studies on plant signaling molecules using these nice cell imaging techniques.

For instance, although single-particle analysis couldn't conclusively link FLS2 and remorin, FLIM-FRET effectively highlighted their ligand-triggered association and the disengagement brought on by mutations. While these methodologies seemed to present differing outcomes, they were described in the manuscript as harmonious. In reality, these differences could highlight distinct protein populations active in immune responses, each accentuated differently by the respective imaging techniques due to their individual spatial and temporal limitations. Addressing these variations is imperative, especially when designing future imaging explorations of immune complexes.

<https://doi.org/10.7554/eLife.91072.2.sa0>

Author Response

The following is the authors' response to the original reviews.

We greatly thank you and the reviewers for your expert comments and valuable suggestions on our manuscript. After reading these comments, we realized that the previous version of the manuscript contained some weak points. Surely, the issues raised by the six reviewers were of great help in the revision of our manuscript.

According to the comments, we have now fully revised the manuscript to address most of the questions and suggestions. In addition, we reworded some parts of the Introduction, Results and Discussion, Figures, Figure legends and Experimental Methods to increase the rigor of our conclusions.

Overall, you will see that we have paid serious attention to all the concerns and criticisms expressed by reviewers. Addressing these various issues has most certainly allowed us to prepare a much-improved manuscript and for this we offer our hearty thanks.

Reviewer #1 (Public Review):

Summary:

The organization of cell surface receptors in membrane nanodomains is important for signaling, but how this is regulated is poorly understood. In this study, the authors employ TIRFM single-molecule tracking combined with multiple analyses to show that ligand exposure increases the diffusion of the immune receptor FLS2 in the plasma membrane and its co-localization with remorin REM1.3 in a manner dependent on the phosphosite S938. They additionally show that ligand increases the dwell time of FLS2, and this is linked to FLS2 endocytosis, also in a manner dependent on S938 phosphorylation. The study uncovers a regulatory mechanism of FLS2 localization in the nanodomain crucial for signaling.

Strengths:

TIRFM single-molecule tracking, FRAP, FRET, and endocytosis experiments were nicely done. The role of S938 phosphorylation is convincing.

Weaknesses:

Question 1: The model suggests that S938 is phosphorylated upon flg22 treatment. This is actually not known.

Reply: Thank you for your expert comments. Although the phosphorylation of Ser-938 upon flg22 treatment is not known, the model presented in the manuscript is based on previous studies that have shown the importance of Ser-938 phosphorylation for the function of FLS2 (Cao et al, 2013). When it is mutated to the phosphorylation-mimicking residues aspartate or glutamate, immune responses remain normal. These findings suggest that the phosphorylation of Ser-938 plays a critical role in activating defense mechanisms upon flagellin detection (Cao et al, 2013). Now we added the results of Cao et al. (2013) to the introduction to strengthen in the revised manuscript.

Question 2: In addition, the S938D mutant does not show constitutively increased diffusion and co-localization with remorin. It is necessary to soften the tone in the conclusion.

Reply: We appreciate the valuable suggestions from the reviewer. Based on our findings, we observed that the phosphorylation of Ser-938 significantly impacts the dynamics of

flg22-induced FLS2. However, it does not alter the diffusion coefficient of FLS2 itself. In the revised manuscript, we have carefully adjusted the conclusion by softening the tone to reflect these findings.

Question 3: The introduction (only two paragraphs) and discussion are not properly written in the context of the current understanding of plant receptors in nanodomains. The authors basically just cited a few publications of their own, and this is not acceptable.

Reply: We accepted the criticisms here. Now, we have reworded the introduction and discussion sections to improve clarity. Furthermore, we have incorporated several new reports on plant receptors in nanodomains into the revised manuscript. Besides, we deleted some publications from our own group, while citing the latest references on plant receptors and nanodomains.

Reviewer #2 (Public Review):

Summary:

The research conducted by Yaning Cui and colleagues delves into understanding FLS2-mediated immunity. This is achieved by comparing the spatiotemporal dynamics of an FLS2-S938A mutant and FLS2-WT, especially in relation to their association with the remorin protein. To delineate the differences between the FLS2-S938A mutant and FLS2-WT, they utilized a plethora of advanced fluorescent imaging techniques. By analyzing surface dynamics and interactions involving the receptor signal co-receptor BAK1 and remorin proteins, the authors propose a model of how FLS2 and BAK1 are assembled and positioned within a remorin-specific nano-environment during FLS2 ligand-induced immune responses.

Strengths:

These techniques offer direct visualizations of molecular dynamics and interactions, helping us understand their spatial relationships and interactions during innate immune responses. Advanced cell biology imaging techniques are crucial for obtaining high-resolution insights into the intracellular dynamics of biomolecules. The demonstrated imaging systems are excellent examples to be used in studying plant immunity by integrating other functional assays. Weaknesses:

It's essential to acknowledge that every fluorescence-based method, just like biochemical assays, comes with its unique limitations. These often pertain to spatial and temporal resolutions, as well as the sensitivity of the cameras employed in each setup. Meticulous interpretation is pivotal to guarantee an accurate depiction and to steer clear of potential misunderstandings when employing specific imaging systems to analyze molecular attributes. Moreover, a discerning interpretation and accurate image analysis can offer invaluable guidance for future studies on plant signaling molecules using these nice cell imaging techniques. For instance, although single-particle analysis couldn't conclusively link FLS2 and remorin, FLIM-FRET effectively highlighted their ligand-triggered association and the disengagement brought on by mutations. While these methodologies seemed to present differing outcomes, they were described in the manuscript as harmonious. In reality, these differences could highlight distinct protein populations active in immune responses, each accentuated differently by the respective imaging techniques due to their individual spatial and temporal limitations. Addressing these variations is imperative, especially when designing future imaging explorations of immune complexes.

Reply: Thank you for your insightful comments and suggestions. We appreciate your expertise in fluorescence-based methods and the importance of careful interpretation and accurate image analysis. We agree with you that different imaging techniques may have their limitations and can highlight distinct aspects of protein dynamics and interactions.

In our study, we used single-particle analysis and FLIM-FRET to investigate the spatiotemporal dynamics of FLS2 and its association with remorin. While single-particle analysis did not conclusively link FLS2 and remorin, FLIM-FRET effectively highlighted their ligand-triggered association and the disengagement caused by mutations. We acknowledge that these techniques may have different spatial and temporal resolutions, leading to the discrepancy in their results. However, after the normalized treatment, we can provide very similar conclusions. Accordingly, we have revised the manuscript.

Reviewer #3 (Public Review):

Summary:

Receptor kinases (RKs) perceive extracellular signals to regulate many processes in plants. FLS2 is an RK that acts as a pattern-recognition receptor (PRR) to recognize bacterial flagellin and activate pattern-triggered immunity (PTI). PRRs such as FLS2 have been previously shown to reside within PM nanodomains, which can regulate downstream PTI signaling. In the current manuscript, Cui et al use single particle tracking to characterize the effect of previously-described phosphosite mutants (FLS2-S938A/D) on the PM organization, endocytosis, and signaling functions of FLS2. The authors confirm that FLS2-S938D but not -S938A is functional for flg22-induced responses, while also demonstrating that phosphodead mutation at this site (S938A) prevents flg22-induced sorting into nanodomains and endocytosis. These results are consistent with S938 being an important phosphorylation site for FLS2 function, however, they fall short of demonstrating that membrane disorganization of FLS2-938A is responsible for downstream signaling defects.

Strengths:

The authors' experiments (single particle tracking, co-localization, etc) do a good job of demonstrating how a non-functional version of FLS2 (S938A) does not alter its spatio-temporal dynamics, nanodomain organization, and endocytosis in response to flg22, suggesting that these require a functional receptor and are regulated by intracellular signaling components.

Weaknesses:

Question 1: The authors do not provide direct evidence that S938 phosphorylation specifically affects membrane organization, rather than FLS2 signaling more generally. All evidence is consistent with S938A being a non-functional version of FLS2, wherein an activated/functional receptor is required for all downstream events including membrane re-organization, downstream signalling, internalization, etc. Furthermore, the authors never demonstrate that this site is phosphorylated in planta in the basal or flg22-elicited state.

Reply: Sorry that we did not describe clearly in the original manuscript. In fact, we found in our study that the phosphorylation of the Ser-938 site influences the efficient sorting of FLS2 into AtRem1.3-associated microdomains rather than membrane organization, as depicted in Figure 2. Furthermore, we found that the immune responses are disrupted when Ser-938 is mutated to alanine, which is consistent with previously reported results (Cao et al, 2013). However, they remain normal when mutated to the phosphorylation-mimicking residues aspartate or glutamate. These results suggest that the phosphorylation of Ser-938 is crucial

for activating defense mechanisms upon flagellin detection. Although the phosphorylation of Ser-938 in plant at the basal or flg22-elicited state is not known, the model presented in the manuscript is based on the results of our current investigation together with those in the previous study that have shown the importance of Ser-938 phosphorylation for FLS2 function (Cao et al, 2013).

Question 2: As written, the manuscript also has numerous scientific issues, including a misleading/incomplete description of plant immune signaling, lack of context from previous work, and extensive use of inappropriate references.

Reply: We accept the criticism here. After reading the comments, we realized the problem. Now we have revised the misleading or incomplete description of plant immune signaling, added the context of previous works and deleted inappropriate references in the revised manuscript.

Reviewer #1 (Recommendations For The Authors):

Question 1: The description of the data has no details. How many biological repeats were done? How were statistical analyses done? What is the concentration of flg22? How was the calcium flux done (Fig. 4A)? The method also lacks details and relevant references.

Reply: We apologize for the lack of detail in presenting the data. Following your suggestion, we added comprehensive figure legends that provide clear explanations for each figure. Additionally, we included supplementary information on the measurement methods and references pertaining to calcium flux in the revised manuscript.

Question 2: Data in Fig. 4 basically repeated the 2013 PLoS Pathog paper. Why were these experiments even performed? Were GFP-tagged FLS2 lines used in these experiments? If this is the case, the data just verified that the GFP-tagged FLS2 functions as expected and should be moved to supporting data.

Reply: Thanks for the expert suggestions. In our study, we utilized GFP-tagged FLS2 lines to generate FLS2-S938 mutants and conducted experiments to investigate the flg22-induced immune response. Although some experiments in Figure 4 are similar to those reported (Cao et al, 2013), we provided a more detailed analysis of the immune response. The comprehensive analysis included early immune responses and late immune responses, e.g., the activation of a calcium burst, mitogen-activated protein kinases (MAPKs), the induction of immune-responsive genes and callose deposition, ultimately resulting in the inhibition of plant growth. As some results are analogous to the previous paper, we transfer some of the experiments as suggested, including the analysis of MAPKs and callose deposition, to the supporting data section of the revised manuscript.

Question 3: Flg22-induced FLS2-BAK1 association does not require S938, this is consistent with prior study that flg22 acts as a molecular glue for the ectodomains of FLS2 and BAK1 (Sun et al., 2013 Science). This needs to be cited.

Reply: Yes, we agree with the comment. Now we added an additional sentence in the revised manuscript: “ This aligns with the previous finding that flg22 acts as a molecular glue for FLS2 and BAK1 ectodomains (Sun et al., 2013).”

Question 4: Line 50, the references cited do not match what they say here.

Reply: We are sorry for the mistake in citing inappropriate references. In the revised manuscript, we deleted this sentence as well as the incorrect reference.

Question 5: Line 105, "flg22 can act as a ligand-like factor". It is a ligand!

Reply: Sorry for the mistake. Now, the sentence was corrected in the revised manuscript by deleting the word "like".

Question 6: Line 107, FLS2/BAK1 heterodimerization, not heterooligomerization.

Reply: Now we used "heterodimerization" to replace "heterooligomerization" in the revised manuscript.

Question 7: Line 114, are these really the best references to cite here?

Reply: After reading the comment, we found the references were not suitable here. Now we changed references by citing "(Martinière et al., 2021)" in the revised manuscript.

Question 8: Lines 123-124, the sentence is incomplete.

Reply: In the revised manuscript, we reworded the sentence to make it complete now. We changed "In a previous investigation, we demonstrated that flg22 induces FLS2 translocation from AtFlot1-negative to AtFlot1-positive nanodomains in the plasma membrane, implying a connection between FLS2 phosphorylation and membrane nanodomain distribution (Cui et al., 2018). To validate this, we assessed the association of FLS2/FLS2S938D/FLS2S938A with membrane microdomains, using AtRem1.3-associated microdomains as representatives (Huang et al., 2019)." in the revised manuscript.

Question 9: Lines 169-170, Why is this "most important"?

Reply: Sorry for the unsuitable description. As we have dramatically changed the manuscript, this sentence was deleted from the new version.

Reviewer #2 (Recommendations For The Authors):

Here are some specific areas of ambiguity in the study to be improved.

Question 1: Clarity in statistical analysis is necessary. Many figure legends omit details such as the sample size "n", and the nature of the measurements, like ROIs, images, and dots, the size of the seedlings, etc.

Reply: We appreciated this suggestion, which was raised by the reviewer I as well. Now, we provided the details for each figure, including the sample size, the nature of the measurements in the revised manuscript.

Question 2: Additional background about the choice of FLS2-S938 mutant would be beneficial, given that this mutant doesn't affect the BAK1 interaction but nullifies several PTI responses.

Reply: Yes, we agreed that some additional background is required for the FLS2-S938 mutant. Therefore, we added a sentence here: "FLS2 Ser-938 mutations impact flg22-induced signaling, while BAK1 binding remains unaffected, thereby suggesting Ser-938 regulates other aspects of FLS2 activity (Cao et al., 2013)." in the revised manuscript.

Question 3: A specific segment "... Using CLSM, Fluorescence Correlation Spectroscopy (FCS) and Western blotting, we found that the endocytic vesicles of FLS2S938D increased significantly after flg22 treatment (Figure 3B-3E)..." is not easy to follow. The author may

want to differentiate these methods and highlight them by indicting them as endocytic vesicle counting, receptor density on PM measurement by FCS, and WB-based protein degradation characterization to understand such mixed descriptions better. By the way, "Number of Endocytosis" should be "number of endocytic vesicles". Endocytosis is a process and uncountable.

Reply: We thank the reviewer for kindly reminding us to differentiate experimental methods. Therefore, we changed the sentences in the revised manuscript: "Employing confocal laser-scanning microscopy (CLSM) during 10 μ M flg22 treatment, we tracked FLS2 endocytosis and quantified vesicle numbers over time (Figure 3B). It is evident that both FLS2 and FLS2S938D vesicles appeared 15 min after-flg22 treatment, significantly increasing thereafter (Figure 3C). Notably, only a few vesicles were detected in FLS2S938A-GFP, indicating Ser-938 phosphorylation's impact on flg22-induced FLS2 endocytosis. Additionally, fluorescence correlation spectroscopy (FCS) (Chen et al., 2009) monitored molecular density changes at the PM before and after flg22 treatment (Figure S3F). Figure 3D shows that both FLS2-GFP and FLS2S938D-GFP densities significantly decreased after flg22 treatment, while FLS2S938A-GFP exhibited minimal changes, indicating Ser-938 phosphorylation affects FLS2 internalization. Western blotting confirmed that Ser-938 phosphorylation influences FLS2 degradation after flg22 treatment (Figure 3E), consistent with single-molecule analysis findings." Besides, we also changed "number of endocytosis" to "the number of endocytic vesicles" in Figure 3C as suggested.

Question 4: In Figure 1 E, a discrepancy exists where the total percentages in the red and black columns don't sum up to 100%, while other groups look right. This needs clarification.

Reply: We are sorry for our carelessness in making the data incomplete. Now we thoroughly supplemented, collated, and rechecked the data in Figure 1E. Due to an oversight during the production of the figure, some data was inadvertently omitted, resulting in the red column not reaching 100%. Besides, we checked the data in the black column again, and the total percentage indeed added up to 100%.

Question 5: Although Figure 1F uses UMAP analysis to differentiate between FLS2WT and A mutants, only data pertaining to the "D" mutant is shown.

Reply: Thank you for the expert comments. Because there are several images in Figure 1, we only selected the data related to the "D" mutant as a representative for display. As suggested, we have added all the UMAP images in the revised supplement figure S1F.

Question 6: There are apparent inconsistencies in the FRAP results, particularly regarding the initial recovery points post-bleaching. A detailed statistical analysis, supplemented with FRAP images over time, should be included for clarity. Were they bleached to a similar ground level before monitoring their recovery? The data points from "before" and "after" bleaching were not shown. I found the red and blue curves showed similar recovery slop, which suggests no long-distance movement changes for all three FLS2 versions, with or without flg22. This is opposite from the conclusions made by the author.

Reply: Thank you for the expert comments. After reading the comments, we recognized this terrible problem. Therefore, we carried out a new FRAP experiment. The new results showed that, following complete bleaching of three samples of FLS2 to ground level, the recovery rates of FLS2 and FLS2S938D under flg22 treatment were significantly higher compared to the control group (Fig. 1G). In contrast, the recovery rates of the FLS2S938A-GFP after flg22 treatment remain similar to that before treatment (Fig. 1G), indicating that the Ser-938 phosphorylation site indeed affects the flg22-induced lateral diffusion of FLS2 at the PM. The

new results are basically consistent with the motion range of single-molecule results, which is not contradictory to long-distance movement changes. Accordingly, we incorporated the new time-lapse FRAP images into Figure 1G and S1B.

Question 7: There's a potential typo in Figure 1B regarding the bar size. It could neither possibly be 200 μm nor 200 nm. Figure 1A also needs a scale bar.

Reply: Apologies for the mistake. We now corrected “200 μm ” to “2 μm ”. Besides, we also included a scale bar in Figure 1A in the revised manuscript.

Question 8: Due to the unreliable tracking for a long-time by Imaris, the authors analyzed the tracks within 10s and quantified very short live particles under 4s. Such 4s surface retention for a receptor does not seem to match functional endocytic internalization time for cargo. Even after the endocytic adaptor module recruitment, it would take at least more than 10s to finish the internalization. In the field of endocytosis, these events are often described as abortive endocytic events. However, the disappearance of cargoes, FLS2 in this case, indicates internalization into the cytoplasm, which is interesting. May the author discuss more on how these short events analyzed enhance our understanding of the functional behavior of FLS2?

Reply: We greatly appreciated the valuable comments provided by the reviewer. After thorough consideration, we acknowledged that in our original manuscript, we failed to distinguish the short-lived from the long-lived particles and vaguely put them collectively into the internalized particles. We realized that and it is inappropriate to ambiguously categorize all particles as internalized. Therefore, we added the sentence “Additionally, numerous FLS2 exhibited short-lived dwell times, indicating abortive endocytic events associated with the endocytic pathway and signal transduction (Bertot et al., 2018)” in the revised manuscript.

Question 9: Figure 2D should be comprehensive, presenting data for the WT, A, and D versions.

Reply: Yes, we agreed with the suggestions. Now, we added several representative images for the WT, A, and D versions in the revised manuscript.

Question 10: In Figure 2D, TIRM-SIM should be a typo and rectified to TIRF-SIM. Also, a detailed explanation of the TIRF-SIM setup and its specifics would be important. The imaging approach of SIM, especially the time duration for finishing all frames before reconstruction, is essential to rationalize its use in capturing and measuring an appropriate speed range of particle movement. May the author elaborate on the technique details and the use of TIRF-SIM for colocalization analysis? To clarify these, the author may provide additional TIRF-only movies of FLS2 (WT, A, D) and AtRem1.3 for comparison with TIRF-SIM still images.

Reply: Sorry for the mistake. In the revised manuscript, we have corrected “TIRM-SIM” to “TIRF-SIM”. In order to rationalize its use in capturing and measuring an appropriate speed range of particle movement, we included a more detailed description of the imaging approach and the colocalization analysis of TIRF-SIM in the Materials and Methods section as follows: “The SIM images were taken by a 60 × NA 1.49 objective on a structured illumination microscopy (SIM) platform (DeltaVision OMX SR) with a sCMOS camera (Camera pixel size, 6.5 μm). The light source for TIRF-SIM included diode laser at 488 nm and 568 nm with pixel sizes (μm) of 0.0794 and 0.0794 (Barbieri et al., 2021). For the dual-color imaging, FLS2/FLS2S938A/FLS2S938D-GFP (488 nm/30.0%) and AtRem1.3-mCherry (561 nm/30.0%) were excited sequentially. The exposure time of the camera was set at 50 ms throughout

single-particle imaging. The time interval for time-lapse imaging was 100 ms, the total time was 2s, and the total time points were 21s. The Imaris intensity correlation analysis plugin was used to calculate the co-localization ratio.” in the revised manuscript. Furthermore, we provided additional TIRF-SIM movies of FLS2 (WT, A, D) and AtRem1.3.

Question 11: The colocalization displayed in Figure 2D is hard to tell. A colocalization ratio of FLS2-AtRem1.3 is shown as ~0.8%, which has only ~0.2% difference from the flg22-treated condition. "n" of Figure 2F should be specified in the legend, such as a line with a specific length, or an ROI with a specific area size.

Reply: Thank you for the expert comments. Although the increased colocalization after flg22 treatment is not high, the change is statistically significant as compared with the wild type. We agreed that every fluorescence-based method, like biochemical analysis, has its own unique limitations, which were raised by the Reviewer #2 (Public Review) as well. In order to provide strong evidence, we also carried out the FLIM-FRET experiment as a supplement, which can effectively detect their ligand-triggered association or disassociation. From figure 2G and H, we clearly found that the co-localization of FLS2/FLS2S938D-GFP with AtRem1.3-mCherry significantly increase in response to flg22 treatment (FLS2-GFP control: 2.45 ± 0.019 s; FLS2-GFP flg22-treated: 2.39 ± 0.016 s; FLS2S938D-GFP control: 2.42 ± 0.010 ns; FLS2S938D-GFP flg22-treated: 2.35 ± 0.028 ns). In contrast, FLS2S938A-GFP shows no significant changes (control: 2.53 ± 0.011 ns; flg22-treated: 2.56 ± 0.013 ns), indicating that Ser-938 phosphorylation influences efficient sorting of FLS2 into AtRem1.3-associated microdomains. Following the suggestion of the reviewer, we now rearranged the order of 2E and 2F, in which N represents the entire image region used for analysis rather than a specific region of interest.

Question 12: I appreciate the nice results of the FLIM-FRET results for FLS2-Rem1.3. Figure 2H should be supplemented with additional representative images of all FLS2 variants including WT and mutants.

Reply: Thanks for your warm encouragement. As suggested, we added all the representative images in the revised manuscript.

Question 13: The unit of the X-axis of Figure 2E can not be pixel. Should it be, μm ? In the method, the author could specify the camera model and magnification for TIRF-SIM to understand pixel size of the image better.

Reply: Sorry for the mistake here. Indeed, the unit of the X-axis in Figure 2E should be μm . Now we correct this mistake in Figure 2E in the revised manuscript. Besides, we included a detailed description of the imaging approach of TIRF-SIM in the Materials and Methods section as follows: “The SIM images were taken by a $60 \times$ NA 1.49 objective on a structured illumination microscopy (SIM) platform (DeltaVision OMX SR) with a sCMOS camera (Camera pixel size, $6.5 \mu\text{m}$)”.

Question 14: "... as shown in A..." in Figure Legend 2E should be "... as shown in D..."

Reply: Thanks for pointing out this mistake. In the revised manuscript, we used “as shown in D” to replace “as shown in A”.

Question 15: I recommend that the authors exercise caution when drawing conclusions based on the Rem1.3 data and when representing the "microdomain" concept in their final model. While Rem1.3 punctate is a nanometer-sized protein cluster specific to its identity, its shape can be categorized as a nanodomain. Conceptually, however, it neither

universally represents all nanodomains nor microdomains, as depicted in Figure 4. We should exercise caution to prevent providing misleading information to the field.

Reply: We thank the reviewer for expert comments. To avoid misleading conclusions, we changed “nanodomains” to “AtRem1.3-associated microdomains” in the revised manuscript. Besides, we have also made modifications to Figure 4.

Reviewer #3 (Recommendations For The Authors):

Question 1: The manuscript needs to be extensively re-written and has severe issues as-is. Many references are either not quite appropriate or are completely unrelated to the use in the text. In general, the current state-of-the-art of PTI and RK signaling is not correctly described or incorporated.

Reply: We accepted the criticisms here. As suggested, we thoroughly rewrote the manuscript to address the concerns raised. Furthermore, we have thoroughly checked and revised the manuscript by removing 21 irrelevant references and adding 30 relevant references. We also incorporated the most up-to-date descriptions of the PTI and RK signaling pathways.

Question 2: Receptor-like kinase (RLK) should generally be receptor kinase (RK) as receptor functions are now well established.

Reply: Yes, we agreed with your expert comment here. Now, we changed “Receptor-like kinase (RLK)” into “receptor kinase (RK)” in the revised manuscript.

Question 3: Line 20 - is this really true?

Reply: Sorry for the mistake. In the revised manuscript, we changed “However, the mechanisms underlying the regulation of FLS2 phosphorylation activity at the plasma membrane in response to flg22 remain largely enigmatic.” to “However, the dynamic FLS2 phosphorylation regulation at the plasma membrane in response to flg22 needs further elucidation.”

Question 4: S938D sorts better in response to Flg22; S938A is unaffected - suggests phosphorylation of S938 is not dynamic in response to Flg 22 but is required for pre-elicitation sorting. Overall, there is a chicken-and-egg problem in this paper: which comes first, immune/signalling functionality or nanodomain sorting? And which is explaining the defects of S938A?

Reply: We thank the reviewer for expert suggestions. In fact, the previous studies showed that membrane microdomains serve as signaling platforms that mediate cargo protein sorting and protein-protein interactions in a variety of contexts (Goldfinger et al. 2017). Since our previous research showed that the disruption of membrane microdomains affected flg22-induced immune signaling (Cui et al. 2018), we speculate that the immune signal occurred after entering the membrane microdomains.

As shown in Figure 1 and 2, ligand exposure leads to an increase in diffusion coefficient and enhanced co-localization with REM1.3, both of which are dependent on the phosphorylation of the Ser-938 site. Deducing from these results, we inferred that the defects in S938A resulted largely from its failure to sort into membrane microdomains. The phosphorylation of the Ser-938 site can regulate FLS2 into functional AtRem1.3-associated microdomains, thereby affecting flg22-induced plant immunity.

Question 5: Line 37 conserved, not conservative (though not technically true - the domain organization is conserved but the ECDs are not conserved).

Reply: Thank you for pointing this mistake out. In the revised manuscript, we used “conserved” to replace “conservative”.

Question 6: Lines 40-42 - not all phosphorylation sites are within the kinase domain, for example, sites are well-described on the JM and/or C-tail regions outside of the kinase domain.

Reply: We accepted the criticisms here. We have corrected the sentence to “with phosphorylation sites mainly located in PKC” in the revised manuscript.

Question 7: Line 42 - what is BIK1? Intro to relevant topics is severely lacking.

Reply: Sorry for the incomplete introduction here. We added the relevant introduction of BIK1 by adding that “Upon recognizing flg22, FLS2 interacts with the co-receptor Brassinosteroid-Insensitive 1-associated Kinase 1 (BAK1), initiating phosphorylation events through the activation of receptor-like cytoplasmic kinases (RLCKs) such as BOTRYTIS-INDUCED KINASE 1 (BIK1) to elicit downstream immune responses (Chinchilla et al., 2006; Li et al., 2016b; Majhi et al., 2021).” in the revised manuscript.

Question 8: Lines 42-44 - not sure this sequence of events is being properly described (e.g. BIK1 release is unlikely to precede activation by BAK1/SERKs).

Reply: We apologize for not expressing this sentence clearly. Now, we reworded the sentence: “Upon recognizing flg22, FLS2 interacts with the co-receptor Brassinosteroid-Insensitive 1-associated Kinase 1 (BAK1), initiating phosphorylation events through the activation of receptor-like cytoplasmic kinases (RLCKs) such as BOTRYTIS-INDUCED KINASE 1 (BIK1) to elicit downstream immune responses (Chinchilla et al., 2006; Li et al., 2016b; Majhi et al., 2021).” in the revised manuscript.

Question 9: Line 61 - S938 was identified by Cao et al (2013) based on in vitro MS, but was functionally validated using genetic assays, not based on MS.

Reply: Thank you for your comments. Now, we changed the sentence: “In vitro mass spectrometry (MS) identified multiple phosphorylation sites in FLS2. Genetic analysis further identified Ser-938 as a functionally important site for FLS2 in vivo (Cao et al., 2013).” in the revised manuscript.

Question 10: Line 68-69 - phospho-dead and phospho-mimic, not phosphorylated/non-phosphorylated.

Reply: We thank the reviewer for expert suggestions. In the revised manuscript, we changed the sentence by replacing “phosphorylated/non-phosphorylated” with “phospho-mimic” and “phospho-dead”.

Question 11: Lines 104-106 - this is wildly misleading. Flg22 is more than a ligand-like factor, as it is a bona fide ligand, and the heterodimerization with BAK1/SERKs is extremely well-established (and relevant foundational papers should be cited here in place of the authors' previous work).

Reply: We apologize for the incorrect expression here. After reading the comments, we realized the problem which was raised by the reviewer I as well. Now, we changed “ligand-like factor” to “ligand”. Besides, we cited the new references “(Orosa et al., 2018)” to replace the references of our group in the revised manuscript.

Question 12: Lines 107-112 - again, this is confusing. There is a decade of (uncited, undiscussed) work previously establishing that heterodimerization of RK-co-receptor complexes is mediated by extracellular ligand binding and independent of intracellular phosphorylation.

Reply: We thank the reviewer for expert suggestions. Now, we added several sentences in the revised manuscript: “Therefore, we further investigated if Ser-938 phosphorylation affects FLS2/BAK1 heterodimerization. Tesseler segmentation, FRET-FLIM, and smPPI analyses revealed no impact of Ser-938 phosphorylation on FLS2/BAK1 heterodimerization (Figure 2A-C and S2). This aligns with the previous finding that flg22 acts as a molecular glue for FLS2 and BAK1 ectodomains (Sun et al., 2013), confirming the independence of FLS2/BAK1 heterodimerization from phosphorylation, with these events occurring sequentially.”

Question 13: Line 119 - this is the wrong citation - Yu et al 2020 is a review and does not cover RALFs; correct citation is Gronnier et al 2022 eLife.

Reply: In the revised manuscript, we updated the reference from “(Yu et al., 2020)” to “(Gronnier et al., 2022)”.

Question 14: Lines 123-124 - this sentence is incomplete.

Reply: Sorry for the incomplete sentence. Now we reworded the sentence to “In a previous investigation, we demonstrated that flg22 induces FLS2 translocation from AtFlot1-negative to AtFlot1-positive nanodomains in the plasma membrane, implying a connection between FLS2 phosphorylation and membrane nanodomain distribution (Cui et al., 2018). To validate this, we assessed the association of FLS2/FLS2S938D/FLS2S938A with membrane microdomains, using AtRem1.3-associated microdomains as representatives (Huang et al., 2019).” in the revised manuscript.

Question 15: Line 126 - this requires a reference.

Reply: Yes, we added a new reference: “(Huang et al., 2019)” in the revised manuscript.

Question 16: Lines 125-128 - should clarify that the authors are not looking at direct interaction between FLS2 and REM1.3.

Reply: Sorry for the inappropriate expressions here. In the revised manuscript, we reworded the sentence as follows: “To validate this, we assessed the association of FLS2/FLS2S938D/FLS2S938A with membrane microdomains, using AtRem1.3-associated microdomains as representatives (Huang et al., 2019)”.

Question 17: Line 138 - these are odd references to use for such a broad statement.

Reply: Now the inappropriate references cited here have been deleted.

Question 18: Line 161 - incorrect reference, again.

Reply: Sorry for this mistake. In the revised manuscript, we reworded the sentence and changed the reference.

Question 19: Lines 160-165 - this is very confusing and misleading. I would suggest just having a short section introducing PTI earlier on (with appropriate references).

Reply: As suggestion, we reworded and added a section in the revised manuscript as follows: “PTI plays a pivotal role in host defense against pathogenic infections (Lorrai et al., 2021; Ma et al., 2022). Previous studies demonstrated that FLS2 perception of flg22 initiates a complex signaling network with multiple parallel branches, including calcium burst, mitogen-activated protein kinases (MAPKs) activation, callose deposition, and seedling growth inhibition (Baral et al., 2015; Marcec et al., 2021; Huang et al., 2023). Our focus was to investigate the significance of Ser-938 phosphorylation in flg22-induced plant immunity. Figure 4A-F illustrates diverse immune responses in FLS2 and FLS2S938D plants following flg22 treatment. These responses encompass calcium burst activation, MAPKs cascade reaction, callose deposition, hypocotyl growth inhibition, and activation of immune-responsive genes. In contrast, FLS2S938A (Figure S4A-D) exhibited limited immune responses, underscoring the importance of Ser-938 phosphorylation for FLS2-mediated PTI responses”.

Question 20: Line 166 - these are not appropriate references, again.

Reply: Thank you for the suggestion. In the revised manuscript, we removed the inappropriate references. Besides, we added new references by citing: “(Baral et al., 2015; Marcec et al., 2021)”.

Question 21: Lines 169-173 - this is not relevant, the inhibition of growth by elicitors is extremely well-documented (though not by the refs cited here).

Reply: We reworded the sentence and deleted the inappropriate reference in the revised manuscript.

Question 22: Lines 174-175 - I don't see why this is unexpected, as nanodomain organization of PRRs has been previously described.

Reply: Sorry for the inappropriate expressions here. As we have dramatically changed the manuscript, this sentence was deleted from the new version.

References we added into the revised manuscript

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