S-acylation stabilizes ligand-induced receptor kinase complex formation during plant pattern-triggered immune signalling.

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Summary

Plant receptor kinases are key transducers of physical extracellular stimuli, such as the presence of beneficial or pathogenic microbes or secreted signalling molecules. Receptor kinases are regulated by numerous post-translational modifications. Here, using the bacterial flagellin perceiving receptor kinase FLS2, we show that S-acylation at an evolutionarily conserved cysteine is crucial for function. S-acylation involves the addition of long-chain fatty acids to cysteine residues within proteins, altering their biophysical properties and behaviour within the membrane environment. We observe S-acylation of FLS2 at C-terminal kinase domain cysteine residues within minutes of treatment with flg22 ligand, and in a BAK1 co-receptor dependent manner. We demonstrate that S-acylation is essential for FLS2-mediated immune signalling, including anti-bacterial immunity. Similarly, mutating the corresponding conserved cysteine residue in the immune receptor kinase EFR supressed elf18 mediated signalling. Biochemical analysis of unstimulated and activated FLS2 containing complexes using detergents and native membrane DIBMA nanodiscs indicates that S-acylation assists the stabilization of activated receptor kinase complexes withing the membrane environment to increase signalling efficiency.

Key words

S-acylation; palmitoylation; Receptor-kinase; Receptor-like kinase; FLS2; EFR; microdomain; nanodomain; membrane; Arabidopsis

Introduction

The plasma membrane defines the boundary between the cell interior and the external environment. Receptor kinases (RKs) found in the plasma membrane act as the principle means of perception for most of the stimuli that a plant encounters, such as hormones, signalling peptides or microbe associated molecular patterns (MAMPs). RKs comprise the largest single gene family in plants [1, 2] and are central to current efforts to breed or engineer crops able to withstand emerging pathogen threats, interact with beneficial microbes or better tolerate abiotic stress [3-6]. Understanding the mechanisms and principles underlying the formation and activation of RKs complexes is therefore critical to informing these approaches.

The RK FLAGELLIN SENSING 2 (FLS2) is the receptor for bacterial flagellin and the flagellin-derived peptide flg22 [7], and is an archetype for RK research, particularly in the area of host-microbe interactions. flg22 binding to the extracellular FLS2 leucine-rich repeats promotes interaction with the extracellular leucine-rich repeats of the co-receptor BAK1/SERK3, with flg22 acting as molecular glue between FLS2 and BAK1. Subsequent transphosphorylation of FLS2 by BAK1 initiates a cascade of immune signalling to activate anti-bacterial defence responses. As part of this overall process, flg22 binding by FLS2 has been shown to dictate FLS2 phosphorylation, SUMOylation and ubiquitination state, indicating a high degree of post-translational regulation. FLS2 activation also alters overall complex composition [7-17], biophysical properties [18] and behaviour [19, 20] of the complex. However, the underlying mechanisms and functional relevance of these changes remain unknown.

S-acylation is a reversible post-translational modification, whereby long chain fatty acids are added to cysteine residues by protein S-acyl transferases [21] and removed by acyl-protein thioesterases [22]. This modification can lead to changes in protein trafficking, stability, and turnover. S-acylation has been proposed to drive membrane phase partitioning [23, 24] while changes in protein S-acylation state have been hypothesised to modulate protein-protein and protein-membrane interactions, or even alter protein activation states [25]. However, direct experimental evidence to support these ideas is lacking. We recently discovered that FLS2, alongside all other plant RKs tested, is post-translationally modified by S-acylation [26]. Here we demonstrate that S-acylation of FLS2, at a site conserved in all RKs across plants, acts as a positive regulator of signal transduction. Mechanistically, this appears to be driven by S-acylation induced changes to the physical properties of the protein complex, resulting in enhanced stability within its lipid environment and more efficient signal propagation.

Results

FLS2 undergoes ligand responsive S-acylation.

Our previous analysis of FLS2 identified the juxta-transmembrane (TM) domain cysteines (Cys830,831) as being constitutively S-acylated, but this modification was dispensable for FLS2 function [26]. All RK superfamily members subsequently tested, with or without a juxta-TM S-acylation site homologous to FLS2 $C^{830,831}$, also appear to be S-acylated [26]. This indicates that non-juxta-TM S-acylation sites, potentially conserved in all RKs, exist. Other post-translational modifications affecting FLS2, and the broader RK superfamily, including phosphorylation [27], ubiquitination [11] and SUMOylation [17] are all responsive to ligand binding. Given the dynamic nature of S-acylation [22] we were interested to determine whether FLS S-acylation state is also ligand responsive. In Col-0 wild type plants treated with the eliciting peptide flg22, FLS2 S-acylation increased by almost 60% above basal levels following 20-min exposure to flg22. FLS2 S-acylation subsequently returned to basal levels within 1 h (figures 1A and S1A). Consistent with its ligand-dependency, FLS2 S-acylation was contingent upon the FLS2 co-receptor BAK1 (BRI1-ASSOCIATED KINASE) (figure 1B). Additionally, flg22 induced S-acylation of FLS2 was unaffected in *chc2-1* mutants [15] of clathrin heavy chain 2, indicating that S-acylation occurs before endocytosis (figure 1C). Treatment of Arabidopsis Col-0 plants with elf18, a conserved peptide derived from bacterial elongation factor Tu, recognised by the RK EFR (ELONGATION FACTOR-Tu RECEPTOR) that acts similarly to FLS2 [28], failed to elevate FLS2 S-acylation (figure 1D). This demonstrates that the increase in FLS2 S-acylation is specifically linked to activation of FLS2 signalling and not a general phenomenon related to activation of RK-mediated defence responses.

FLS2 flg22 responsive S-acylation sites are conserved in the wider Receptor kinase superfamily.

FLS2 $C^{830,831}S$ mutants [26] showed a similar elevation of S-acylation in response to flg22 (figure S1B). FLS2 therefore contains S-acylation sites in addition to $C^{830,831}$ that are responsive to ligand perception. While FLS2 $C^{830,831}S$ expressed at native levels in unstimulated Arabidopsis is not S-acylated [26], we noted that FLS2 $C^{830,831}S$ is weakly S-acylated in the absence of flg22 when overexpressed in *Nicotiana benthamiana*. Mutation of FLS2 Cys 1132 and 1135 in addition to Cys 830 and 831 (FLS2 $C^{830,831,1132,1135}S$) abolished FLS2 S-acylation compared to FLS2 $C^{830,831}S$ (figure

109 1E) when expressed in *N. benthamiana*, suggesting that Cys 1132 and 1135 are sites of S-acylation.
110 Accordingly, *fls2c/proFLS2:FLS2 $C^{1132,1135}S$* Arabidopsis plants (figure S1C) showed no increase in S-
111 acylation following flg22 treatment (figure 1F). Interestingly, 1-2 conserved cysteine residues at
112 the C-terminus of the kinase domain (corresponding to FLS2 Cys 1132 and/or 1135) are conserved
113 across RKs in *Arabidopsis thaliana* (figure S2) and the broader Streptophyte lineage, suggesting a
114 conserved and important role for these cysteines.
115
116 **Receptor kinase C-terminal S-acylation enhances early immune signalling through FLS2 and EFR**
117 Consistent with the evolutionarily conserved nature of the FLS2 S-acylated cysteines amongst RKs,
118 *fls2c/proFLS2:FLS2 $C^{1132,1135}S$* plants are impaired in several aspects of early immune signalling,
119 such as reactive oxygen species production, MAP kinase activation and pathogen responsive gene
120 expression (figure 2A, B, C). Both FLS2 and FLS2 $C^{1132,1135}S$ show similar accumulation at the plasma
121 membrane (figure S3A), lateral membrane mobility (figure S3B, C), and association with REM1.3
122 nanodomains (figure S3D, E, F), indicating that there is no aberrant basal cellular behaviour of the
123 FLS2 $C^{1132,1135}S$ mutant that could be impacting responses to flg22. To determine whether the
124 conserved C-terminal cysteines have a general role in RK function, we mutated the equivalent
125 cysteine in EFR (Cys975, figure S2) to serine and transiently expressed EFR-GFP [29] and EFR $C^{975}S$-
126 GFP in *N. benthamiana.* Elicitation of EFR action with elf18 demonstrated that MAP kinase
127 activation and immune gene expression was reduced in EFR $C^{975}S$-GFP expressing plants
128 compared to EFR-GFP (figure 2C, D). This indicates that mutation of the conserved C-terminal
129 cysteine in both FLS2 and EFR has a similar effect on early outputs. Structural homology modelling
130 of FLS2 indicates that the $C^{1132,1135}S$ mutation does not affect FLS2 kinase domain structure (figure
131 S4). Kinase activity is also dispensable for activation of signalling by EFR [30]. The observed effects
132 of the FLS2 $C^{1132,1135}S$ and EFR $C^{975}S$ mutation on early signalling therefore cannot readily be
133 explained by effects on kinase activity or structure.
134
135 **FLS2 kinase domain S-acylation is required for sustained signalling and bacterial immunity**
136 Early signalling outputs resulting from bacterial perception by FLS2 lead to longer term sustained
137 responses to promote immunity. In line with decreased early immune responses, later flg22-
138 induced gene expression and physiological outputs, such as *PR1* (PATHOGENESIS-RELATED GENE
139 1) expression and seedling growth inhibition, were affected in *fls2c/proFLS2:FLS2 $C^{1132,1135}S$* plants
140 (figure 3A, B). As a result of these cumulative signalling defects, FLS2 $C^{1132,1135}S$ failed to
141 complement the hyper-susceptibility of *fls2* mutant plants to the pathogenic bacterium
142 *Pseudomonas syringae* pv. tomato (*Pto*) DC3000 (figure 3C).
143
144 **S-acylation of FLS2 stabilizes flg22 induced FLS2-BAK1 signalling complexes within the**
145 **membrane**
146 Differential solubility in cold non-ionic detergents such as IGEPAL CA-630, leading to formation of
147 detergent soluble or resistant membrane fractions (DSM and DRM respectively), has been used
148 to characteries changes to protein biophysical properties, particularly in the context of protein S-
149 acylation [18, 31]. Following flg22 treatment, FLS2 abundance in cold IGEPAL CA-630 derived
150 DRMs showed a slight reduction, while FLS2 $C^{1132,1135}S$ DRM abundance decreased by ~50% (figure

151 4A, B). Overall, while FLS2 containing complex solubility in cold IGEPAL CA-630 fractionally
152 increases upon activation, suggesting a change in complex protein composition and/or
153 surrounding membrane order, loss of S-acylation has a dramatic effect. These data indicate that
154 FLS2 S-acylation is a major contributor to the overall biophysical properties of the stimulated FLS2
155 containing protein complex when considered in the context of its membrane environment.
156
157 Assessment of flg22-induced FLS2-BAK1 complex formation by co-immunoprecipitation following
158 solubilisation with cold IGEPAL CA-630 [32] indicated that FLS2-BAK1 interaction was reduced or
159 less stable in FLS2 $C^{1132,1135}S$ mutants (figure 4C). Furthermore, flg22-induced BAK1 S^{612} auto-
160 phosphorylation [33], used as a marker of *in vivo* complex formation, was also consistently weaker
161 in FLS2 $C^{1132,1135}S$-expressing plants (figure 4C), supporting these biochemical observations. DRM
162 and co-immunoprecipitation data combined suggests a role for DRM stabilization of observed
163 FLS2-BAK1 complexes during co-immunoprecipitation. This indicates that FLS2 S-acylation alters
164 the biophysical properties of FLS2 that will in turn impact upon the strength or stability of FLS2-
165 BAK1 interactions in the context of cellular membranes.
166
167 In contrast to IGEPAL CA-630, diisobutylene/maleic acid (DIBMA) copolymer disrupts cellular
168 membranes in an unbiased manner and does not form DRM-like fractions. DIBMA disrupts all
169 lipid-lipid, but not protein-protein or protein-lipid, interactions to form native membrane
170 nanodiscs containing proteins and their higher order complexes. Loose conformations of protein
171 transmembrane domains within a complex will therefore show reduced co-purification compared
172 to tightly packed transmembrane domains [34]. Comparing the behaviour of proteins and
173 complexes following solubilization with either IGEPAL CA-630 or DIBMA allows for biochemical
174 character and properties to be determined. Using DIBMA to solubilize flg22-induced FLS2-BAK1
175 complexes prior to co-immunoprecipitation (figure 4D) indicates that FLS2-BAK1 complexes are
176 stabilized by protein-protein and protein-lipid interactions that are reduced or absent from FLS2
177 $C^{1132,1135}S$-BAK1 complexes. This indicates that S-acylation induced changes to the physical
178 character of FLS2 promote assembly of a DIBMA resistant complex with tightly packed
179 transmembrane domains.
180
181 **Discussion**
182 FLS2, a prototypical RK, has been shown here to require flg22 ligand-induced S-acylation at
183 Cys1132,1135 for efficient flg22-triggered signalling and resistance to bacterial infection. FLS2 S-
184 acylation occurs within minutes of flg22 perception and requires the co-receptor BAK1, but
185 precedes FLS2 entry into the endocytic pathway (figure 1). Supporting this timescale, preventing
186 FLS2 S-acylation from occurring impairs early signalling outputs, such as the phosphorylation of
187 MAPK and the production of ROS (figure 2). This failure to activate initial signalling also explains
188 the defects in subsequent signalling outputs such as PR1 induction, growth inhibition and,
189 ultimately, resistance to pathogenic bacteria (figure 3). Sequence analysis of RKs from across the
190 Streptophyte lineages indicate that the S-acylation site identified here at the C-terminus of the
191 FLS2 kinase domain is conserved throughout evolutionary history. Mutation of the equivalent
192 cysteine in EFR (Cys975) recapitulates the defects observed in S-acylation defective FLS2,

193 indicating a conserved function for this site. Recently the P2K1/DORN1/LecRK-I.9 RK was
194 proposed to undergo de-S-acylation followed by re-S-acylation during immune responses [35].
195 However, the site proposed is unique to the LecRK family, being distinct in function, location,
196 sequence, and structure to the universally conserved cysteine identified here that is also present
197 in P2K1 but was not considered in the previous work. These data demonstrate that, in common
198 with other post-translational modifications, S-acylation may affect multiple sites within an RK with
199 differing effects on RK function. The position and effect of the S-acylation site identified here at
200 the C-terminus of the FLS2 and EFR kinase domains is highly conserved. This opens up the exciting
201 possibility that S-acylation at the conserved C-terminal kinase site may potentially regulate the
202 function of all RKs across plants in a similar manner to FLS2 and EFR.
203
204 RK signalling is initiated by binding of a ligand (e.g., flg22) to its receptor (e.g., FLS2), which then
205 facilitates the binding of a co-receptor (e.g., BAK1/SERK3). While this constitutes the minimal
206 ligand recognition complex, substantial evidence supports a far larger number of proteins being
207 intimately associated with both unstimulated and activated receptors and co-receptors. Indeed,
208 existing data indicates that during the process of activation RKs recruit or eject specific proteins
209 from their complexes [10, 16, 36, 37], but precise molecular mechanisms determining these
210 changes are not known. Live cell imaging of unstimulated FLS2 and BAK1 indicates that complex
211 composition, specifically presence or absence of the RK FERONIA (FER), has marked effects on
212 nanoscale organisation and mobility of in the plasma membrane. In addition, activation of the RK
213 FERONIA (FER) by its ligand RALF23 alters BAK1 organisation and mobility [20]. This indicates that
214 both complex composition, and the activation state of individual components, affects behaviour
215 of the whole complex. Changes in direct protein-protein interaction can be explained by allosteric
216 effects. However, it is also possible that alteration of the immediate lipid environment
217 composition, micro-curvature, or structure, brought about by changes in the biophysical
218 properties of the complex, would act to recruit or exclude proteins based on their solubility and
219 packing in the membrane environment surrounding the complex. This is, in essence, one of the
220 biochemical principles proposed to underlie the formation of membrane nanodomains [38].
221 Activation of FLS2 by flg22 has been reported to decrease overall plasma membrane fluidity and
222 increase plasma membrane order [39], while changing sterol abundance in the plasma membrane
223 affects all stages of FLS2 signalling [40]. This indicates that membrane composition and structure
224 have profound effects on receptor complex function and supports the principle of protein-lipid
225 interactions affecting or effecting RK function. S-acylation, being a fatty acid-based modification
226 of proteins, has been shown to affect protein biophysical character and behaviour in membrane
227 environments [31, 41]. S-acylation also affects membrane micro-curvature [24], a key theoretical
228 determinant of membrane component partitioning required for nanodomain formation [38]. S-
229 acylation therefore represents an ideal mechanism to not only modulate interactions between RK
230 complexes and their proximal membrane components, but also to effect changes in the
231 composition of both.
232
233 Altogether our data suggest that flg22-induced, BAK1-dependent S-acylation influences the
234 biophysical properties of FLS2. Our data supports a model where FLS2 S-acylation changes the

transmembrane domain packing or order within the FLS2 complex and alters the FLS2 complex lipid microenvironment. This process stabilises signalling active FLS2 molecular assemblies. These data therefore provide a mechanistic basis for the observed phenomenon of activation state dependent changes in membrane nanodomain content and organisation described for various RKs, including FLS2 [18, 20, 40, 42].

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Author Contributions:

CRediT statement

CHH: Conceptualization, Methodology, Validation, Formal analysis (Equal), Investigation (Lead), Data curation (Equal), Writing - Review & Editing, Visualization. **DT**: Methodology, Validation, Investigation (Equal), Writing - Review & Editing. **JG**: Methodology, Formal analysis (Equal), Investigation (Equal), Data curation (Equal), Writing - Review & Editing, Visualization, Funding acquisition (Equal). **SM**: Validation, Investigation. **MK**: Investigation. **SJ**: Methodology, Software, Investigation. **SR**: Resources, Writing - Review & Editing, Supervision, Funding acquisition (Equal). **CZ**: Resources, Writing - Review & Editing, Supervision, Funding acquisition (Equal). **PAH**: Conceptualization (Lead), Methodology (Lead), Validation, Formal analysis (Lead), Investigation, Data curation (Lead), Resources, Writing - Original Draft (Lead), Writing - Review & Editing (Lead), Visualization (Lead), Supervision (Lead), Project administration (Lead), Funding acquisition (Equal).

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Figure Legends

Figure 1. FLS2 S-acylation increases upon flg22 perception. **A.** Quantification of changes in FLS2 S-acylation following flg22 (n = 5, green solid line) or water only control (n = 2, blue short dashed line) treatment. S-acylation state is shown relative to T0 (black, long dashed line). Error bars show SEM. Significance of difference between flg22 and water treated at 20 minutes is shown as determined by Student's t-test. **B.** S-acylation of FLS2 in response to flg22 requires BAK1. S-acylation state is shown relative to water treated plants of the same genotype (dashed line). Error bars show SEM, Col-0 +flg22 n=5, Col-0 + H2O n = 2, *bak1-4* n = 4, *chc2-1* n = 2, significant difference to flg22 treated Col-0 as determined by Student's t-test are shown. Data shown in

panels A and B are derived from the same biological repeats, Col-0 controls are therefore shared between panels. **C.** FLS2 undergoes S-acylation in response to flg22 treatment but not elf18. S-acylation state is shown relative to untreated plants (black, dashed line). Error bars show SEM, n = 2, significant differences to flg22 treated Col-0 as determined by Student's t-test are shown. **D.** Mutation of FLS2 Cys1132,1135 to serine abolishes residual S-acylation observed in the FLS2 $C^{830,831}S$ background. EX - indicates S-acylation state, LC - loading control, Hyd - indicates presence (+) or absence (-) of hydroxylamine. **E.** FLS2 $C^{1132,1135}S$ mutants are blocked in flg22 mediated increases in S-acylation. S-acylation state is shown relative to water treated plants of the same genotype (black, dashed line). Error bars show SEM, n=3, significant difference to flg22 treated Col-0 as determined by Student's t-test are shown.

Figure 2. Acute responses to flg22 perception are reduced in FLS2 $C^{1132,1135}S$ expressing plants. **A.** ROS production induced by 100 nM flg22 treatment. Data points are the sum of the 3 highest consecutive readings per sample. n = 10 per genotype. Statistical outliers are shown as open circles. Box shows median and IQR, whiskers show +/- 1.5 x IQR. Statistically significant differences at $p < 0.01$ are indicated (a, b) and were calculated using ANOVA and Tukey HSD tests. **B.** MAPK activation in *fls2/FLS2pro:FLS2* $C^{1132,1135}S$ seedlings in response to 100 nM flg22 as determined over time by immunoblot analysis. pMAPK6/pMAPK3 show levels of active form of each MAPK. MAPK6 indicates total levels of MAPK6 as a loading control. Upper shadow band in MAPK6 blot is RUBISCO detected non-specifically by secondary antibody. **C.** Induction of WRKY40 gene expression after 1 hour treatment with 1 mM flg22 in *fls2/FLS2pro:FLS2* $C^{1132,1135}S$ seedlings as determined by qRT-PCR. **D.** Induction of NbACRE31 gene expression after 3-hour treatment with 1 mM elf18 in EFR-GFP and EFR $C^{975}S$-GFP expressing *N. benthamiana* plants as determined by qRT-PCR. Values were calculated using the $\Delta\Delta_{CT}$ method, error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's t-test. **E.** MAPK activation in EFR-GFP and EFR C975S-GFP expressing N. benthamiana plants in response to 15 minutes treatment with 1 mM elf18 as determined by immunoblot analysis. pSIPK/pWIPK show levels of active form of each MAPK. WIPK indicates total levels of WIPK as a loading control. EFR-GFP and EFR $C^{975}S$-GFP levels are shown as a control for dosage effects on MAPK activation.

Figure 3. FLS2 S-acylation is required for long term immune response outputs **A.** Induction of PR1 gene expression after 24 hours treatment with 1 mM flg22 in *fls2/FLS2pro:FLS2* $C^{1132,1135}S$ seedlings as determined by qRT-PCR. Values were calculated using the $\Delta\Delta_{CT}$ method, error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's t-test. Significant differences in transcript mRNA detected in *fls2/FLS2pro:FLS2* $C^{1132,1135}S$ seedlings compared to Col-0 levels in flg22 treated samples are indicated. Similar data were obtained over 3 biological repeats. **B.** Inhibition of growth after 10 days of 1 mM flg22 treatment is reduced in *fls2/FLS2pro:FLS2* $C^{1132,1135}S$ seedlings. Data are averages of 3 biological replicates, error bars are SEM, significant differences at $p < 0.01$ are indicated (a, b, c) and calculated by ANOVA with Tukey HSD test. **C.** Resistance to *P. syringae* DC3000 infection is impaired by loss of FLS2 S-acylation. Box and whisker plots show data from 7

319 biological repeats (box denotes median and IQR, whiskers show +/- 1.5 x IQR, outliers are shown
320 as open circles), significant differences at $p < 0.05$ are indicated (a, b, c) and calculated by ANOVA
321 with Tukey HSD test.
322
323 **Figure 4.** FLS2 $C^{1132,1135}S$ shows reduced interaction with BAK1 following flg22 stimulation. **A.**
324 Arabidopsis flg22 treated seedlings were lysed in cold IGEPAL CA-630 buffer and separated into
325 detergent soluble (S) and detergent resistant (R) fractions. Relative partitioning of FLS2 into each
326 fraction was determined by western blotting with anti-FLS2 rabbit polyclonal antibody. Loading
327 and purity of fractions is shown by Ponceau S staining of the membrane. **B.** Quantification of data
328 shown in A from 3 biological repeats. Error bars show SEM, significance was calculated using
329 Student's t-test. **C.** FLS2 was immunoprecipitated from IGEPAL CA-630 solubilised flg22 treated
330 Arabidopsis seedling lysates using anti-FLS2 rabbit polyclonal antibody. BAK1 recovery was
331 assessed using rabbit polyclonal anti-BAK1 antibody. flg22 induced BAK1 autophosphorylation at
332 Ser612 was assessed in input samples using rabbit polyclonal anti-BAK1 pS612 antibody. **D.** FLS2
333 was immunoprecipitated from DIBMA solubilised flg22 treated Arabidopsis seedling lysates using
334 anti-FLS2 rabbit polyclonal antibody. BAK1 recovery was assessed using rabbit polyclonal anti-
335 BAK1 antibody.
336
337 **Materials and Methods**
338 **Cloning and constructs**
339 Fully functional *$FLS2_{pro}$:FLS2* was made using the described FLS2 promoter and open reading frame
340 [43] with stop codon cloned into pENTR D-TOPO [44]. All *FLS2* mutant variants used were based
341 on this construct and were generated using Q5 site directed mutagenesis kit (NEB) according to
342 the manufacturer's guidelines. *$FLS2_{pro}$:FLS2-3xMYC-EGFP* and *$FLS2_{pro}$:FLS2 $C^{1132,1135}$S-3xMYC-EGFP*
343 were made by yeast recombinatorial cloning using a 3xMYC-EGFP PCR fragment amplified from
344 *$FLS2_{pro}$:FLS2-3xMYC-EGFP* [45] recombined with pENTR D-TOPO $FLS2_{pro}$:FLS2 or pENTR D-TOPO
345 $FLS2_{pro}$:FLS2 $C^{1132,1135}S$. Entry clones were recombined into pK7WG,0 [46] using Gateway
346 technology (ThermoFisher) to generate expression constructs. Expression constructs were
347 transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90 [47] for transformation of
348 either Arabidopsis or *Nicotiana benthamiana*.
349
350 **Plant lines and growth conditions**
351 All Arabidopsis lines were in the Col-0 accession background. The *fls2* [43], *bak1-4* [48] and *chc2-*
352 *1* [15] mutants have all been described previously. Transgenic *fls2/$FLS2_{pro}$:FLS2* [44] and
353 *fls2/$FLS2_{pro}$:FLS2 $C^{1132,1135}S$* mutant variant lines were generated by Agrobacterium-mediated floral
354 dip transformation [49]. T_3 homozygous plants were used for all experiments. Plant material for
355 experiments was grown on 0.5x MS medium, 0.8% phytagar under 16:8 light:dark cycles at 20 °C
356 in MLR-350 growth chambers (Panasonic). For transient expression *Nicotiana benthamiana* plants
357 were grown in 16:8 light:dark cycles at 24 °C and used at 4-5 weeks old. A. tumefaciens mediated
358 transient expression was performed as described [50] using an OD600 of 0.1 of each expression
359 construct alongside the p19 silencing suppressor at an OD600 of 0.1. Tissue was harvested 48-60
360 hours post infiltration.

361
362 **Eliciting peptides**
363 Flg22 peptide (QRLSTGSRINSAKDDAAGLQIA) was synthesised by Dundee Cell Products (Dundee,
364 UK). Elf18 peptide (Ac-SKEKFERTKPHVNVGTIG) was synthesised by Peptide Protein Research Ltd.
365 (Bishops Waltham, UK).
366
367 **Seedling growth inhibition**
368 For each biological replicate four days post-germination, 10 seedlings of the named genotypes
369 were transferred to 12-well plates (5 seedlings per well), ensuring the cotyledons were not
370 submerged. Wells contained 2 mL of 0.5x MS liquid medium with or without 1 µM flg22. Seedlings
371 were incubated for 10 days and the fresh weight of pooled seedlings in each genotype for each
372 treatment measured and an average taken. Flg22- treated/untreated weights for each genotype
373 were calculated and presented data is an average of these data over three biological repeats. Fully
374 independent biological repeats were performed over a period of 6 months with each genotype
375 only being present once in each repeat.
376
377 **MAPK activation**
378 Essentially as for [51]; 6 Arabidopsis seedlings of each genotype 10 days post germination were
379 treated with 100 nM flg22 for the indicated times in 2 mL 0.5x MS medium. The 6 seedlings from
380 each genotype at each time point for each treatment were pooled before further analysis. Fully
381 independent biological repeats were performed over a period of 2 years with each genotype only
382 being present once in each repeat. To assess EFR induced MAPK activation in *N. benthamiana*
383 leaves from 5-week-old plants were transiently transformed by agrobacterium infiltration (OD600
384 0.1 of each construct plus p19 at OD600 0.1). 60 hours after transformation, 1 µM elf18 peptide
385 in water or water only was infiltrated into the leaf and samples harvested after 15 minutes.
386 Samples were subsequently processed as described [51].
387
388 **Reactive oxygen species production**
389 Protocol based on Mersmann et al. (2010). Essentially, 10 seedlings of each genotype were grown
390 for 14 days in 100 µL of 0.5x MS medium with 0.5% sucrose, in 96-well plates (PerkinElmer).
391 Conditions were maintained at 22 °C with 12:12 light:dark cycles. Growth medium was exchanged
392 for water with 10 nM flg22 for 1 hour, before replacing with water for a further 1 hour. ROS burst
393 was then induced by replacing with a solution containing 100 nM flg22, 400 nM luminol (Fluka),
394 and 20 µg/mL peroxidase (Sigma). Luminescence in each well was measured every 2 minutes in a
395 Varioskan Lux (Thermo Fisher) for 30 cycles (approx. 1 hour total).
396
397 **Gene expression analysis**
398 Ten seedlings of each genotype 10 days post-germination were treated with 1 µM flg22 or water
399 for the indicated times. The 10 seedlings from each genotype/treatment at each time point for
400 each treatment were pooled before further analysis. RNA was extracted using RNAeasy Plant kit
401 with on column DNAse digestion according to the manufacturer's instructions (Qiagen). Two
402 micrograms RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription kit

403 (Applied Biosystems). All transcripts were amplified using validated gene-specific primers [44].
404 Expression levels were normalized against *PEX4* (At5g25760) [52]. Each sample was analyses in
405 triplicate (technical repeats) for each primer pair within each biological repeat. Relative
406 quantification (RQ) was achieved using the $\Delta\Delta_{CT}$ (comparative cycle threshold) method [53].
407 Significant differences between samples were determined from a 95% confidence interval
408 calculated using the t-distribution. Fully independent biological repeats were performed over a
409 period of 2 years with each genotype only being present once in each repeat.
410
411 **Bacterial infection assays**
412 Infection assays of Arabidopsis lines by *Pseudomonas syringae* pv. tomato DC3000 were
413 performed using seedling flood inoculation assays as described [54].
414
415 **Western blotting**
416 FLS2 was detected using rabbit polyclonal antisera raised against the C-terminus of FLS2 as
417 previously described [9, 55]. Anti-p44/42 MAPK (Erk1/2) (Cell Signalling Technology #9102) was
418 used to detect phosphorylated MAPK3/6 according to manufacturer's recommendations at
419 1:2000 dilution. Total Arabidopsis MAPK6 or *N. benthamiana* WIPK was detected using anti-
420 Arabidopsis MPK6 (Sigma A7104) at 1:2000. Rabbit polyclonal antibodies against BAK1 were as
421 described [32] or obtained from Agrisera (AS12 1858) and used at 1:5000 dilution. BAK1 phospho-
422 S612 was detected using polyclonal rabbit antisera as described [33]. HRP conjugated secondary
423 antibodies were used to visualise antibody reacting proteins, and Clean-Blot HRP (Thermo Fisher)
424 secondary antibody was used for immunoprecipitation experiments. Western blots were
425 developed using SuperSignal West pico and femto in a 3:1 ratio by volume and signal captured
426 using a Syngene G:box storm imager and quantitative photon count data stored as Syngene SGD
427 files. Signal intensity was quantified from SGD files using Syngene GeneTools software.
428
429 **S-acylation assays**
430 S-acylation assays using acyl-biotin exchange (ABE) were performed exactly as described [55]. For
431 flg22-dependent changes in FLS2 S-acylation, 7 seedlings 10 days post germination were
432 transferred to each well of 12-well plates. Each well contained 2 mL 0.5 x MS liquid medium.
433 Seedling were incubated for 24 hours on an orbital mixer (Luckham R100/TW Rotatest Shaker, 38
434 mm orbit at 75 RPM). Thereafter, 100 µL of 0.5 x MS media containing flg22 was added to give a
435 final flg22 concentration of 10 µM. Seedlings were incubated with continued mixing for the
436 indicated times before harvesting.
437
438 **Co-immunoprecipitation assays using IGEPAL CA-630**
439 Seedlings grown on solid ½ MS for 30-35 days were transferred to wells of a 6-well plates and
440 grown for 7 days in ½ MS 2 mM MES-KOH, pH 5.8. Thereafter, the seedlings were transferred in
441 beakers containing 40 mL of ½ MS 2 mM MES-KOH, pH 5.8 and subsequently treated with sterile
442 mQ water with or without flg22 (final concentration of 100 nM) and incubated for 10 minutes.
443 The seedlings were then frozen in liquid nitrogen and proteins extracted in 50 mM Tris-HCl pH
444 7.5, 150 mM NaCl, 10% glycerol, 5 mM dithiothreitol, 1% protease inhibitor cocktail (Sigma

445 Aldrich), 2 mM Na_2MoO_4, 2.5 mM NaF, 1.5 mM activated Na_3VO_4, 1 mM phenylmethanesulfonyl
446 fluoride and 0.5% IGEPAL for 40 minutes at 4 °C. Lysates were clarified at 10,000 g for 20 minutes
447 at 4 °C and the supernatants were filtered through miracloth. For immunoprecipitations, α-rabbit
448 Trueblot agarose beads (eBioscience) coupled with α-FLS2 antibodies [8] were incubated with the
449 crude extract for 3 hours at 4 °C. Subsequently, beads were washed 3 times (50 mM Tris-HCl pH
450 7.5, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 0.1% IGEPAL) before adding Laemmli
451 buffer and incubating for 10 minutes at 95 °C. Protein samples were separated in 10%
452 bisacrylamide gels at 150 V for approximately 2 hours and transferred into activated PVDF
453 membranes at 100 V for 90 minutes. Immunoblotting was performed with antibodies diluted in
454 blocking solution (5% fat-free milk in TBS with 0.1% (v/v) Tween-20). Antibodies used in this study:
455 α-BAK1 [32] (1:5000); α-FLS2 [8] (1:1000); α-BAK1 pS612 [33] (1:3000). Blots were developed with
456 Pierce ECL/ ECL Femto Western Blotting Substrate (Thermo Scientific). The following secondary
457 antibodies were used: anti-rabbit IgG-HRP Trueblot (Rockland, 18-8816-31, dilution 1:10000) for
458 detection of FLS2-BAK1 co-immunoprecipitation or anti-rabbit IgG (whole molecule)–HRP (A0545,
459 Sigma, dilution 1:10000) for all other western blots.
460
461 **Co-immunoprecipitation assays using Diisobutylene-maleic acid (DIBMA)**
462 For each genotype, 2 x 10 seedlings 10 days post-germination were transferred to each well of
463 12-well plate containing 2 mL 0.5 x MS liquid medium and incubated for 24 hours on an orbital
464 mixer (Luckham R100/TW Rotatest Shaker, 38 mm orbit at 75 RPM). Thereafter, 100 µL of 0.5 x
465 MS media containing flg22 was added to give a final flg22 concentration of 10 µM. The seedlings
466 were further incubated with continued mixing for 20 minutes prior to harvesting and blotting dry.
467 Tissue was lysed in 500 µl of lysis buffer (50 mM Tris-HCl pH 7.2, 10% v/v glycerol, 150 mM NaCl,
468 1% w/v DIBMA (Anatrace BMA101), with protease inhibitors (1% v/v, Sigma P9599)) and
469 incubated at room temperature for 1 hour with gentle end-over-end mixing. The lysate was
470 centrifuged at 5,000 g for 1 minute and the supernatant filtered through 2 layers of miracloth and
471 combined with an additional 500 µl of filtered lysis buffer (without DIMBA). The clarified lysate
472 was further centrifuged at 16,000 g for 1 minute and the supernatant applied to Amicon 0.5 mL
473 100 kDa MWCO spin filtration columns and centrifuged at 14,000 g until the retentate was <50
474 µl. The retentate was diluted to 500 µl with IP buffer (50 mM Tris-HCl pH 7.2, 10% glycerol, 200
475 mM L-arginine, with protease inhibitor (0.5% v/v, Sigma P9599) and centrifuged at 14,000 g until
476 the retentate was <50 µl. The spin column was inverted and eluted into a 1.5 mL microfuge tube
477 by centrifugation at 100 g for 1 minute The eluate was diluted to 500 µl with IP buffer, of which
478 20 µl was retained as an input control. Magnetic protein A beads (20 µl per IP reaction) were
479 coated with 5 µg αFLS2 antibody overnight at 4 °C. The resulting beads were washed for 5 minutes
480 with IP buffer containing 0.5 M NaCl followed by 2 washes with IP buffer and resuspended in IP
481 buffer to 100 µl per IP reaction. The resulting FLS2-coated magnetic protein A beads were added
482 to the DIBMA solubilised protein solution and incubated for 3 hours at room temperature with
483 end-over-end mixing. Thereafter, the beads were washed three times with IP buffer, resuspended
484 in 30 µl 2x LDS sample buffer with 2-mercaptoethanol and incubated at 65 °C for 5 minutes with

485 shaking at 1000 RPM. The samples were separated on a 7.5% SDS-PAGE gel prior to transfer to
486 PVDF and western blotting.
487
488 **Detergent resistant membrane preparation**
489 To evaluate flg22-dependent changes in FLS2 detergent resistant membrane occupancy, 7
490 seedlings 10 days post-germination were transferred to each well of a 12-well plate, of which each
491 well contained 2 mL 0.5 x MS liquid medium. Seedlings were incubated for 24 hours on an orbital
492 mixer (Luckham R100/TW Rotatest Shaker, 38 mm orbit at 75 RPM), after which 100 µL of 0.5 x
493 MS media containing flg22 was added to give a final flg22 concentration of 10 µM. The seedlings
494 were further incubated with continuous mixing as before for 20 minutes before harvesting and
495 snap freezing in liquid nitrogen. All subsequent steps were performed at 4 °C or on ice. The
496 seedlings were then lysed in 0.5 mL ice cold 1% (v/v) IGEPAL CA-630 in 25 mM Tris-HCl pH 7.4,
497 150 mM NaCl, 2 mM EDTA, and 0.1% (v/v) protease inhibitors (Sigma-Aldrich, P9599). Lysates
498 were clarified at 500 g and filtered through 1 layer of miracloth. The filtrate was centrifuged at
499 16,000 g for 30 minutes and the supernatant retained as a detergent soluble fraction (DSM) and
500 mixed 3:1 with 4x reducing (2-mercaptoethanol) LDS sample buffer. The detergent resistant pellet
501 (DRM) was gently washed with 1 mL lysis buffer, centrifuged at 16,000 g for 5 minutes, and the
502 supernatant discarded. The resulting pellet was resuspended in 27 µL of 3:1 lysis buffer: 4x
503 reducing LDS sample buffer, after which 25 µL of the DRM and DSM were separated by 7.5% SDS-
504 PAGE and probed using anti-FLS2 polyclonal antibody as described [55].
505
506 **Variable Angle - Total Internal Reflection Fluorescence (VA-TIRF) microscopy**
507 VA-TIRF microscopy was performed using an inverted Leica GSD equipped with a 160x objective
508 (NA = 1.43, oil immersion), and an Andor iXon Ultra 897 EMCCD camera. Images were acquired
509 by illuminating samples with a 488 nm solid state diode laser, a cube filter with an excitation filter
510 488/10 and an emission filter 535/50 for FLS2-GFP, and a 532 nm solid state diode laser, a cube
511 filter with an excitation filter 532/10 and an emission filter 600/100 for mRFP-REM1.3. Optimum
512 critical angle was determined as giving the best signal-to-noise.
513
514 **Single particle tracking analysis**
515 *Nicotiana benthamiana* plants (14-21 days old) were infiltrated with *Agrobacterium tumefaciens*
516 (strain GV3101) solution of OD = 0.15 and imaged 32 to 40 hours post infiltration. For single
517 particle tracking experiments, image time series were recorded at 20 frames per second (50 ms
518 exposure time) by VA-TIRFM. Analyses were carried out as previously described [19], using the
519 plugin TrackMate 2.7.4 in Fiji. Single particles were segmented frame-by-frame by applying a
520 Laplacian of Gaussian filter and estimated particle size of 0.4 µm. Individual single particle were
521 localized with sub-pixel resolution using a built-in quadratic fitting scheme. Single particle
522 trajectories were reconstructed using a simple linear assignment problem [56] with a maximal
523 linking distance of 0.4 µm and without gap-closing. Only tracks with at least ten successive points
524 (tracked for 500 ms) were selected for further analysis. Diffusion coefficients of individual
525 particles were determined using TraJClassifier [57]. For each particle, the slope of the first four
526 time points of their mean square displacement (MSD) plot was used to calculate their diffusion

527 coefficient according to the following equation: MSD = (x-x0)2+(y-y0)2 and D = MSD/4t, where x0
528 and y0 are the initial coordinates, x and y are the coordinates at any given time, and t is the time
529 frame.
530
531 **Co-localization analyses**
532 *Nicotiana benthamiana* plants (14-21 days old) were infiltrated with *Agrobacterium tumefaciens*
533 (strain GV3101) solution of OD = 0.2 and imaged 48 hours post infiltration. Images were recorded
534 by VA-TIRFM using 250 ms exposure time. As previously reported [42], we emphasised cluster
535 formation in the presented images by using the 'LoG3D' plugin [58]. Quantitative co-localization
536 analyses of the FLS2-GFP and mRFP-REM1.3 were carried out as previously described [42], with
537 minor modification. Using FiJi, images were subjected to a background subtraction using the
538 "Rolling ball" method (radius = 20 pixels) and smoothed. We selected regions of TIRF micrographs
539 with homogeneous illumination for both FLS2-GFP and mRFP-REM1.3. The Pearson co-localization
540 coefficients were assessed using the JACoP plugin of FIJI [59]. For comparison, we determined
541 values of correlation, which could be observed by chance by calculating the Pearson coefficient
542 after flipping one of the two images.
543
544 **Structural modelling of FLS2 kinase domain**
545 The FLS2 intracellular domain (amino acids 831-1173) was submitted to the phyre2 [60] server
546 (http://www.sbg.bio.ic.ac.uk/phyre2/) in default settings. The solved BIR2 kinase domain
547 structure (PDB 4L68, residues 272-600) [61] was identified as the best match and FLS2 residues
548 841-1171 were successfully modelled onto the BIR2 structure (confidence 100%, coverage 89%).
549 Cys to Ser mutational effects were modelled using Missense3D [62] in default settings.
550
551 **Supplemental Figure Legends**
552 **Supplemental figure 1. A.** Example western blot from Col-0 plants treated with or without flg22
553 used to generate data shown in Figure 1. FLS2 S-acylation state is shown as a function of recovery
554 on thiopropyl-Sepharose beads in the presence of hydroxylamine (EX+). Samples lacking
555 hydroxylamine (EX-) demonstrate completeness of blocking and lack of background or non-
556 specific binding. LC lanes act as input loading controls for standardisation. **B.** Quantification of
557 changes in FLS2 and FLS2 $C^{830,831}S$ S-acylation following flg22 treatment or water only control (n =
558 2, blue short dashed line) treatment. flg22 induced changes in S-acylation state are shown relative
559 to water only treatment (black dashed line). Col-0 n = 5, FLS2 $C^{830,831}S$ n = 2, error bars show SEM.
560 Significance of difference in S-acylation state change between FLS2 (Col-0) and FLS2 $C^{830,831}S$ is
561 shown as determined by Student's t-test. **C.** Expression levels of FLS2 $C^{1132,1135}S$ in
562 *fls2/FLS2pro:FLS2 FLS2* $C^{1132,1135}S$ transgenic lines used in this study. 50 mg total protein from 7-
563 day old seedlings was loaded per lane. MYH9.5 is a previously reported cross-reacting protein
564 with the primary anti-FLS2 antibody used.
565
566 **Supplemental figure 2.** Alignment of a representative member from each of the wider Arabidopsis
567 RK superfamilies, centred on the conserved C[X]7RP motif found in the loop between the G- and
568 H-helices of the kinase domain. Putative S-acylation site cysteines are highlighted in teal.

569
570 **Supplemental figure 3.** FLS2-3xMyc-GFP and FLS2 $C^{1132,1135}$S-3xMyc-GFP behave similarly when
571 expressed in N. benthamiana in the absence of flg22. **A.** Fluorecence intensity measurements at
572 the plasma membrane of single cells using TIRF microscopy. Box plot shows median and IQR,
573 whiskers indicate 1.5 x IQR. FLS2-3xMyc-GFP n = 59, FLS2 $C^{1132,1135}$S-3xMyc-GFP n = 42. p value
574 calculated using Student's t-test. Statistical outliers are indicated by open circles. **B.** Single particle
575 tracking of FLS2-3xMyc-GFP and FLS2 $C^{1132,1135}$S-3xMyc-GFP at the plasma membrane using TIRF
576 microscopy. **C.** Quantification of data in B. Box plot shows median and IQR, whiskers indicate 1.5
577 x IQR. n = 13, p value calculated using Student's t-test. Statistical outliers are indicated by open
578 circles. **D.** FLS2-3xMyc-GFP and FLS2 $C^{1132,1135}$S-3xMyc-GFP form nanodomains in the plasma
579 membrane and show similar co-localisation with mRFP-REM1.3 nanodomains when transiently
580 expressed in N. benthamiana in the absence of flg22. Representative micrographs of FLS2-3xMyc-
581 GFP and FLS2 $C^{1132,1135}$S-3xMyc-GFP (green) co-localisation with mRFP-REM1.3 (magenta) at the
582 plasma membrane of single epidermal cells using TIRF microscopy. **E.** Quantification of FLS2-
583 3xMyc-GFP or FLS2 $C^{1132,1135}$S-3xMyc-GFP co-localisation with mRFP-REM1.3 at the plasma
584 membrane of single epidermal cells. FLS2-3xMyc-GFP n = 14, FLS2 $C^{1132,1135}$S-3xMyc-GFP n = 12.
585 Box plot shows median and IQR, whiskers indicate 1.5 x IQR. p value calculated using Student's t-
586 test. **F.** To determine whether measured co-localisation values shown in B (original) were
587 significant, co-localisation analysis was repeated after rotation of the mRFP-REM1.3 image by 90
588 degrees (rotated). In all cases, co-localisation was reduced and overall, significantly different,
589 indicating that the co-localisation observed in B is both specific and significant. p values were
590 calculated using Student's t-test.
591
592 **Supplemental figure 4.** Mutation of kinase domain S-acylation site cysteines to serine in FLS2 is
593 not predicted to affect kinase domain structure. **A.** Superimposition of the modelled structures of
594 FLS2 (white) and FLS2 $C^{1132,1135}$S (blue) kinase domains. B. Zoomed in view of Cys1132,1135 in FLS2
595 (yellow) and substituted serine (red) residues in FLS2 $C^{1132,1135}$S. Only the proton of Ser1132 is
596 predicted to diverge from the FLS2 structure, being rotated by ~110 degrees compared to the
597 original cysteine. This rotation does not affect the position or packing of any other amino acid.
598
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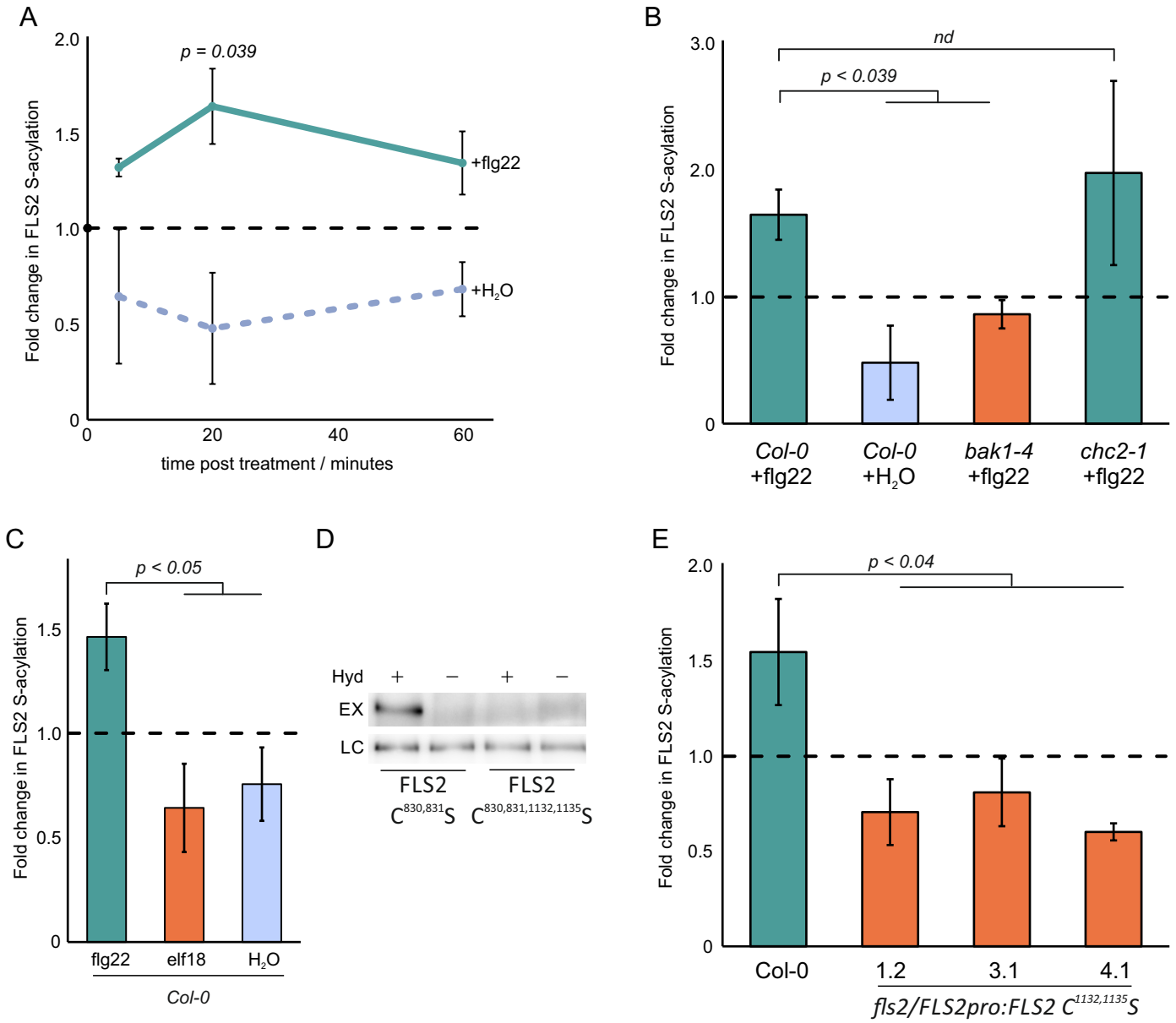


Figure 1. FLS2 S-acylation increases upon flg22 perception. A. Quantification of changes in FLS2 S-acylation following flg22 (n = 5, green solid line) or water only control (n = 2, blue short dashed line) treatment. S-acylation state is shown relative to T_0 (black, long dashed line). Error bars show SEM. Significance of difference between flg22 and water treated at 20 minutes is shown as determined by Student's t-test. **B.** S-acylation of FLS2 in response to flg22 requires BAK1. S-acylation state is shown relative to water treated plants of the same genotype (dashed line). Error bars show SEM, *Col-0* +flg22 n=5, *Col-0* + H2O n = 2, *bak1-4* n = 4, *chc2-1* n = 2, significant difference to flg22 treated *Col-0* as determined by Student's t-test are shown. Data shown in panels A and B are derived from the same biological repeats, *Col-0* controls are therefore shared between panels. **C.** FLS2 undergoes S-acylation in response to flg22 treatment but not elf18. S-acylation state is shown relative to untreated plants (black, dashed line). Error bars show SEM, n = 2, significant differences to flg22 treated *Col-0* as determined by Student's t-test are shown**. D.** Mutation of FLS2 Cys1132,1135 to serine abolishes residual S-acylation observed in the FLS2 $C^{830,831}S$ background. EX - indicates S-acylation state, LC - loading control, Hyd - indicates presence (+) or absence (-) of hydroxylamine. **E.** FLS2 $C^{1132,1135}S$ mutants are blocked in flg22 mediated increases in S-acylation. S-acylation state is shown relative to water treated plants of the same genotype (black, dashed line). Error bars show SEM, n=3, significant difference to flg22 treated *Col-0* as determined by Student's t-test are shown.

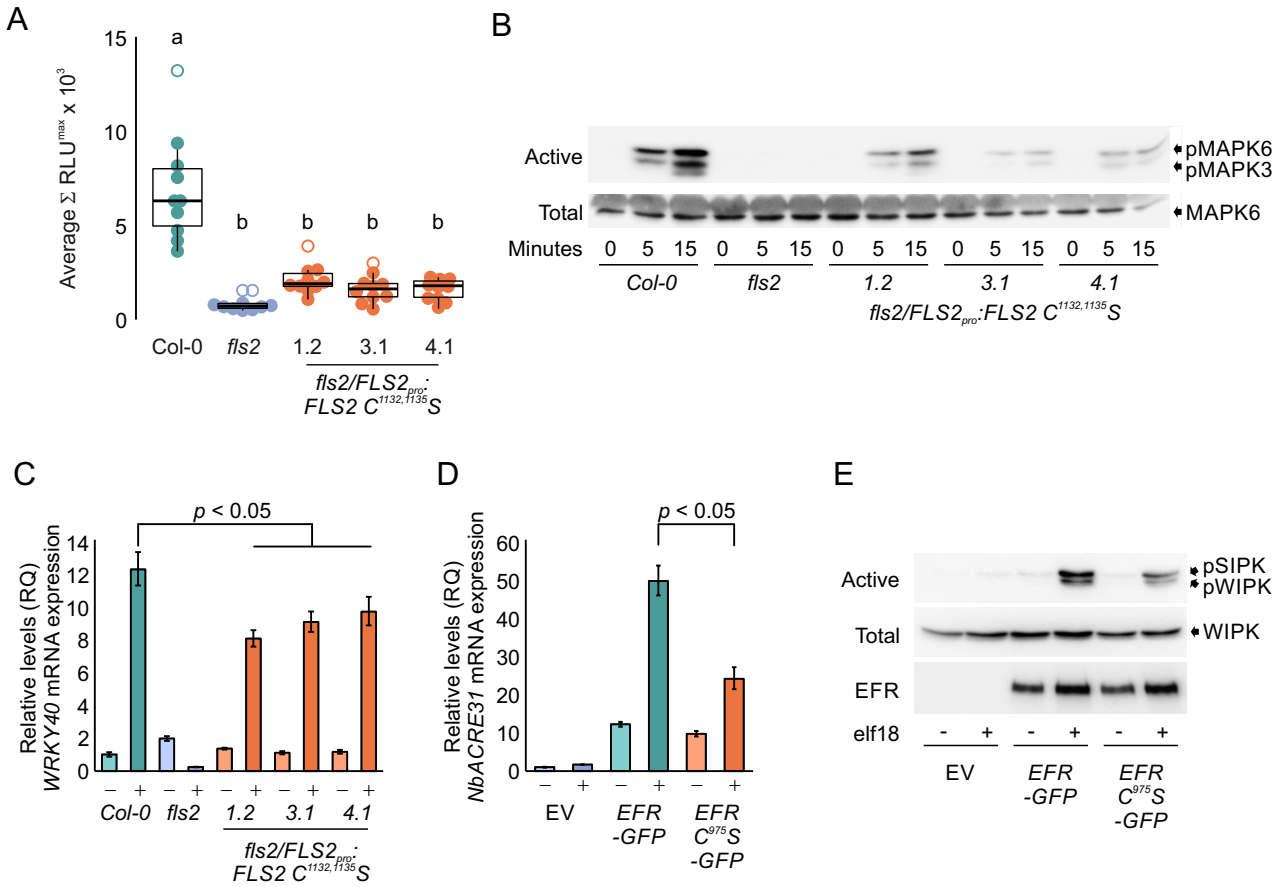


Figure 2. Acute responses to flg22 perception are reduced in FLS2 $C^{1132,1135}S$ expressing plants. A. ROS production induced by 100 nM flg22 treatment. Data points are the sum of the 3 highest consecutive readings per sample. n = 10 per genotype. Statistical outliers are shown as open circles. Box shows median and IQR, whiskers show +/- 1.5 x IQR. Statistically significant differences at $p < 0.01$ are indicated (a, b) and were calculated using ANOVA and Tukey HSD tests. **B**. MAPK activation in *fls2/FLS2pro:FLS2 $C^{1132,1135}S$* seedlings in response to 100 nM flg22 as determined over time by immunoblot analysis. pMAPK6/pMAPK3 show levels of active form of each MAPK. MAPK6 indicates total levels of MAPK6 as a loading control. Upper shadow band in MAPK6 blot is RUBISCO detected non-specifically by secondary antibody. **C.** Induction of *WRKY40* gene expression after 1 hour treatment with 1 μM flg22 in *fls2/FLS2pro:FLS2 $C^{1132,1135}S$* seedlings as determined by qRT-PCR. **D.** Induction of *NbACRE31* gene expression after 3 hour treatment with 1 μM elf18 in *EFR-GFP* and *EFR $C^{975}S$-GFP* expressing *N. benthamiana* plants as determined by qRT-PCR. Values were calculated using the $\Delta\Delta C_T$ method, error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence interval according to Student's t-test. **E.** MAPK activation in *EFR-GFP and EFR $C^{975}S$*-GFP expressing *N. benthamiana* plants in response to 15 minutes treatment with 1 μM elf18 as determined by immunoblot analysis. pSIPK/pWIPK show levels of active form of each MAPK. WIPK indicates total levels of WIPK as a loading control. EFR-GFP and EFR $C^{975}S$-GFP levels are shown as a control for dosage effects on MAPK activation.

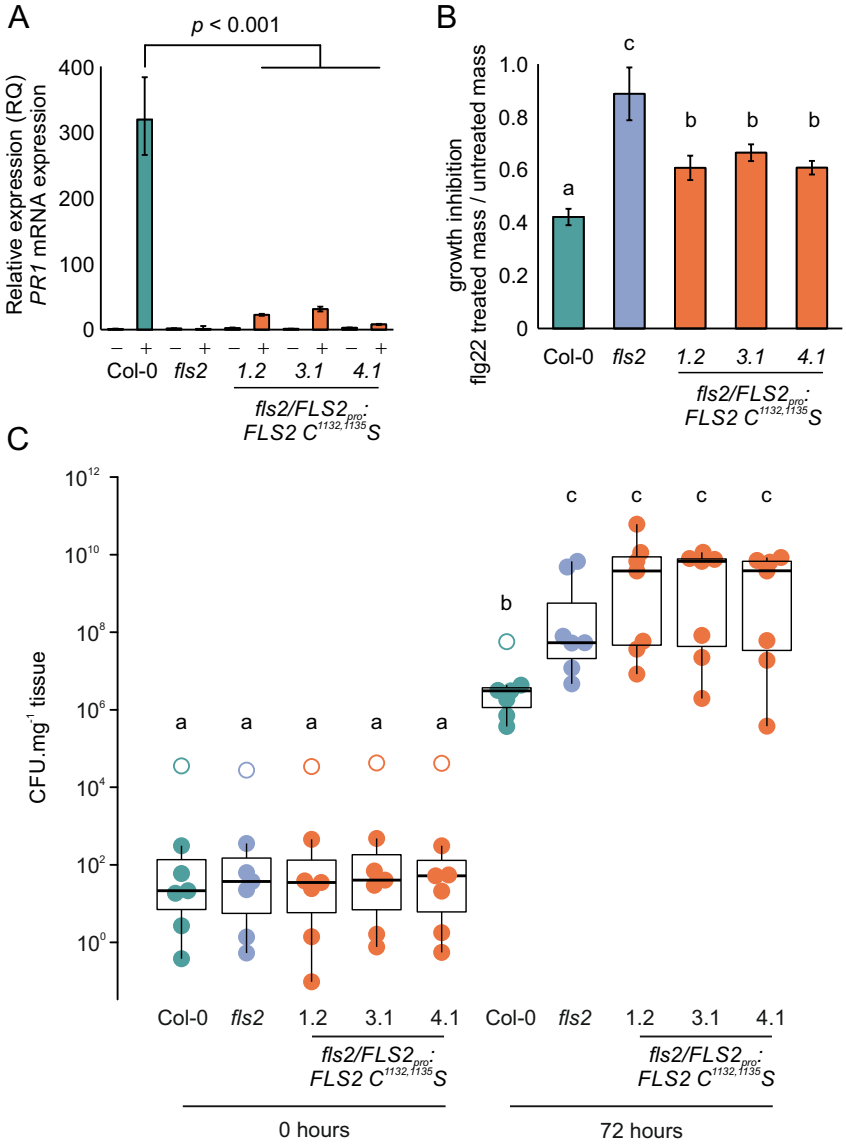


Figure 3. FLS2 S-acylation is required for long term immune response outputs (A). Induction of *PR1* gene expression after 24 hours treatment with 1 μM flg22 in *fls2/FLS2pro:FLS2 C*^{1132,1135}*S* seedlings as determined by qRT-PCR. Values were calculated using the $\Delta\Delta C_T$ method, error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's t-test. Significant differences in transcript mRNA detected in *fls2/FLS2pro:FLS2 C*^{1132,1135}*S* seedlings compared to Col-0 levels in flg22 treated samples are indicated. Similar data were obtained over 3 biological repeats. **(B).** Inhibition of growth after 10 days of 1 μM flg22 treatment is reduced in *fls2/FLS2pro:FLS2 C*^{1132,1135}*S* seedlings. Data are averages of 3 biological replicates, error bars are SEM, significant differences at $p < 0.01$ are indicated (a, b, c) and calculated by ANOVA with Tukey HSD test. **(C).** Resistance to *P. syringae* DC3000 infection is impaired by loss of FLS2 S-acylation. Box and whisker plots show data from 7 biological repeats (box denotes median and IQR, whiskers show +/- 1.5 x IQR, outliers are shown as open circles), significant differences at $p < 0.05$ are indicated (a, b, c) and calculated by ANOVA with Tukey HSD test.

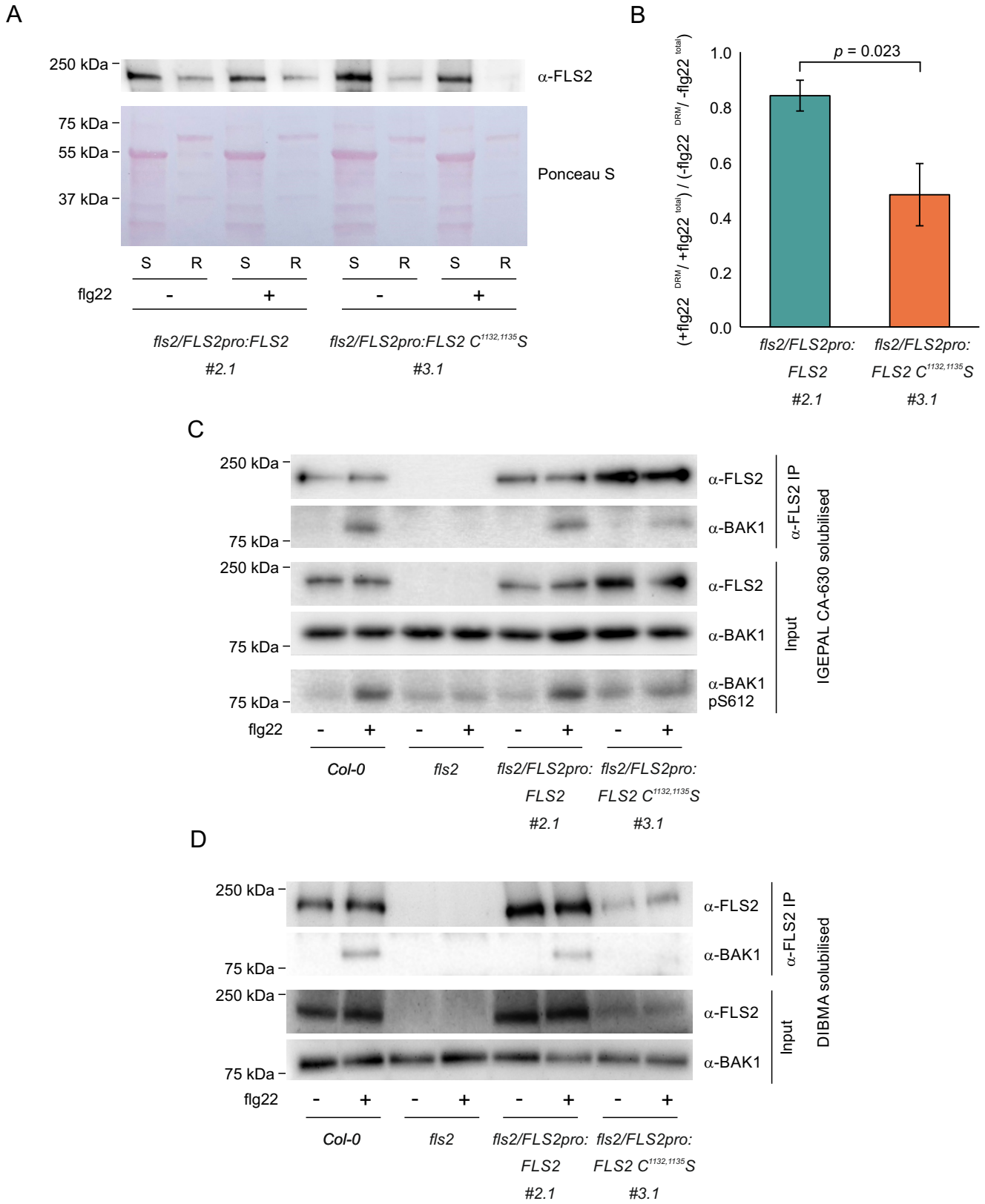
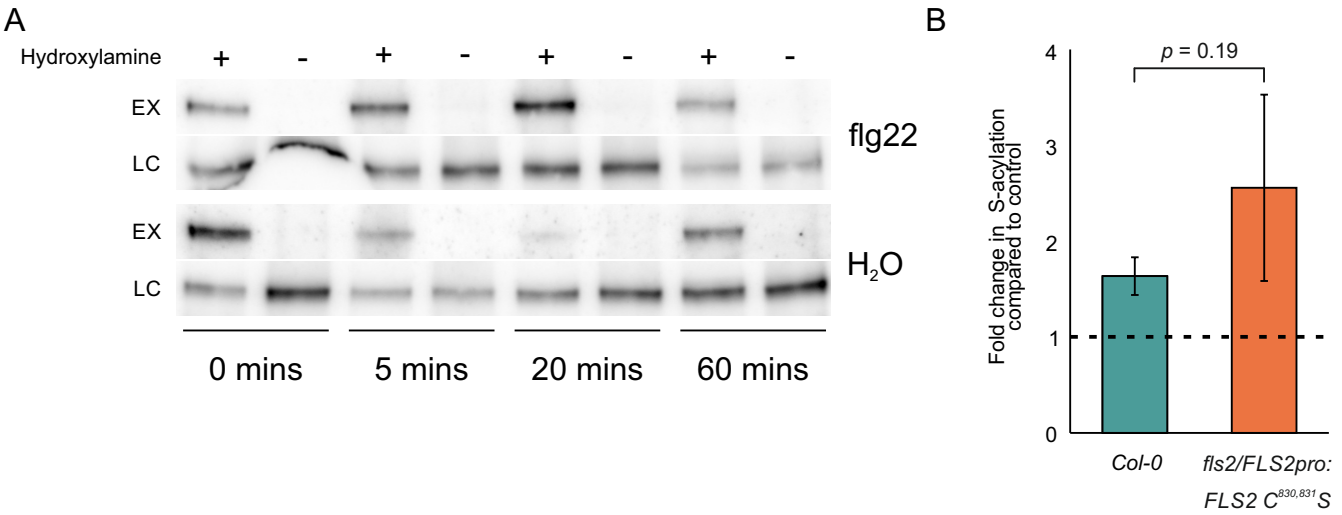


Figure 4. FLS2 $C^{1132,1135}S$ shows reduced interaction with BAK1 following flg22 stimulation. **A.** Arabidopsis flg22 treated seedlings were lysed in cold IGEPAL CA-630 buffer and separated into detergent soluble (S) and detergent resistant (R) fractions. Relative partitioning of FLS2 into each fraction was determined by western blotting with anti-FLS2 rabbit polyclonal antibody. Loading and purity of fractions is shown by Ponceau S staining of the membrane. **B.** Quantification of data shown in A from 3 biological repeats. Error bars show SEM, significance was calculated using Student's t-test. **C.** FLS2 was immunoprecipitated from IGEPAL CA-630 solubilised flg22 treated Arabidopsis seedling lysates using anti-FLS2 rabbit polyclonal antibody. BAK1 recovery was assessed using rabbit polyclonal anti-BAK1 antibody. flg22 induced BAK1 autophosphorylation at Ser612 was assessed in input samples using rabbit polyclonal anti-BAK1 pS612 antibody. **D.** FLS2 was immunoprecipitated from DIBMA solubilised flg22 treated Arabidopsis seedling lysates using anti-FLS2 rabbit polyclonal antibody. BAK1 recovery was assessed using rabbit polyclonal anti-BAK1 antibody.



Supplemental figure 1. A. Example western blot from *Col-0* plants treated with or without flg22 used to generate data shown in Figure 1. FLS2 S-acylation state is shown as a function of recovery on thiopropyl-Sepharose beads in the presence of hydroxylamine (EX+). Samples lacking hydroxylamine (EX-) demonstrate completeness of blocking and lack of background or non-specific binding. LC lanes act as input loading controls for standardisation. **B.** Quantification of changes in FLS2 and FLS2 $C^{830,831}S$ S-acylation following flg22 treatment or water only control (n = 2, blue short dashed line) treatment. flg22 induced changes in S-acylation state are shown relative to water only treatment (black dashed line). *Col-0* n = 5, FLS2 $C^{830,831}S$ n = 2, error bars show SEM. Significance of difference in S-acylation state change between FLS2 (*Col-0*) and FLS2 $C^{830,831}S$ is shown as determined by Student's t-test.

```
At5g46330 FLS2             1125 IEDFLKLCLFCTSSRPEDRPDMNEILTHLM 1154
At5g20480 EFR               965 LRLVLQVGIKCSEEYPRDRMRTDEAVRELI 994
At3g24550 AtPERK1           525 MARMVACAAACVRHSARRRPRMSQIVRALE 554
At2g48010 RKF3              529 LEKYVLIAVLCSHPQLHARPTMDQVVKMLE 558
At3g51550 FERONIA           779 FKKFAETAMKCVLDQGIERPSMGDVLWNLE 808
At1g18390 AtLRK10L-1.2      582 VIAVAELAFQCLQSDKDLRPCMSHVQDTLT 611
At2g20300 AtALE2            588 MAKVAAIASMCVHQEVSHRPFMGEVVQALK 617
At1g52310                   514 VQKVVDLVYSCTQNVPSMRPRMSHVVHQLQ 543
At3g26700                   320 VEELITLTLRCVDVSSEKRPTMSFVVTELE 349
At1g21250 WAK1              651 IQEAARIAAECTRLMGEERPRMKEVAAKLE 680
At5g38280 AtPR5K1           577 AKKLVLVALWCIQMNPSDRPPMIKVIEMLE 606
At5g60300 AtP2K1            584 VEMVMKLGLLCSNIVPESRPTMEQVVLYLN 613
At1g19090 AtCRK1            543 ALKVLQIGLLCVQSSVELRPSMSEIVFMLQ 572
At1g11330                   765 IEKCVHIGLLCVQEVANDRPNVSNVIWMLT 794
At3g59420 AtCR4             567 LKRIVSVACKCVRMRGKDRPSMDKVTTALE 596

consensus                       ----------C-------RP----------
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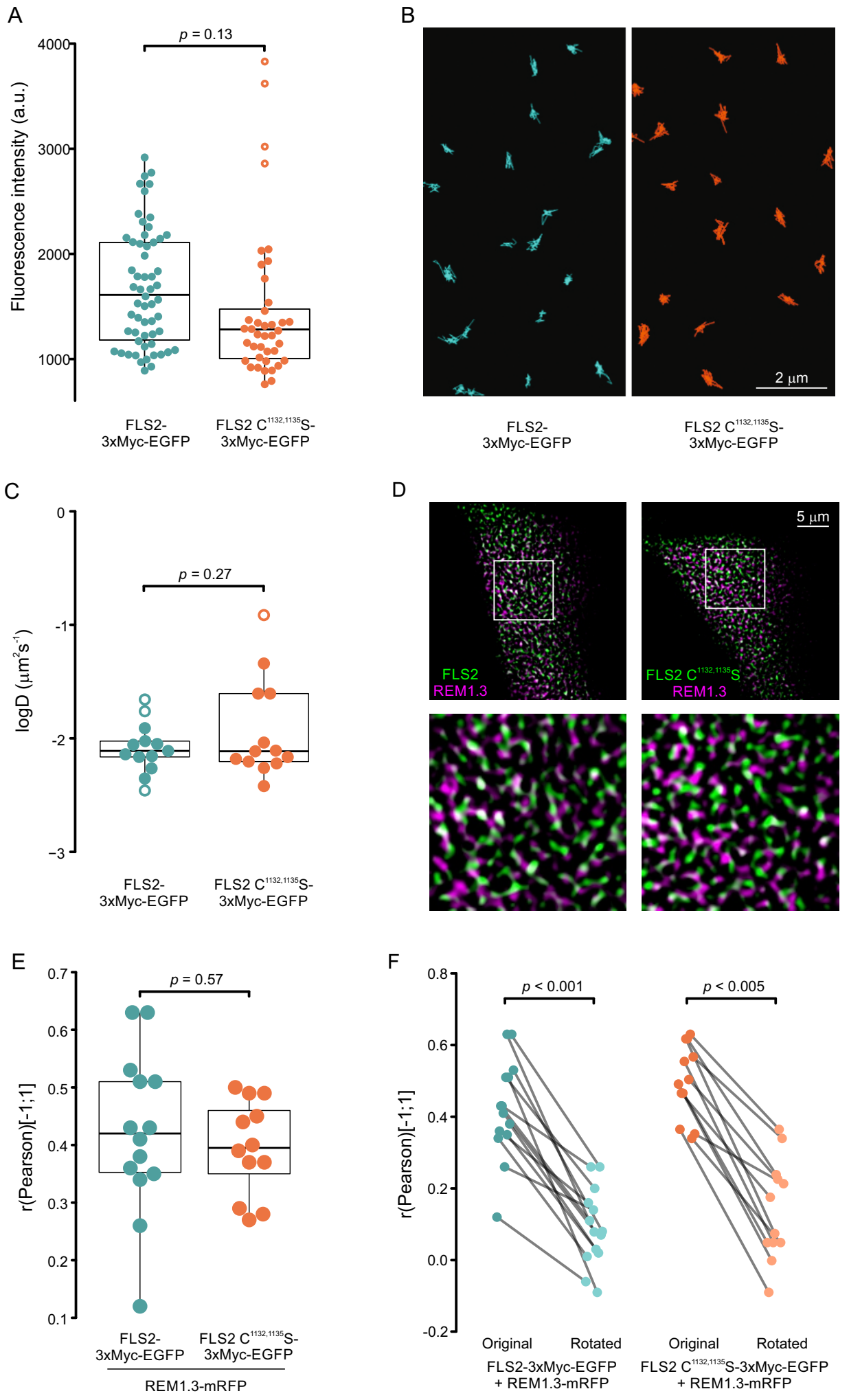
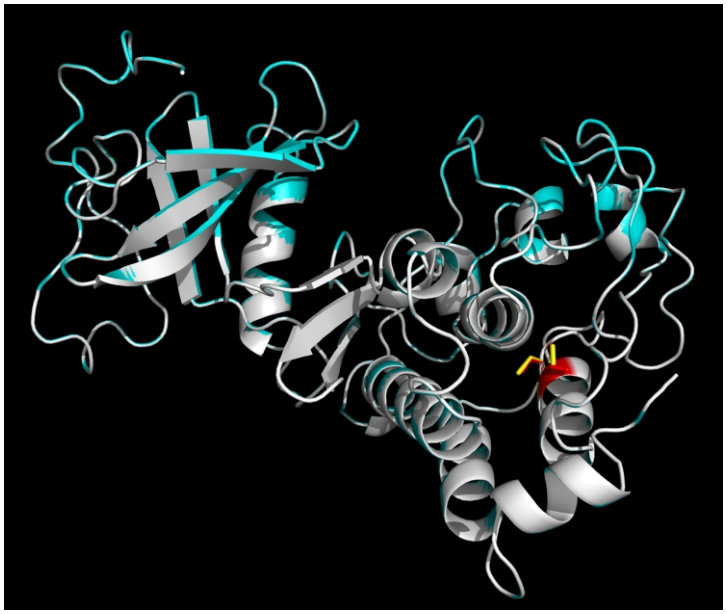
Supplemental figure 2. Alignment of a representative member from each of the wider Arabidopsis RK superfamilies, centred on the conserved C[X]$_7$RP motif found in the loop between the G- and H-helices of the kinase domain. Putative S-acylation site cysteines are highlighted in teal.

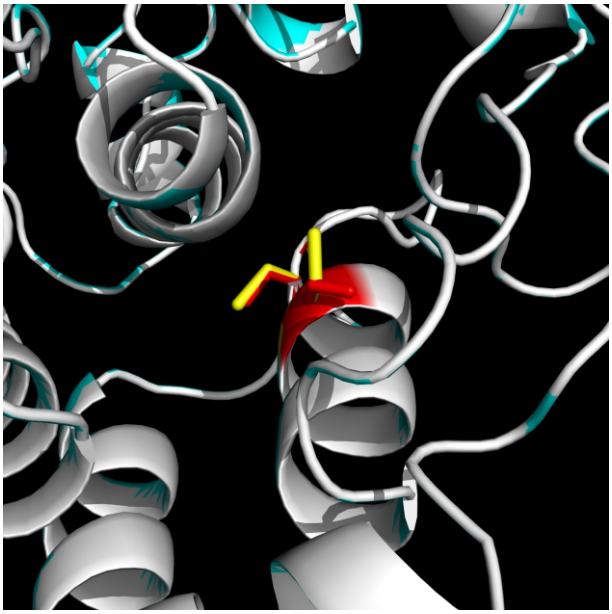


Supplemental figure 3. FLS2-3xMyc-GFP and FLS2 C^{1132,1135}S-3xMyc-GFP behave similarly when expressed in *N. benthamiana* in the absence of flg22. **A.** Fluorecence intensity measurements at the plasma membrane of single cells using TIRF microscopy. Box plot shows median and IQR, whiskers indicate 1.5 x IQR. FLS2-3xMyc-GFP n = 59, FLS2 C^{1132,1135}S-3xMyc-GFP n = 42. *p* value calculated using Student's t-test. Statistical outliers are indicated by open circles. **B.** Single particle tracking of FLS2-3xMyc-GFP and FLS2 C^{1132,1135}S-3xMyc-GFP at the plasma membrane using TIRF microscopy. **C.** Quantification of data in B. Box plot shows median and IQR, whiskers indicate 1.5 x IQR. n = 13, *p* value calculated using Student's t-test. Statistical outliers are indicated by open circles. **D.** FLS2-3xMyc-GFP and FLS2 C^{1132,1135}S-3xMyc-GFP form nanodomains in the plasma membrane and show similar co-localisation with mRFP-REM1.3 nanodomains when transiently expressed in *N. benthamiana* in the absence of flg22. Representative micrographs of FLS2-3xMyc-GFP and FLS2 C^{1132,1135}S-3xMyc-GFP (green) co-localisation with mRFP-REM1.3 (magenta) at the plasma membrane of single epidermal cells using TIRF microscopy. **E.** Quantification of FLS2-3xMyc-GFP or FLS2 C1132,1135S-3xMyc-GFP co-localisation with mRFP-REM1.3 at the plasma membrane of single epidermal cells. FLS2-3xMyc-GFP n = 14, FLS2 C^{1132,1135}S-3xMyc-GFP n = 12. Box plot shows median and IQR, whiskers indicate 1.5 x IQR. *p* value calculated using Student's t-test. **F.** To determine whether measured co-localisation values shown in B (original) were significant, co-localisation analysis was repeated after rotation of the mRFP-REM1.3 image by 90 degrees (rotated). In all cases, co-localisation was reduced and overall significantly different, indicating that the co-localisation observed in B is both specific and significant. *p* values were calculated using Student's t-test.

A



B



Supplemental figure 4. Mutation of kinase domain S-acylation site cysteines to serine in FLS2 is not predicted to affect kinase domain structure. A. Superimposition of the modelled structures of FLS2 (white) and FLS2 $C^{1132,1135}S$ (blue) kinase domains. **B.** Zoomed in view of Cys1132,1135 in FLS2 (yellow) and substituted serine (red) residues in FLS2 $C^{1132,1135}S$. Only the proton of Ser1132 is predicted to diverge from the FLS2 structure, being rotated by ~110 degrees compared to the original cysteine. This rotation does not affect the position or packing of any other amino acid.