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Running title: Variable outcomes of FLS2 epitope tagging

Corresponding author:
Piers A. Hemsley
Division of Plant Sciences
School of Life Sciences
University of Dundee
Dow Street, Dundee
DD1 5EH
Scotland, UK
p.a.hemsley@dundee.ac.uk +44 844 9285428 ext:7012

Variable effects of C-terminal fusions on FLS2 function – not all epitope tags are created equal

Charlotte H. Hurst [1, 2], Dionne Turnbull [1], Sally M. Myles [1], Kerry Leslie [1, 2], Nana F. Keinath [3, 4], Piers A. Hemsley [1, 2, *]

1. Division of Plant Sciences, School of Life Sciences, University of Dundee, Dow Street, Dundee, DD1 5EH, Scotland, UK

2. Cell and Molecular Sciences, The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, Scotland, UK

3. Department of Plant Developmental Biology, Centre for Organismal Studies, Heidelberg University, 69120 Heidelberg, Germany

4. Present address: BioQuant, Im Neuenheimer Feld 267, Heidelberg University, D-69120 Heidelberg, Germany

* Author for correspondence – p.a.hemsley@dundee.ac.uk

One sentence summary:
Complementation analysis reveals that C-terminal epitope tags have varied and unpredictable effects on FLS2 function.

Author contributions
P.H. and N.K. conceived the original research plan and designed the experiments; P.H., N.K. C.H. and D.T. supervised the experiments; C.H., S.M., D.T., N.K., K.L. and PH performed the experiments; P.H., C.H., S.M., D.T. and N.K. analysed the data; P.H. and N.K. wrote the article with contributions from all co-authors.

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Abstract

Receptor-like kinases (RLKs) are the largest family of proteins in plants and are responsible for perceiving the vast majority of extracellular stimuli. Thus, RLKs function in diverse processes, including sensing pathogen attacks, regulating symbiotic interactions, transducing hormone and peptide signals, and monitoring cell wall status. However, despite their fundamental role in plant biology, very few antibodies are available against RLKs, which necessitates the use of epitope tags and fluorescent protein fusions in biochemical analyses such as immunoblot analysis and intracellular visualisation. Epitope tags are widely used and are typically assumed to be benign with no influence on protein function. FLS2 is the receptor for bacterial flagellin and is often used as a model for RLK function. Previous work implies that C-terminal epitope fusions to FLS2 maintain protein function. Here, a detailed complementation analysis of Arabidopsis *fls2* mutant plants expressing various FLS2 C-terminal epitope fusions revealed highly variable and unpredictable FLS2-mediated signalling outputs. In addition, only one out of four FLS2 epitope fusions maintained the ability to inhibit plant growth in response to flg22 treatment comparable to that in wild type or control untagged transgenic lines. These results raise concerns over the widespread use of RLK epitope tag fusions for functional studies. Many of the subtleties of FLS2 function, and by extension that of other RLKs, may have been overlooked or inappropriately interpreted through the use of RLK epitope tag fusions.

Introduction

Receptor-like kinases (RLKs) form the largest gene family in plants (Shiu and Bleecker, 2001) and are the principal sensing mechanism for physical extracellular signals. Their roles in governing processes such as plant-microbe interactions (Macho and Zipfel, 2014), cell wall integrity (Voxeur and Hofte, 2016), hormonal status (Belkhadir et al., 2014; Xu et al., 2014), peptide signalling (Hara et al., 2007), and developmental processes (Clark et al., 1997; Fisher and Turner, 2007) are particularly well studied. Due to their core importance in plant biology, a very large body of work on RLKs has accrued, ranging from studies on their fundamental functions to transferral of RLKs to new species to provide novel pathogen resistance (Lacombe et al., 2010) or synthetic

biology approaches to alter plant behaviour (Brutus et al., 2010). Much of the work done to examine RLK function relies on biochemical approaches. In the absence of specific antibodies against the RLK of interest, protein sequences (epitopes) recognised by other antibodies must be fused to RLKs to enable detection. For this purpose, C-terminal fusion tags such as fluorescent proteins (Robatzek et al., 2006; Mbengue et al., 2016; Bucherl et al., 2017) and peptides, including HA (YPYDVPDYA) (Dunning et al., 2007), MYC (EQKLISEEDL) (Zipfel et al., 2004), or FLAG (DYKDDDDK) (Sun et al., 2012), have proven popular. For the purposes of this report, any genetically encoded proteinaceous sequence added to a protein to enable detection by an antibody is referred to as an epitope tag. Epitope tags are often presumed to have no effect on function; however, a number of reports now suggest that N-, C-, or internal epitope placements can affect both protein subcellular localisation and/or function. For instance, 1 in 5 mammalian proteins tagged with GFP do not co-localise with their native forms (Stadler et al., 2013). Moreover, any tag renders the potato resistance protein R3a non-functional (Engelhardt et al., 2012) and GFP-tagged α-tubulin disrupts microtubule formation in Arabidopsis leading to right-handed helical growth (Abe and Hashimoto, 2005). One report even details how epitope effects on protein function vary depending on cell type, further complicating functional validation in one system being used to inform another (Jiang et al., 2012). Detailed work also indicates that multiples of an epitope tag, such as FLAG, MYC, or HA repeats, are more likely to disrupt function than a single tag (Georgieva et al., 2015).

FLAGELLIN SENSITIVE 2 (FLS2) and BRASSINOSTEROID INSENSITIVE 1 (BRI1), in conjunction with their co-receptor BRASSINOSTEROID-ASSOCIATED KINASE 1 (BAK1), are the receptors for bacterial flagellin (Gomez-Gomez and Boller, 2000; Zipfel et al., 2004; Chinchilla et al., 2007; Heese et al., 2007) and brassinosteroids (Friedrichsen et al., 2000; Li et al., 2002; Nam and Li, 2002), respectively. They are the most well-characterised leucine-rich repeat-RLK (LRR-RLK) pairings, with a range of tools and known signalling outputs available for their investigation. In particular, FLS2 and BAK1 are frequently used as the model system for LRR-RLK-mediated signalling during immune responses. FLS2 and BAK1 rapidly hetero-dimerise upon perception of

flagellin by FLS2 and downstream signalling responses are initiated, which include reactive oxygen species (ROS) bursts, mitogen-activated protein kinase (MAPK) activation, and changes in gene expression (Chinchilla et al., 2007; Heese et al., 2007). Previous work has shown that BAK1, fused to various C-terminal epitopes, is able to interact with FLS2 in a flagellin-dependant manner but shows greatly reduced potency in terms of activating downstream signalling outputs (Ntoukakis et al., 2011). Interestingly, brassinosteroid signalling is largely unaffected by C-terminal tags on either BAK1 or BRI1 (Geldner et al., 2007; Ntoukakis et al., 2011) indicating that the effect of epitope tags on function is not easily predictable. Studies using BAK1 C-terminal fusions during examination of pathogen-associated molecular pattern (PAMP)-triggered immune responses should therefore be planned and interpreted appropriately (Ntoukakis et al., 2011). Historically FLS2-epitope fusions have been assumed to be functional as they confer flagellin responsiveness to the natural *fls2* mutant accession Ws-0 (Zipfel et al., 2004; Robatzek et al., 2006) and reportedly complement *fls2* mutant lines (Chinchilla et al., 2006). However, comparative assays of functionality or quantitative complementation analyses have not been explicitly shown and, to the best of our knowledge, there is no published data on *fls2* loss-of-function mutant defects being restored to wild-type levels by physiologically relevant expression of FLS2 epitope fusions. We recently began working on FLS2 signalling, using our own and others' Arabidopsis lines expressing epitope-tagged FLS2 in *fls2* mutant backgrounds, and have discovered that C-terminally tagged FLS2 constructs do not behave in a consistent or predictable manner with respect to signalling outputs. Here, we demonstrate that three out of four FLS2 C-terminal epitope fusions are greatly impaired in their ability to restore flg22-mediated growth inhibition, suggesting that many FLS2 C-terminal epitope fusions are, at best, only partially functional. We present complementation assays for FLS2 C-terminal fusions as a resource for the community and identify the best constructs to use in future work.

Results

The effect of C-terminal epitope tags on FLS2-mediated signalling is unpredictable.

FLS2 recognises flg22, the elicitor-active epitope of bacterial flagellin (Felix et al., 1999), and subsequently forms a dimer with BAK1 (Chinchilla et al., 2007; Heese et al., 2007). This interaction leads to increased activation of MAPK cascades, which is one of the earliest observable signalling outputs of PAMP-triggered immunity (PTI) (Nühse et al., 2000). During work to characterise the flg22-induced responses of various forms of FLS2, we consistently observed that all lines expressing FLS2-mGFP6 fusions exhibited greatly reduced MAPK activation compared to that in wild-type Col-0 plants. We therefore set out to test whether C-terminal epitope tags impair FLS2 signalling, or if these observations were a peculiarity of the mGFP6 tag. To this end, we used the previously published *fls2-101/FLS2$_{pro}$:FLS2-3xHA* line (Dunning et al., 2007) and two *fls2/FLS2$_{pro}$:FLS2-3xMYC-EGFP* lines (Mbengue et al., 2016). We also generated *fls2/FLS2$_{pro}$:FLS2-EGFP* lines, as well as *fls2/FLS2$_{pro}$:FLS2* control lines without epitope tags. The constructs in these two lines, and the *FLS2$_{pro}$:FLS2-mGFP6* constructs described above, use the same promoter region and open reading frame described previously (Zipfel et al., 2004). For comparative purposes, linker, peptide epitope, and GFP sequences appended to the FLS2 C-terminus used in this study are shown in Supplemental Figure S1 and S2. All generated epitope-tagged FLS2 transgenic lines were tested for *FLS2* expression and those lines showing a range of mRNA expression to control for expression level effects were selected for further study. *fls2/FLS2$_{pro}$:FLS2* control lines were selected to cover the range of mRNA expression observed between the tagged lines under investigation and Col-0 to control for expression-level effects (Fig. 1, Supplemental Fig. S3). All plant lines displayed 3:1 antibiotic selection segregation in the T_2 and qPCR analysis indicated that each line carried the transgene integrated at a single locus. Comparison of transgene copy number in T_3 lines to *FLS2* expression level showed very little correlation, suggesting that expression levels are likely dictated by transgene insertion site rather than transgene copy number (Fig. 1, Supplemental Fig. S3).

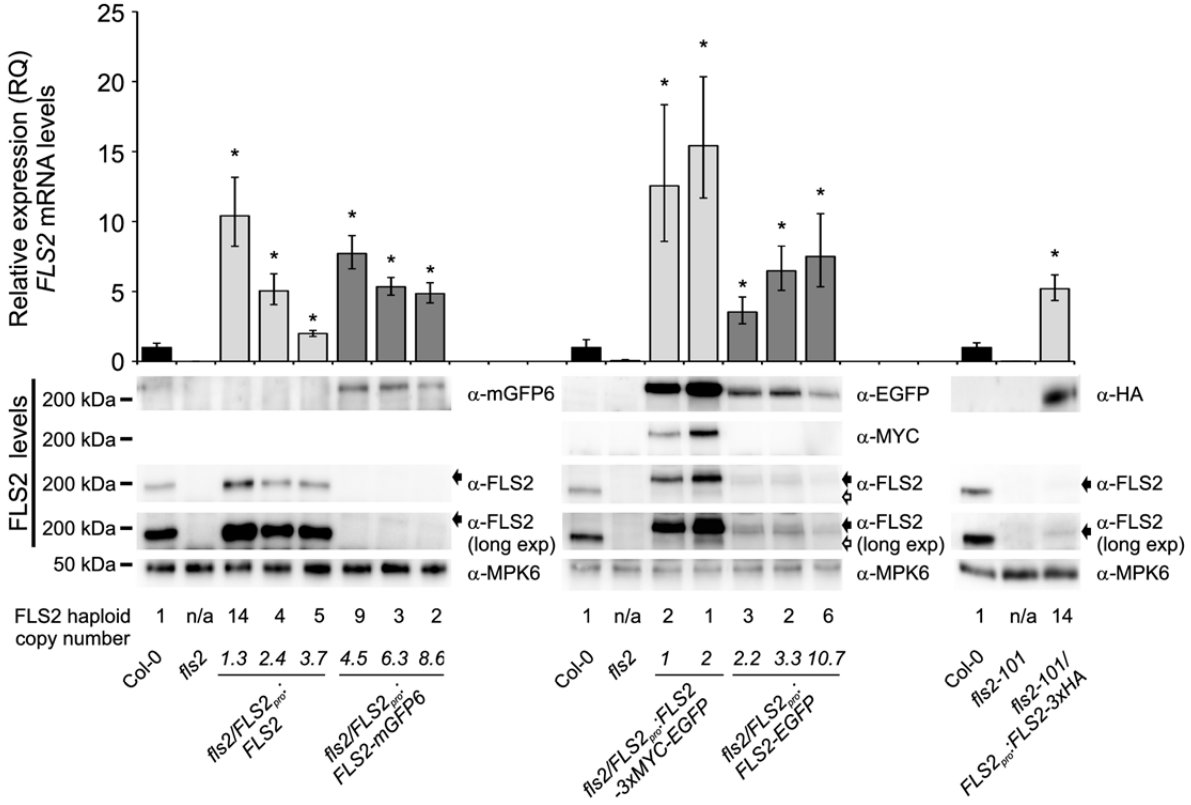

Figure 1. Expression of tagged and untagged forms of FLS2 in *fls2* mutant backgrounds. Upper - Graph shows qRT-PCR analysis of *FLS2* expression levels after normalization to *PEX4* mRNA and relative to that in Col-0. Values were calculated using the $\Delta\Delta C_T$ method. Error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's *t*-test. Graph shows data from 1 of 2 biological replicates and * indicates significant increase in *FLS2* mRNA levels compared to that in Col-0 in both biological replicates ($p<0.05$). Lower – Immunoblot analysis of levels of FLS2 (α-FLS2 antibody against the FLS2 C-terminus) or tagged FLS2 (antibody directed against mGFP6, HA, EGFP, or MYC tags). MPK6 levels are shown as a loading control (α-MPK6). Long exp indicates long exposure for FLS2 (some signal will be saturated). Black arrow indicates FLS2 epitope fusion detected using α-FLS2 antibody. White arrow indicates FLS2-sized band likely originating from FLS2 epitope fusion cleavage.

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170 It was not possible to directly compare protein levels between the different tagged and
171 untagged forms of FLS2 as the only α-FLS2 antibody available, raised against the
172 extreme C-terminus of FLS2 (Chinchilla et al., 2006; Hurst et al., 2017), shows variable

and apparently reduced sensitivity towards epitope-tagged FLS2 (e.g. compare relative signal intensity for α-FLS2 and α-GFP in Fig. 1 and Supplemental Fig. S3 for *fls2/FLS2$_{pro}$:FLS2-3xMYC-EGFP* and *fls2/FLS2$_{pro}$:FLS2-EGFP*). This is likely due to the tags, by virtue of being attached to the C-terminus of FLS2, partially disrupting the epitope recognised by the α-FLS2 antibody, with different tags affecting antibody binding to varying degrees. It is therefore not appropriate to draw quantitative comparisons between FLS2 levels in different lines using this antibody, which is important to note for future studies comparing different tagged and untagged FLS2 lines. A similar situation was observed for BAK1 when an antibody raised against the C-terminus was used to probe tagged and untagged BAK1-expressing lines (Ntoukakis et al., 2011). Despite this, immunoblot analysis using antibodies against either mGFP6, EGFP, MYC, or HA epitopes revealed signal at the appropriate molecular weight from the epitope-tagged FLS2-expressing lines, indicating that FLS2 protein was present. Relative *FLS2* mRNA and FLS2 protein levels within each set of lines correlated, with the exception of *fls2/FLS2$_{pro}$:FLS2-EGFP* #10.7, where high mRNA levels did not translate into higher protein levels. Although we do not have an explanation for this, sufficient FLS2-EGFP protein was produced to be detected by both anti-EGFP and anti-FLS2 immunoblot analysis. Interestingly in FLS2-3xMYC-EGFP-expressing lines, an untagged FLS2 sized band was frequently observed, albeit weaker than the α-FLS2 signal from Col-0-derived FLS2 or full-length FLS2-3xMYC-EGFP, which is reactive with the FLS2 antibody but not MYC or GFP antibodies. This suggests the presence of a cleavage product where both 3xMYC and EGFP have been removed leaving a form of FLS2 that closely resembles wild-type FLS2.

After flg22 treatment, FLS2-3xHA- and FLS2-mGFP6-expressing lines showed little MAPK6/3 activation, whereas MAPK6/3 activation in FLS2-EGFP-, FLS2-3xMYC-EGFP-, and untagged FLS2-expressing lines were essentially indistinguishable from that in Col-0 (Fig. 2A). These data indicate that the presence of a C-terminal tag can have an impact on flg22-mediated FLS2 signalling outputs. RLKs have been shown to act in large multicomponent complexes (Jorda et al., 2016; Yeh et al., 2016), raising the possibility that the presence of the epitope tag in mGFP6- or HA-tagged lines could be

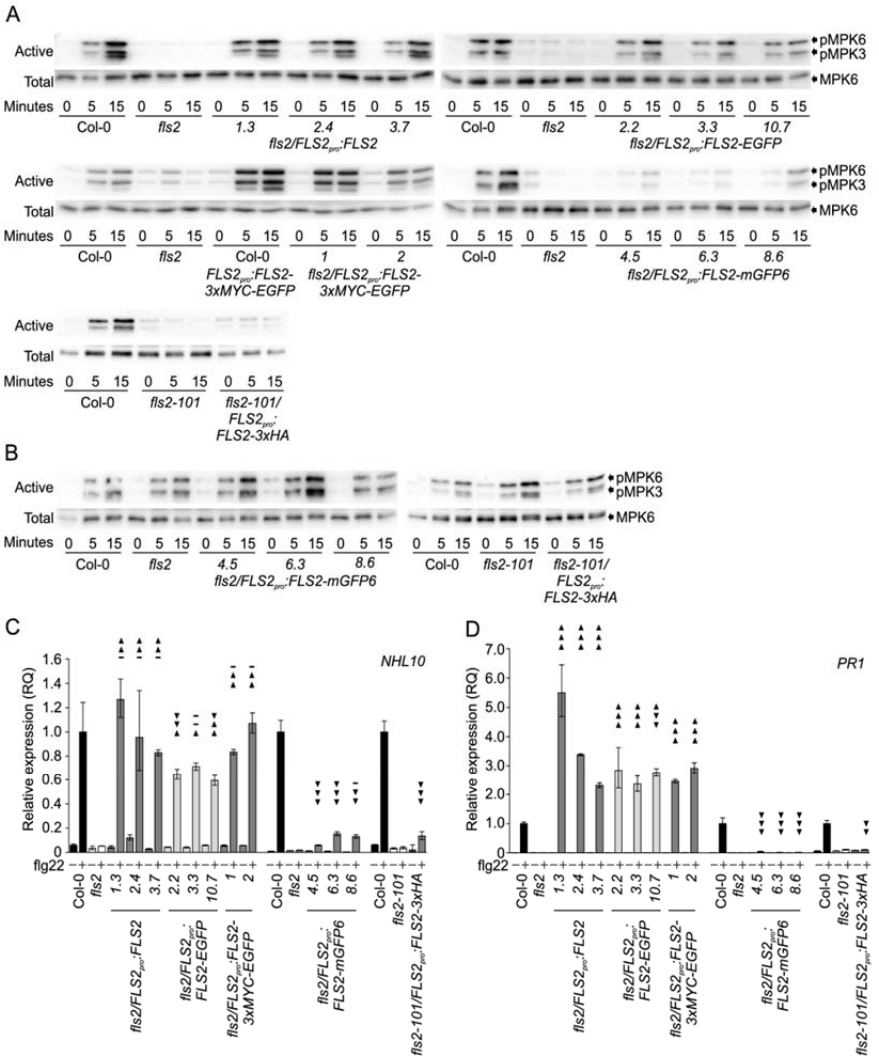


Figure 2. Plants expressing epitope-tagged FLS2 show variable MAPK activation and gene induction in response to elicitation by flg22. A, MAPK activation in *fls2* mutant seedlings expressing FLS2 tagged with 3xHA, mGFP6, EGFP, or 3xMYC-EGFP or untagged FLS2 in response to 100 nM flg22 as determined over time by immunoblot analysis. MAPK assays of Col-0 *FLS2*$_{pro}$*:FLS2-3xMYC-EGFP* lines were included for completeness but were not taken further as no deleterious effects were observed and no conclusion about complementation could be drawn. B, MAPK activation in seedlings expressing 3xHA- or mGFP6-tagged FLS2 in response to 100 nM elf18 as determined over time by immunoblot analysis. Blots in A and B were probed with α-p42/44 (active MAPK; pMPK6/3) and α-MPK6 (total) as a loading control. C and D, Expression analysis of *NHL10* (C) and *PR1* (D). Expression levels were normalized to *PEX4* mRNA and relative to gene expression in Col-0 at 1h (*NHL10*) or 24h (*PR1*). 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. Data shown are representative of three independent biological replicates; significant increases (▲, $p<0.05$), decreases (▼, $p<0.05$) or no difference (-) in expression compared to that in Col-0 in each of the individual biological replicates are shown above each sample to illustrate consistency between biological replicates. The remaining two data sets are shown in Supplemental Figure 4.

acting as a general suppressor of PTI signalling. The elongation factor-Tu receptor, EFR, activates MAPK cascades in an almost identical manner to FLS2 during

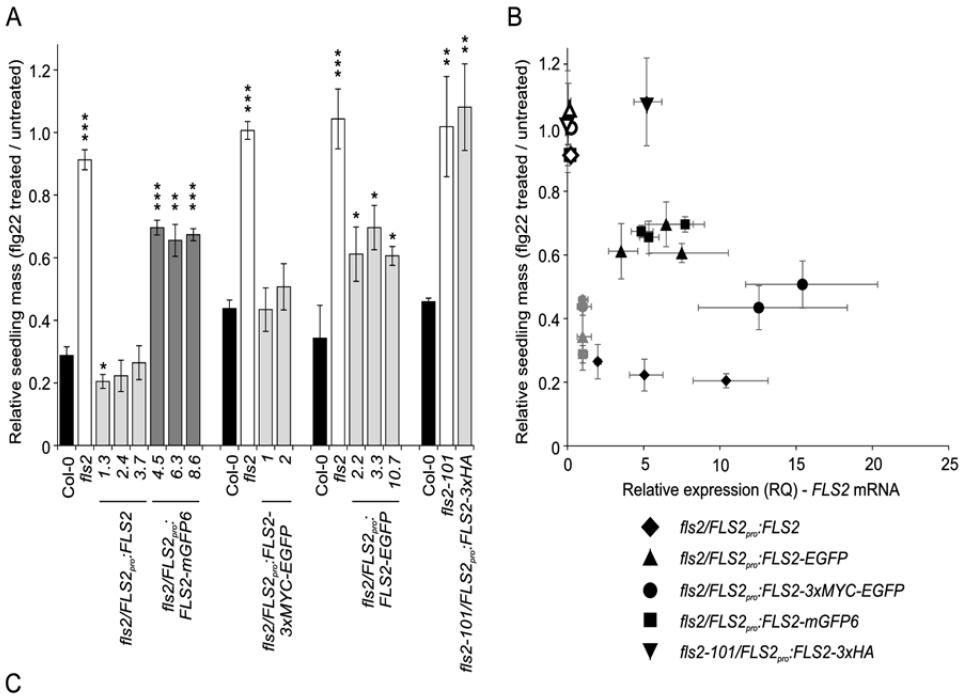
perception of bacterial pathogens (Zipfel et al., 2006). FLS2-3xHA- and FLS2-mGFP6-expressing lines were therefore treated with the elongation factor-Tu-derived peptide elf18 and showed normal MAPK induction when compared to that in Col-0 (Fig. 2B). This demonstrates that the effect of mGFP6 or HA epitope tags on FLS2 is restricted to outputs of FLS2-mediated signalling rather than PTI responses in general.

PAMP perception leads to transcriptional reprogramming and defence gene expression (Asai et al., 2002). In light of our conflicting MAPK activation data, we decided to test whether epitope-tagged FLS2-expressing lines exhibit changes in PAMP-induced gene expression. After flg22 treatment, typical early (*NHL10*; Zipfel et al., 2004) and late (*PR1*; Robatzek and Somssich, 2002) PAMP-induced genes were upregulated in Col-0 but not in *fls2* mutants (Fig. 2C and D, Supplemental Fig. S4). However, *fls2* plants expressing epitope-tagged FLS2 variants showed variable PAMP-triggered gene induction when compared to that in Col-0 (Fig. 2C and D, Supplemental Fig. S4). Lines expressing FLS2-3xHA or FLS2-mGFP6 showed little to no gene induction compared to that in Col-0, whereas lines expressing untagged FLS2 or 3xMYC-EGFP-tagged FLS2 appeared capable of activating all tested genes in response to flg22 (Fig. 2C and D, Supplemental Fig. S4). FLS2-EGFP-expressing lines were capable of inducing gene expression but did not appear to be quite as effective as untagged or 3xMYC-EGFP-tagged lines at inducing early MAPK mediated response genes such as NHL10 (Fig. 2C; 1 hour induction), whereas late salicylic acid mediated response genes such as PR1 were induced similar to that in Col-0 (Fig. 2D, Supplemental Fig. S4 C and D; 24 hours induction). These data further substantiate the hypothesis that epitope tags can impact upon FLS2 functionality in terms of activating PAMP responsive outputs, regardless of the mediating pathway, but not in a manner that can be readily predicted. Basal expression levels of *NHL10* and *PR1* are almost nil under non-flg22 challenged conditions; the presence of any *FLS2* transgene tested here, regardless of expression level or copy number, failed to elevate either *PR1* or *NHL10* expression compared to that in either unchallenged Col-0 or *fls2* mutant plants (Supplemental Fig. S5). This is similar to the observations made using MAPK activation and suggests that the transgenes do not affect plant physiology or outputs in the absence of flg22.

C-terminal epitope tags can impair FLS2-mediated growth inhibition.

One of the responses to prolonged PAMP-treatments such as flg22 is the inhibition of both seedling shoot and root growth, which is often used as an assay for the overall combined outputs and long-term effects of receptor pathway activation (Gomez-Gomez et al., 1999; Gomez-Gomez and Boller, 2000). In light of our unexpected data above on the varied functionality of FLS2 epitope fusions, we therefore determined whether the lines tested also show variation in overall output resulting from long-term PAMP exposure. Interestingly, all FLS2 C-terminal fusions, with the exception of that in FLS2-3xMYC-EGFP-expressing lines, show impaired ability to inhibit seedling growth after flg22 treatment when compared to that in Col-0. Untagged FLS2-expressing lines essentially showed slightly greater or comparable growth inhibition to that in Col-0 (Fig. 3A), despite FLS2 being present at lower levels than that in FLS2-3xMYC-EGFP-expressing lines. This implies that untagged FLS2 is still more active than the most potent tagged form of FLS2. These data indicate that the presence of a tag contributes more to the observed outcome of FLS2-mediated growth inhibition after flg22 treatment than FLS2 expression level (Fig. 3B).

Combined, these data indicate that C-terminal tagging of FLS2 can affect several PTI responses in a manner that cannot be easily predicted based solely on which epitope tag is used. Furthermore, the overall outcome of FLS2 signalling is consistently reduced in all FLS2 C-terminal fusion-expressing lines, with the potential exception of FLS2-3xMYC-EGFP-expressing plants. It is theoretically possible that the lines with mGFP6-, EGFP-, and HA-tagged FLS2, despite expressing *FLS2* and producing detectable FLS2 protein, do not produce stable FLS2 protein and this leads to diminished responses compared to that in Col-0. Arguing against this is the strong MAPK activation and gene induction in the EGFP-tagged FLS2 lines, and partial rescue of growth inhibition in mGFP6- and EGFP-tagged FLS2 lines. This again suggests that the FLS2 antibody recognising the C-terminal epitope, as used here and previously described (Chinchilla et al., 2006), is not suitable for comparing levels of the various tagged and untagged forms of FLS2. These data combined make the effect of a given tag on FLS2 outputs very difficult to predict.



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Genotype	MAPK activation	NHL10 induction	PR1 induction	Growth inhibition
fls2/FLS2$_{pro}$*:FLS2*	o	++	++	o/+
fls2/FLS2$_{pro}$*:FLS2-mGFP6*	– –	– –	– –	–
fls2/FLS2$_{pro}$*:FLS2-3xMYC-EGFP*	o	++	++	o
fls2/FLS2$_{pro}$*:FLS2-EGFP*	o	o/–	++	–
fls2-101/FLS2$_{pro}$*:FLS2-3xHA*	– –	– –	– –	– –

Figure 3. flg22-mediated growth suppression can be impaired in plant lines expressing epitope-tagged FLS2. A, Average relative seedling mass (flg22 treated/untreated) of seedlings grown in 1 µM flg22 for 10 days (14 days post germination). Data are averages of at least 2 independent biological replicates. Error bars show SEM. */**/*** denotes statistically significant difference compared to that of Col-0 control ($p<0.1$ / $p<0.05$ / $p<0.01$, respectively) determined by one-way ANOVA and Tukey's HSD test. B, Growth inhibition data shown in A plotted against FLS2 mRNA expression data from Figure 1A. Grey and white data points represent Col-0 and *fls2*/*fls2-101* mutants, respectively, used as controls for each indicated genotype/experiment. X-axis (*FLS2* expression) error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval according to Student's *t*-test. Y-axis (growth inhibition) error bars show SEM. Statistically significant differences are as for A and Figure 1A but are omitted here for clarity. C, Summary table of FLS2 responses in all lines used in this study. Difference compared to that in Col-0 for each response is indicated: **++** strong increase, **+** mild increase, **o** no change, **–** mild decrease, **– –** strong decrease.

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Discussion

Epitope fusions are a common tool in molecular biology, and are indispensable for many cell biology applications or where antibodies are not available for a protein of interest. As the use of epitope tagging has become standard practice, it is easy to forget that the epitope itself may impact upon function by altering protein conformation, obscuring ligand binding surfaces, or hindering protein-protein interactions. Deleterious or anomalous effects of fused epitopes on protein function are not limited to FLS2 as reported here, but have also been identified for 20% of tested mammalian proteins (Stadler et al., 2013). The effects of fused epitope tags on RLK function have been shown in a study on BAK1 (Ntoukakis et al., 2011), and seem likely to also affect ERECTA (ER). An *ER_{pro}:ER-LUC* construct only showed complementation in 27% of recovered transformants compared to 100% complementation for an *ER_{pro}:ER* construct, but expression levels of ER-LUC fusions compared to wild-type ER levels were not determined and the significance or possible implications were not discussed further (Kosentka et al., 2017). Alongside BAK1 and BRI1, FLS2 is probably the best studied RLK in plants and much of the published work utilises epitope-tagged forms. FLS2 has been reported to be functional when transformed into Ws-0 accession plants (a natural *fls2* mutant) as 3xMYC-EGFP- or 3xMYC-fusions (Zipfel et al., 2004; Robatzek et al., 2006) but, as these papers state, these data are gain of function analyses not complementation assays; there is no reference for wild-type activity. In our work, we found that FLS2-3xHA and FLS2-mGFP6 expressing lines were broadly impaired in all FLS2 responses tested, FLS2-EGFP expressing lines showed an intermediate phenotype, while only those lines expressing FLS2-3xMYC-EGFP were able to complement the gene induction, MAPK activation, and seedling growth inhibition phenotypes of *fls2* mutants. Interestingly, FLS2-mGFP6- and FLS2-EGFP-expressing lines show similar growth inhibition despite FLS2-mGFP6 conferring minimal MAPK activation and reduced gene induction and FLS2-EGFP conferring wild-type levels of MAPK activation and only mildly affected gene induction. This suggests that whereas MAPK and gene induction are differentially affected in these lines, other aspects of the flg22 response leading to growth inhibition not measured here are possibly less affected in FLS2-mGFP6-expressing lines. Alternatively, absolute levels of MAPK activation and

gene induction may not be closely correlated with outcomes of growth inhibition experiments. This makes the effects of tags on each specific aspect or outcome of FLS2 function very difficult to predict. These unexpected and potentially variable effects of tags on FLS2 appear to have gone un-reported, likely because constructs are at least partially functional (Dunning et al., 2007; Hemsley et al., 2013; Fig. 1–3), unlike in the case of BAK1 where all tested C-terminal tags abolish function during PTI signalling (Ntoukakis et al., 2011). Effects of FLS2 expression levels on functional outcomes is reported to be non-linear, with several orders of magnitude increase in expression required to increase measured outputs a few fold (Gomez-Gomez and Boller, 2000). In WT plants, FLS2 receptors have an EC_{50} of ~0.2 nM but a K_d of ~1.3 nM for flg22 (Bauer et al., 2001). This indicates that FLS2 signalling responses are saturated long before all receptor binding sites are occupied, and a 10-fold increase in FLS2 expression is therefore not going to increase outputs 10-fold, particularly under treatment conditions commonly used where flg22 concentrations range from 100 nM – 10 µM flg22. In fact, our observations suggest that a 10-fold increase in FLS2 expression (e.g. *$FLS2_{pro}$:FLS2* line 1.3) has, at most, a 1.2-fold effect on growth inhibition and minimal effect on MAPK activation or gene expression compared to that in Col-0 or other *$FLS2_{pro}$:FLS2* lines with lesser untagged FLS2 expression. It is also a formal possibility that other factors, such as BAK1 or downstream components, are present in rate limiting amounts. Elevated FLS2 expression would therefore have limited additional effect as extra activated FLS2 receptor would be reduced in its ability to transduce signal. As the *FLS2* promoter and coding sequence used in all of the lines studied here is identical and multiple independent transformants for each construct show the same phenotype regardless of FLS2 expression level, the observed variation must be a result of either the epitope tag or the vector T-DNA sequence. The vector effect can likely be excluded as our own *fls2/$FLS2_{pro}$:FLS2-EGFP* and *fls2/$FLS2_{pro}$:FLS2* lines use the same vector series (Karimi et al., 2002).

Interestingly, a 3xHA tag (Dunning et al., 2007) of ~3 kDa adopting a largely disordered conformation (Georgieva et al., 2015) is apparently more effective at reducing FLS2 responses compared to the ~27 kDa, highly stable and structured mGFP6 or EGFP

fusions. While this is outwardly counterintuitive, the effects of exposed, terminal regions of disorder, such as MYC or HA repeat peptide tags, have been suggested to have deleterious effects on protein function (Georgieva et al., 2015). BAK1-3xHA constructs were also unexpectedly impaired in BR responses despite being strongly expressed (Ntoukakis et al., 2011), indicating that C-terminal 3xHA tags may impair RLK function more than would otherwise be expected given their size. The linker between FLS2 and mGFP6 contains 2 prolines (Supplemental Fig. S2), and proline is an amino acid known to produce inflexible linkers with reduced mobility (Radford et al., 1987). This may explain the difference in functionality observed between FLS2-mGFP6 and FLS2-EGFP, as the EGFP linker is predicted to be highly flexible and therefore less likely to sterically hinder downstream interactions (Supplemental Fig. S2). The most widely used and published FLS2 epitope fusion, and demonstrated to be most active based on our data, is the FLS2-3xMYC-EGFP construct (Robatzek et al., 2006). In this construct, the highly disordered 3xMYC epitope (Georgieva et al., 2015) separates EGFP from FLS2 more than that in any other construct tested (Supplemental Fig. S2). This may reduce steric hindrance by GFP to a greater extent than that resulting from any of the other constructs used here and allow for a more native conformation of the FLS2 C-terminus and better access for interacting proteins. However, it is worth noting that in FLS2-3xMYC-EGFP-expressing lines, an α-FLS2 reactive band is frequently observed at the size expected for full length FLS2. This band is not detected by either α-MYC or α-GFP antibodies, suggesting that this cleavage product contains a C-terminus very similar to, or only slightly longer than, that of native FLS2. It cannot therefore be ruled out that this cleavage product may be able to function like untagged FLS2 and provide a greater degree of flg22 responsiveness in FLS2-3xMYC-EGFP-expressing lines than would be expected given the behaviour of other epitope-tagged FLS2 lines. It should be noted that we have not tested this construct for complementation of every FLS2 mediated output described in the literature.

A possible explanation for the deleterious effects of epitope tagging on RLK outputs identified here or previously reported (Ntoukakis et al., 2011; Kosentka et al., 2017) involve a weak kinase activity (e.g. FLS2 or ER; Schwessinger et al., 2011) in the

receptor/co-receptor pairing. It may well be the case that epitope tags impact upon all RLKs, but the effects are minor or unnoticed if both RLK partners are strong kinases (e.g. BRI1, BAK1; Schwessinger et al., 2011) and able to compensate for the negative effects of the tag. The use of RLK C-terminal fusions may therefore reduce researchers' ability to properly observe and differentiate the effects of various mutant forms of RLKs on signalling or interactions. As a result, many intricacies and details of RLK signalling may have been obscured (Dunning et al., 2007; Sun et al., 2012; Hemsley et al., 2013; Kosentka et al., 2017), and potentially false negative, uninterpretable, or inappropriate conclusions may have been reached in other studies that have therefore gone unpublished.

While this study indicates that untagged forms of FLS2 provide the best possible approach for testing functionality of FLS2 variants, it is of course impossible to avoid epitope tags altogether. Much of the vital cell biology data on RLK localisation and trafficking (Robatzek et al., 2006; Beck et al., 2012; Choi et al., 2013; Liang et al., 2013; Spallek et al., 2013; Smith et al., 2014; Mbengue et al., 2016) could not have been achieved without the presence of GFP or similar marker proteins, and the field would be much poorer for their absence. However, as FLS2 (this study), BAK1 (Ntoukakis et al., 2011), and ER (Kosentka et al., 2017) function have now all been shown to be affected by C-terminal epitope tags, we suggest that any functional study on RLKs using epitope tags should be designed and interpreted with care; functionality of epitope tag fusions should not be assumed and full quantitative complementation studies of each output of interest should precede further evaluation. Possible alternative solutions to C-terminal tagging not yet considered by the RLK field is the use of tags internal to the protein sequence or the use of "Innocuous" tags (Georgieva et al., 2015). In the case of FLS2, the obvious and immediate solution is to base all future constructs on FLS2-3xMYC-EGFP (Robatzek et al., 2006), but it would be interesting to determine whether a 3xMYC or other long and flexible linker between the RLK of interest and GFP provides a generic solution to the issue of C-terminal RLK tags.

Methods

Plant lines and growth conditions

All Arabidopsis lines used are of the Col-0 ecotype. The *FLS2* mutant alleles, *fls2* (SAIL_691C4) (Zipfel et al., 2004) and *fls2-101,* as well as *fls2-101/FLS2$_{pro}$:FLS2-3xHA* lines (Dunning et al., 2007) and *fls2/FLS2$_{pro}$:FLS2-3xMYC-EGFP* (Mbengue et al., 2016) have been described previously. *fls2/FLS2$_{pro}$:FLS2-mGFP6* (pMDC107 based; Curtis and Grossniklaus, 2003) and *fls2/FLS2$_{pro}$:FLS2-EGFP* (pH7FWG0 based; Karimi et al., 2002) were created for this study using a construct with the same promoter region and open reading frame of FLS2 lacking a stop codon as found in the *FLS2$_{pro}$:FLS2-3xMYC-EGFP* construct and previously described (Zipfel et al., 2004; Robatzek et al., 2006). *fls2/FLS2$_{pro}$:FLS2* constructs were created using the same *FLS2* fragment as for mGFP6- and EGFP-tagged lines but with a stop codon introduced and cloned into pK7WG0 (Karimi et al., 2002). Transgenic Arabidopsis plants were generated by Agrobacterium-mediated floral dip transformation (Clough and Bent, 1998) and selected for homozygosity at T_3. Plant material for experiments was grown on 0.5x MS medium, 0.8% phytagar under 16:8 light:dark cycles at 20°C in MLR-350 growth chambers (Panasonic).

Transgene copy number determination

Genomic DNA was extracted from Arabidopsis plate-grown seedlings (Edwards et al., 1991). Real time PCR determination of copy number was performed using SYBR green and the $\Delta\Delta C_T$ method as previously described (Bubner and Baldwin, 2004). Validated primer pairs used were against *NHL10* (endogenous control) and *FLS2* (target for copy number determination).

Gene expression analysis

Gene expression levels were analysed using reverse transcription quantitative PCR (RT-qPCR). For this, 10 seedlings of each genotype 10 days post germination were treated with 1µM flg22 for the indicated times. The 10 seedlings from each genotype at each time point for each treatment were pooled before further analysis. RNA was extracted using RNAeasy kit with on column DNAse digestion according to the

manufacturer's instructions (Qiagen). 2 µg RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). All transcripts were amplified using validated gene-specific primers. Expression levels were normalized against *PEX4* (At5g25760) (Wathugala et al., 2012). Each sample was analysed in triplicate (technical replicates) for each primer pair within each biological replicate. Relative quantification (RQ) was achieved using the $\Delta\Delta C_T$ (comparative cycle threshold) method (Yuan et al., 2006; Schmittgen and Livak, 2008). Significant differences between samples in a biological replicate were determined from a 95% confidence interval calculated using the t distribution. Fully independent biological replicates were performed over a period of 2 years with each genotype only being present once in each replicate.

MAPK activation

Performed essentially as previously described (Schwessinger et al., 2011); 6 seedlings of each genotype 10 days post germination were treated with 100nM flg22 for the indicated times in 2 ml 0.5x MS medium. The 6 seedlings from each genotype at each time point for each treatment were pooled before further analysis. Fully independent biological replicates were performed over a period of 2 years with each genotype only being present once in each replicate.

Immunoblot analysis of protein levels

Proteins were extracted from pooled whole seedlings as previously described (Hurst et al., 2017) and blotted for active and total MAPK (Schwessinger et al., 2011) or FLS2 (Martinez-Garcia et al., 1999; Hurst et al., 2017). α-HA: Roche 11867423001, α-mGFP6: Santa Cruz Biotechnologies sc-9996, α-EGFP: Roche 11814460001, α-MYC: Thermo MA1-21316, α-p42/44: CST #9101, α-MPK6: Sigma-Aldrich A7104.

Seedling growth inhibition

Performed essentially as previously described (Gomez-Gomez et al., 1999). 4 days post-germination, 10 seedlings with green cotyledons, erect hypocotyls, and emergent root of the named genotypes were transferred to 12-well plates (2 seedlings per well),

ensuring the cotyledons were not submerged. Wells contained 2 ml of 0.5x MS liquid medium with or without 1 µM flg22. Seedlings were incubated for a further 10 days and the fresh weight of pooled seedlings in each genotype for each treatment was measured and an average was calculated. flg22-treated and -untreated weights for each genotype were calculated and data are averages of these measurements for at least 2 biological replicates. Fully independent biological replicates were performed over a period of 18 months with each genotype only being present once in each replicate.

Accession numbers

FLS2 – At5g46330, BAK1 – At4g33430, ER – At2g26330, NHL10 – At2g35980, PR1 – At2g14610, PEX4 - At5g25760, MPK6 – At2g43790, MPK3 – At3g45640.

Supplemental data

Supplemental Data Titles

Supplemental Figure S1. Alignment of GFP sequences found in vectors used in this study.

Supplemental Figure S2. Alignment of FLS2 and appended C-terminal sequences in the constructs used in this study.

Supplemental Figure S3. Supporting data for Figure 1: biological replicates.

Supplemental Figure S4. Supporting data for Figure 2: biological replicates.

Supplemental Figure S5. Supporting data for Figure 2 and Supplemental Figure S4.

Supplemental Figure S1. Alignment of GFP sequences found in vectors used in this study.

Supplemental Figure S2. Alignment of FLS2 and appended C-terminal sequences in the constructs used in this study.

Supplemental Figure S3. Supporting data for Figure 1: biological replicates. Expression of tagged and untagged forms of FLS2 in *fls2* mutant backgrounds.

Supplemental Figure S4. Supporting data for Figure 2: biological replicates. flg22-mediated gene expression induction in epitope-tagged FLS2 expressing plants – biological replicates to support Figure 2.

Supplemental Figure S5. Supporting data for Figure 2 and Supplemental Figure S4. FLS2 constructs do not promote marker gene expression in the absence of flg22.

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Figure legends

Figure 1. Expression of tagged and untagged forms of FLS2 in *fls2* mutant backgrounds. Upper - Graph shows qRT-PCR analysis of *FLS2* expression levels after normalization to *PEX4* mRNA and relative to that in Col-0. 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. Graph shows data from 1 of 2 biological replicates and * indicates significant increase in *FLS2* mRNA levels compared to that in Col-0 in both biological replicates ($p<0.05$). Lower – Immunoblot analysis of levels of FLS2 (α-FLS2 antibody against the FLS2 C-terminus) or tagged FLS2 (antibody directed against mGFP6, HA, EGFP, or MYC tags). MPK6 levels are shown as a loading control (α-MPK6). Long exp indicates long exposure for FLS2 (some signal will be saturated). Black arrow indicates FLS2 epitope fusion detected using α-FLS2 antibody. White arrow indicates FLS2-sized band likely originating from FLS2 epitope fusion cleavage.

Figure 2. Plants expressing epitope-tagged FLS2 show variable MAPK activation and gene induction in response to elicitation by flg22. A, MAPK activation in *fls2* mutant seedlings expressing FLS2 tagged with 3xHA, mGFP6, EGFP, or 3xMYC-EGFP or untagged FLS2 in response to 100 nM flg22 as determined over time by immunoblot analysis. MAPK assays of Col-0 $FLS2_{pro}$*:FLS2-3xMYC-EGFP* lines were included for completeness but were not taken further as no deleterious effects were observed and no conclusion about complementation could be drawn. B, MAPK activation in seedlings expressing 3xHA- or mGFP6-tagged FLS2 in response to 100 nM elf18 as determined over time by immunoblot analysis. Blots in A and B were probed with α-p42/44 (active MAPK; pMPK6/3) and α-MPK6 (total) as a loading control. C and D, Expression analysis of *NHL10* (C) and *PR1* (D). Expression levels were normalized to *PEX4* mRNA and relative to gene expression in Col-0 at 1h (*NHL10*) or 24h (*PR1*). 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. Data shown are representative of three independent biological replicates; significant increases (▲, $p<0.05$), decreases (▼, $p<0.05$) or no difference (-) in expression compared to that in Col-0 in each of the individual biological replicates are shown above each sample to illustrate consistency between biological replicates. The remaining two data sets are shown in Supplemental Figure 4.

Figure 3. flg22-mediated growth suppression can be impaired in plant lines expressing epitope-tagged FLS2. A, Average relative seedling mass (flg22 treated/untreated) of seedlings grown in 1 μM flg22 for 10 days (14 days post germination). Data are averages of at least 2 independent biological replicates. Error bars show SEM. */**/*** denotes statistically significant difference compared to that of Col-0 control ($p<0.1$ / $p<0.05$ / $p<0.01$, respectively) determined by one-way ANOVA and Tukey's HSD test. B, Growth inhibition data shown in A plotted against FLS2 mRNA expression data from Figure 1A. Grey and white data points represent Col-0 and *fls2*/*fls2-101* mutants, respectively, used as controls for each indicated genotype/experiment. X-axis (*FLS2* expression) error bars represent RQMIN and RQMAX and constitute the acceptable error level for a 95% confidence interval

according to Student's *t*-test. Y-axis (growth inhibition) error bars show SEM. Statistically significant differences are as for A and Figure 1A but are omitted here for clarity. C, Summary table of FLS2 responses in all lines used in this study. Difference compared to that in Col-0 for each response is indicated: **++** strong increase, **+** mild increase, **o** no change, **–** mild decrease, **– –** strong decrease.

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