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1 **Title: Subcellular localization requirements and specificities for plant immune receptor Toll-**
2 **interleukin-1 receptor (TIR) signaling**

3

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28 **SUMMARY**

29 Recent work shed light on how plant intracellular immune receptors of the nucleotide-binding
30 leucine-rich repeat (NLR) family are activated upon pathogen effector recognition to trigger immune
31 responses. Activation of Toll-interleukin-1 receptor (TIR) domain-containing NLRs (TNLs) induces
32 receptor oligomerization and close proximity of the TIR domain, which is required for TIR enzymatic
33 activity. TIR-catalysed small signaling molecules bind to EDS1 family heterodimers and
34 subsequently activate downstream helper NLRs, which function as Ca²⁺ permeable channel to
35 activate immune responses eventually leading to cell death. Subcellular localisation requirements of
36 TNLs and signaling partners are not well understood, although they are required to fully understand
37 the mechanisms underlying NLR early signaling. TNLs show diverse subcellular localization while
38 EDS1 shows nucleocytosolic localization. Here, we studied the impact of TIR and EDS1
39 mislocalization on the signaling activation of different TNLs. In *N. benthamiana*, our results suggest
40 that close proximity of TIR domains isolated from flax L6 and Arabidopsis RPS4 and SNC1 TNLs
41 drives signaling activation from different cell compartments. Nevertheless, both Golgi-membrane
42 anchored L6 and nucleocytosolic RPS4 have the same requirements for EDS1 subcellular localization
43 in *Arabidopsis thaliana*. By using mislocalized variants of EDS1, we found that autoimmune L6 and
44 RPS4 TIR domain can induce seedling cell death when EDS1 is present in the cytosol. However,
45 when EDS1 is restricted to the nucleus, both induce a stunting phenotype but no cell death.
46 Our data point out the importance of thoroughly investigating the dynamics of TNLs and signaling
47 partners subcellular localization to fully understand TNL signaling.

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50

51 **INTRODUCTION**

52

53 Plant immunity relies on a combination of cell-surface and intracellular receptors that perceive
54 infection-associated molecules (Dodds and Rathjen, 2010). Cell surface receptors, also called
55 pattern-recognition receptors (PRRs), are anchored to the cell plasma membrane to recognize
56 extracellular signals via their ectodomain. Intracellular receptors belong to the nucleotide-binding
57 leucine-rich repeat receptor (NLR) family, whose modular architecture is similar to animal receptors
58 involved in innate immunity and apoptosis (Bentham et al., 2016; Duxbury et al., 2016). Most NLRs
59 specifically sense, directly or indirectly, the presence of pathogen effectors that are secreted inside
60 the plant cell (Zhang et al., 2017a). Although cell surface and intracellular immune receptors are
61 structurally different and localize to different subcellular compartments, their activation induces
62 largely overlapping immune responses (reactive oxygen species production, MAPKinase cascade

63 activation, ion fluxes, a massive transcriptional reprogramming, and sometimes the induction of cell
64 death) (Lu and Tsuda, 2021). Increasing evidence suggest that PRR- and NLR-mediated signaling
65 pathway mutually potentiate each other (Bernoux et al., 2022).

66 Canonical NLRs contain three modules: the C-terminal leucine-rich repeat (LRR) domain often acts
67 as a sensor that allows specific pathogen effector recognition. The central nucleotide binding (NB)
68 domain controls the switch between inactive (OFF) and active (ON) states with a massive
69 conformational change upon activation (Bernoux et al., 2016; Wang et al., 2019b). The N-terminal
70 domain often acts as a signaling domain. In flowering plants, this region commonly contains a coiled-
71 coil (CC) or Toll-interleukin-1 receptor (TIR) domain. The TIR domain is involved in immune
72 signaling pathways across phyla (Ve et al., 2015; Essuman et al., 2022). Upon effector recognition,
73 several NLRs have been shown to associate into oligomeric complexes termed resistosomes, which
74 is required to further activate downstream signaling (Wang et al., 2019a; Ma et al., 2020; Martin et
75 al., 2020). The 3D structure of the Arabidopsis ZAR1 and wheat Sr35 CC-containing NLRs (CNLs)
76 showed that ZAR1 and Sr35 form a pentamer upon activation and the N-terminal CC domain
77 complex forms a funnel-like structure similar to pore-forming proteins (Wang et al., 2019a; Förderer
78 et al., 2022). Further studies revealed that activated ZAR1 targets the plasma membrane and
79 functions as Ca^{2+} permeable channels via its CC domain, to rapidly activate immune responses (Bi
80 et al., 2021). Signaling of TIR-containing NLRs (TNLs) is more complex. Structural analyses of *N.*
81 *benthamiana* TNLs ROQ1 and Arabidopsis RPP1 revealed a tetrameric resistosome upon effector
82 recognition. TNL oligomerization lead to the induced proximity of the N-terminal TIR domains,
83 which create access to the TIR NADase catalytic site (Gerdtts et al., 2015; Horsefield et al., 2019;
84 Wan et al., 2019; Ma et al., 2020; Martin et al., 2020). TIR complex assembly can also result in a
85 helix-like structure. Depending on the TIR self-association interface and protein complex
86 stoichiometry (tetramer or helix), TIR domains display different enzymatic activity by using different
87 substrates (NAD(P) and DNA/RNA, respectively), to release small signaling molecules (Horsefield
88 et al., 2019; Wan et al., 2019; Huang et al., 2022; Jia et al., 2022; Yu et al., 2022). All sensor TNLs
89 studied so far require the nucleocytoplasmic lipase-like EDS1 (ENHANCED DISEASE
90 SUSCEPTIBILITY 1) to activate immune responses (Wiermer et al., 2005; Lapin et al., 2020). EDS1
91 forms mutually exclusive heterodimers with two other members of the EDS1 family, SAG101
92 (SENESCENCE-ASSOCIATED GENE 101) or PAD4 (PHYTOALEXIN DEFICIENT 4) (Lapin et
93 al., 2020). Upon TNL activation, EDS1/PAD4 or EDS1/SAG101 bind distinct TIR-catalysed
94 NAD(P)-derived small molecules and subsequently associate with the downstream helper CC_R-
95 containing NLRs (RNLs), ADR1 and NRG1, respectively (Huang et al., 2022; Jia et al., 2022). Like
96 ZAR1, autoactivated NRG1 also seems to oligomerize and form Ca^{2+} permeable channels at the

97 plasma membrane (Jacob et al., 2021), suggesting the ultimate output of TNLs signaling is CNL-
98 induced Ca^{2+} influx to activate immune responses (Jacob et al., 2021).

99 Nevertheless, it is unclear where in the cell does the activation of TNLs and the first steps of signaling
100 occurs, and whether this is conserved across NLRs. TNLs are distributed in a range of subcellular
101 locations and NLR mislocalization often affects their function (Takemoto et al., 2012).
102 Nucleocytoplasmic transport can also play an important role for regulating immune complex
103 formation and signaling activation upon NLR activation, including for EDS1 function (Lüdke et al.,
104 2022). Arabidopsis SNC1 and RPS4 TNLs both display a nucleocytoplasmic localization while flax
105 L6 is anchored to the Golgi membrane (Wirthmueller et al., 2007; Cheng et al., 2009; Takemoto et
106 al., 2012). Flax L6 confers resistance to the fungal pathogen *Melampsora lini* (flax rust) producing
107 the AvrL567 effector (Dodds et al., 2006). Releasing L6 from the Golgi membrane impacts L6-
108 mediated resistance to flax rust (Takemoto et al., 2012). RPS4 functions in concert with another TNL,
109 RRS1, to activate disease resistance upon recognition of AvrRps4 and PopP2 effectors from
110 *Pseudomonas syringae* and *Ralstonia solanacearum* bacterial pathogens, respectively. RRS1 acts as
111 a sensor to recognize the presence of the effectors via its integrated WRKY domain, while RPS4
112 translates the signal via its TIR domain (Williams et al., 2014; Le Roux et al., 2015; Sarris et al.,
113 2015). Nuclear pools of SNC1 and RPS4 were shown to be required for their signaling function
114 (Wirthmueller et al., 2007; Cheng et al., 2009; Xu et al., 2014). However, both RPS4 and SNC1
115 function depends on RNLs ADR1 and NRG1, which are thought to activate immune responses at the
116 plasma membrane (Dong et al., 2016; Wu et al., 2019; Saile et al., 2020; Saile et al., 2021). Earlier
117 studies showed that EDS1 physically interacts with some NLRs, including RPS4 and SNC1
118 (Bhattacharjee et al., 2011; Heidrich et al., 2011; Kim et al., 2012; Huh et al., 2017; Zhang et al.,
119 2019), and that a balanced cytosolic and nuclear shuttle of EDS1 is important for Arabidopsis basal
120 and TNL-mediated immunity (García et al., 2010). However, a bifurcation between nuclear and
121 cytosolic EDS1 activities was also previously suggested with the nuclear fraction being involved in
122 gene reprogramming while the cytosolic pool is involved in cell death activation (Heidrich et al.,
123 2011; Lapin et al., 2019). Hence, it is intriguing to know how and in which subcellular compartment
124 do TNLs that are not nucleo-cytosolic, such as L6, function with EDS1 to activate downstream
125 signaling defense pathway.

126 In this study, we investigated the subcellular localization requirement of three TIR domains isolated
127 from different TNLs, as well as EDS1, for the activation of TIR signaling. We first studied the
128 signaling function of mislocalized TIR domains isolated from flax L6, and Arabidopsis RPS4 and
129 SNC1 NLR, as these TIR domains function as signaling unit on their own. They can induce pathogen-
130 independent defence signaling when overexpressed *in planta*, including the heterologous model plant
131 *Nicotiana benthamiana* (Swiderski et al., 2009; Bernoux et al., 2011; Williams et al., 2014; Zhang et

132 al., 2017b) and thus can be used as a tool to study TNL early signaling events. We also determined
133 the impact of EDS1 mislocalization on TIR signaling in transgenic Arabidopsis lines co-expressing
134 inducible autoimmune flax L6 variant or RPS4TIR domain with mislocalized EDS1 variants.

135

136 **RESULTS**

137

138 **Membrane attachment of flax L6TIR domain is required for its signaling activity**

139 The flax TNLs L6 and M are targeted to the Golgi and vacuole membrane, respectively, due to the
140 presence of hydrophobic residues in their N-terminal sequence. In contrast, the flax P2 protein lacks
141 such N-terminal extension and does not target cellular membranes (Takemoto et al., 2012). Domain
142 swaps between the N-terminal sequence of L6, M and P2 revealed that membrane attachment
143 contributes to L6 resistance function against flax rust *Melampsora lini* containing corresponding
144 effector AvrL567 in flax (Takemoto et al., 2012).

145 To decipher whether membrane attachment is required for L6 signaling function, we studied the
146 subcellular localization requirements of isolated L6 TIR by using recombinants containing M or P2
147 N-terminal sequences, based on constructs used in Takemoto et al. (2012), fused to the yellow
148 fluorescent protein (YFP). After Agrobacterium-mediated transient expression in flax leaves,
149 confocal analysis showed that L6TIR-YFP displays a punctate pattern of fluorescence similar to that
150 of L6 full-length protein, typical of Golgi membrane labeling (Figure 1A) (Takemoto et al., 2012).
151 As previously reported, L6TIR-YFP signaling activity triggered a cell death/chlorosis phenotype
152 upon transient overexpression in flax, a proxy for NLR-mediated immune response activation (c)
153 (Bernoux et al., 2011). In contrast, expression of P2/L6TIR-YFP variant did not show any Golgi body
154 labelling but instead showed fluorescence in both nuclear and cytosolic compartments and did not
155 induce cell death (Figure 1B, 1F), although it also showed lower protein accumulation (Figure 1I)).
156 Interestingly, mislocalization of L6TIR to a different membrane (tonoplast) (M/L6TIR-YFP) does
157 not affect L6TIR signaling activity (Figure 1C, 1G), whereas a mutation in M signal anchor (M*) that
158 releases most of M*/L6TIR from the vacuole membrane to the nucleocytoplasm (Takemoto et al., 2012)
159 displays much weaker signaling activity compared to M/L6TIR (Figure 1D, 1H). Both M/L6TIR and
160 M*/L6TIR recombinant proteins accumulated at a similar level (Figure 1I), supporting that
161 membrane attachment is important for L6TIR signaling activity. One possible explanation to explain
162 these results is that concentration of the L6TIR domain at the membrane induces its self-association,
163 which is required to activate signaling (Bernoux et al., 2011; Horsefield et al., 2019; Duxbury et al.,
164 2020). Hence, releasing L6TIR to the nucleocytoplasm would reduce L6TIR self-association and prevent
165 signaling. This hypothesis is supported by recent structural evidence that activated TNLs form
166 oligomeric complexes that bring the N-terminal TIR domains into close proximity to activate its

167 NADase enzymatic activity (Wang et al., 2019a; Ma et al., 2020; Martin et al., 2020).

168

169 **Close proximity of L6TIR domain drives signaling activation from the cytosolic compartment**
170 **in flax and *Nicotiana benthamiana***

171 Given that the membrane localisation of L6TIR is likely a requirement for oligomerisation rather than
172 a locational restraint on signaling, we further investigated the subcellular requirement for L6TIR
173 signaling function, by using the nucleocytoplasmic P2/L6TIR recombinant as a tool. When expressed
174 on its own, P2/L6TIR cannot activate signaling (Figure 1F). To test whether close proximity of
175 P2/L6TIR can activate TIR signaling, we fused P2/L6TIR to the flax rust effector AvrM, which was
176 previously reported to form a stable dimer *in planta* (Catanzariti et al., 2010; Ve et al., 2013). When
177 overexpressed in flax leaves of the Hoshangabad flax genotype, AvrM does not trigger any cell death
178 symptoms (Catanzariti et al., 2006). However, overexpression of P2/L6TIR-AvrM fusion induced a
179 similar response to L6TIR-YFP or L6TIR-AvrM (Figure S1A), suggesting that dimerization of AvrM
180 can promote P2/L6TIR signaling. To examine from which compartment (cytosol or nucleus) can
181 L6TIR activate signaling, we generated a series of constructs consisting of signaling active
182 (P2L6TIR-AvrM) or inactive (P2/L6TIR) recombinants fused to YFP, with a nuclear localization
183 signal (NLS) or a nuclear exclusion signal (NES) and corresponding mutants (nls and nes,
184 respectively). Upon transient expression in flax leaves, all constructs from the P2/L6TIR-YFP-
185 NLS/NES series triggered a very weak response (chlorosis) similar to the control P2/L6TIR-YFP. In
186 contrast, all the variants of P2/L6TIR-AvrM-YFP induced a response that was similar to L6TIR-YFP
187 positive control, except the P2/L6TIR-AvrM-YFP-NLS construct (Figure 2A). The same set of
188 constructs trigger similar responses when expressed in the heterologous plant *Nicotiana*
189 *benthamiana*, suggesting that L6 TIR has the same subcellular requirement for signaling activity in
190 both plant species (Figure 2B). To validate this result, we performed ion leakage measurements, a
191 quantitative indicator of cell death intensity. The amount of ion released in *N. benthamiana* leaf
192 samples expressing inactive P2/L6TIR-YFP fusions series was similar to those expressing nuclear
193 restricted P2/L6TIR-AvrM-YFP-NLS or those infiltrated with an empty *A. tumefaciens* strain. In
194 contrast, leaves expressing the NES, nes or nls fusion constructs from the P2/L6TIR-AvrM-YFP
195 series caused significantly higher ion leakage, demonstrating that P2/L6TIR-AvrM-YFP can activate
196 cell death signaling in the cytosolic compartment but not in the nucleus (Figure 2C). Immunoblot
197 analysis confirmed that the inactive P2/L6TIR-AvrM-YFP-NLS fusion was expressed at similar
198 levels to the active versions in both flax and *N. benthamiana* (Figure 2D,E).

199 Confocal microscopy showed that the NLS fusion proteins were detected exclusively in the nucleus
200 and the nucleolus, whereas NES fusions exhibited fluorescence in the cytoplasm and at the nuclear
201 rim, consistent with nuclear exclusion. P2/L6TIR-YFP fusions to nls and nes inactive variants

202 targeted both nuclear and cytosolic compartments (Figure 2F). The P2/L6TIR-AvrM-YFP-nls and
203 nes fusions were mostly or fully excluded from the nucleus, respectively (Figure 2F), which is similar
204 to the localisation of AvrM which is exclusively cytosolic (Figure S1B), likely due to its large size
205 preventing nuclear diffusion in the absence of an NLS (Ve et al., 2013).

206 Finally, to check that the NLS sequence in the AvrM-YFP fusion constructs does not itself disrupt
207 L6TIR signaling activity, we generated the same set of fusion constructs with signaling active L6TIR
208 including its signal anchor. These constructs all induced an immune response in flax that was similar
209 to the control L6TIR-YFP and the fusion proteins, including those with the NLS, were all still tethered
210 to the Golgi membrane and showed similar accumulation (Figure S2). Altogether, these results show
211 that the signaling potent P2/L6TIR-AvrM construct cannot induce cell death when restricted to the
212 nucleus.

213

214 **Close proximity of Arabidopsis RPS4 and SNC1 TIR domains drives signaling activation from** 215 **both nuclear and cytosolic compartments in *N. benthamiana***

216 To decipher whether TIR domains isolated from other NLRs have, like L6TIR, specific subcellular
217 requirements for their signaling activity, we performed a similar analysis with Arabidopsis RPS4TIR
218 and SNC1TIR signaling domains. Unlike L6, RPS4 and SNC1 do not contain a membrane anchor in
219 their N-terminus and show a nucleocytoplasmic distribution (Wirthmueller et al., 2007; Cheng et al.,
220 2009).

221 Overexpression of RPS4 and SNC1 TIR fragments consisting of the structurally defined TIR domain
222 with an additional 42 or 47 residues respectively (RPS4TIR¹⁻²³⁵ and SNC1TIR¹⁻²²⁶) induce a
223 pathogen-independent immune response in *N. benthamiana* or *N. tabacum* (Swiderski et al., 2009;
224 Williams et al., 2014; Zhang et al., 2017b). Interestingly, transient expression of YFP fusions of TIR-
225 only constructs (RPS4TIR¹⁻¹⁹³ and SNC1TIR¹⁻¹⁷⁹) did not induce immune signaling in *N.*
226 *benthamiana* or *N. tabacum* in contrast to the larger TIR fragments RPS4TIR¹⁻²³⁵ and SNC1TIR¹⁻²²⁶
227 (Figure 3A, B). Confocal microscopy and immunoblot analyses showed that all the fusion proteins
228 were present in both the cytosol and the nucleus but not the nucleolus, and accumulated at similar
229 levels (Figure S3, Figure 3C). Hence, like L6, the RPS4 and SNC1 TIR domains alone are not
230 sufficient to activate a proper signaling response and require an extra peptide sequence, here in the
231 C-terminal end of the TIR domain, possibly also to promote oligomerization.

232 To determine in which subcellular compartment RPS4 and SNC1 TIR domains activate signaling, we
233 generated a series of fusion constructs consisting of the signaling inactive (RPS4¹⁻¹⁹³ and SNC1¹⁻¹⁷⁹)
234 and signaling active (RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶) TIRs fused to YFP, with NLS or NES signals, with
235 or without fusion to the dimerizing AvrM. Unlike the P2/L6TIR, fusions of the inactive RPS4¹⁻¹⁹³
236 and SNC1¹⁻¹⁷⁹ TIR alone fragments to AvrM did not lead to any activation of cell death signaling

237 after transient expression in *N. benthamiana*. However, we observed that these inactive RPS4¹⁻¹⁹³ and
238 SNC1¹⁻¹⁷⁹ TIR alone fragments with an NLS fusion induced cell death, with or without the AvrM
239 fusion (Figure 3 D-G). In contrast, RPS4¹⁻¹⁹³ and SNC1¹⁻¹⁷⁹ constructs fused to NES, or nes and nls
240 mutants, did not trigger an immune response with the exception of RPS4¹⁻¹⁹³-YFP-nls. These findings
241 were also supported by ion leakage measurements (Figure 3 H,I).

242 Immunoblot analysis showed that all the fusion proteins were expressed except SNC1¹⁻¹⁷⁹-YFP-nls,
243 which could not be detected. The protein constructs that triggered a cell death response, such as
244 RPS4¹⁻¹⁹³-NLS fusions (with and without AvrM) and RPS4¹⁻¹⁹³-YFP-nls, also accumulated at lower
245 level (Figure 3J,K), likely because protein samples were taken at the onset of cell death and protein
246 degradation.

247 Confocal microscopy revealed that the NLS and NES peptides functioned as expected to sequester
248 the TIR-YFP and TIR-AvrM-YFP constructs to the nuclear or cytosolic compartment, respectively
249 (Figure S4). While the TIR-YFP fusions with the nes fusion showed nucleocytosolic labelling, TIR-
250 AvrM-YFP-nes constructs were restricted to the cytosol, due to the cytosolic retention of AvrM
251 (Figure S4, Figure S1B, (Ve et al., 2013)). In contrast, TIR-AvrM-YFP-nls constructs were mainly
252 cytosolic but also showed weak labelling in the nucleus, while RPS4¹⁻¹⁹³-YFP-nls was mainly
253 targeted to the nucleus (Figure S4A). This could explain why this latter construct induced a similar
254 immune response to RPS4¹⁻¹⁹³-YFP-NLS. This phenotype was not observed with SNC1¹⁻¹⁷⁹-YFP-nls
255 as this protein construct did not express. Altogether, these results indicate that confining the RPS4
256 and SNC1 TIR domains to the nucleus induces signaling, possibly because the increased protein
257 concentration in a smaller compartment may be sufficient to induce TIR oligomerisation. In contrast,
258 a fusion to AvrM did not induce RPS4 and SNC1 TIR-only signaling, in contrast to P2/L6TIR.

259 The above results suggested that the RPS4 and SNC1 TIRs can signal cell death from the nucleus but
260 were inconclusive with regards to potential cytosolic signaling. To address this question, we
261 generated a similar YFP-NLS/NES fusion series for the larger autonomously active RPS4TIR¹⁻²³⁵
262 and SNC1TIR¹⁻²²⁶ proteins. Interestingly, all tested RPS4TIR¹⁻²³⁵ and SNC1TIR¹⁻²²⁶ TIR-YFP
263 constructs fused to NLS or NES and mutated nls and nes induced a strong immune response when
264 overexpressed in *N. benthamiana*, except for SNC1TIR¹⁻²²⁶-YFP-nls (Figure 4 A-B). Immunoblot
265 analysis revealed that the inactive SNC1TIR¹⁻²²⁶-YFP-nls construct did not accumulate at all,
266 similarly to SNC1TIR¹⁻¹⁷⁹-YFP-nls, for unknown reasons. This analysis also showed that NLS
267 fusions of both RPS4TIR¹⁻²³⁵ and SNC1TIR¹⁻²²⁶-YFP accumulate less than the other constructs,
268 although they still induce a cell death response (Figure 4C,D). Examination of the subcellular
269 localization of these constructs showed the expected localizations, although the RPS4TIR¹⁻²³⁵-YFP-
270 NES fusions was not completely excluded from the nucleus, which still showed some weak nuclear
271 fluorescence (Figure S5). To further support these results, we generated fusion constructs consisting

272 of the L6 N-terminal signal anchor (L6s) fused to signaling active RPS4TIR¹⁻²³⁵ and SNC1TIR¹⁻²²⁶
273 to direct these TIR fragments to the Golgi membrane. These constructs both induced cell death
274 responses equivalent to the corresponding constructs without L6s (Figure S6A). Confocal analysis
275 showed that each L6s/TIR-YFP constructs target Golgi bodies (Figures S6 B-E), although some weak
276 YFP fluorescence was also detected in nuclei for L6s/RPS4TIR¹⁻²³⁵ after infiltration by
277 *Agrobacterium* at OD₆₀₀=0.5, but not OD₆₀₀=0.1, which led to lower protein accumulation (Figure
278 S6F). L6s/TIR constructs triggered an immune response that was even stronger than RPS4TIR¹⁻²³⁵
279 and SNC1TIR¹⁻²²⁶ with no signal anchor at OD₆₀₀ 0.1 (Figure 4E) This phenotypical results was
280 validated by ion leakage assays (Figure 4F). Based on our results, we conclude that RPS4 and SNC1
281 TIR domains can activate cell death signaling from both nuclear and cytosolic compartments in *N.*
282 *benthamiana*.

283

284 **Flax L6 can induce cell death responses in Arabidopsis via the cytosolic pool of AtEDS1**

285 EDS1 is required for TNL signaling and displays nucleocytoplasmic subcellular localization (Parker et
286 al., 1996; Wiermer et al., 2005; Wirthmueller et al., 2007). Our results showing that L6 cannot induce
287 signaling from the nuclear compartment (Figure 2B,C) prompted us to test whether L6 signaling
288 depends on EDS1 subcellular localization. Given that L6 can signal in Arabidopsis (Howles et al.,
289 2005), we generated transgenic lines expressing Dexamethasone (Dex)-inducible full-length L6
290 autoimmune variant that contains an aspartate to valine substitution in the MHD motif (L6MHV)
291 with a C-terminal 3-myc epitope in Col-0 ecotype. When grown on a non-inducing MS media,
292 seedlings from two independent pDex:L6MHV-3myc transgenic lines showed no developmental
293 differences compared to wild-type Col-0 seedlings (Figures S7 and S8). In contrast, upon transfer on
294 Dex-containing media, seedlings from both transgenic lines died after several days, a phenotype that
295 is typical of an autoimmune response (Figure S7). We used pDex:L6MHV-3myc line #2 for further
296 characterization. Upon transfer on Dex-containing media, transgenic but not wild-type seedlings,
297 showed chlorotic symptoms from 3 to 4 days after induction (dai), and growth arrest to eventually
298 die by 7/8 dai (Figure 5A). Immunoblot analyses showed that L6MHV-3myc protein only
299 accumulates upon Dex induction (Figure 5B). Hence, this result demonstrates that Dex-induced
300 L6MHV-3myc is functional and triggers immune responses in Arabidopsis. To verify that flax
301 L6MHV initiates signaling via AtEDS1, we crossed our transgenic line with the Col-0 *eds1-2* deletion
302 mutant. In the F2 segregating population of pDex:L6MHV-3myc x *eds1-2*, individual seedlings were
303 selected for the presence of the transgene pDex:L6MHV-3myc (hygromycin selection) and PCR
304 screened to select plants that were homozygous for *eds1-2* mutation (*eds1/eds1*) or which contain a
305 wild-type allele of *EDS1*. When transferred to Dex-inducing media, pDex:L6MHV-3myc x *eds1-2*
306 (*eds1/eds1*) F2 seedlings showed normal development whereas plants with an intact *EDS1* allele

307 (EDS1/EDS1 or EDS1/*eds1*) were stunted and displayed cell death phenotypes, demonstrating that
308 L6MHV-mediated signaling is dependent on AtEDS1 (Figure S9).

309 In order to test whether L6 signaling depends on EDS1 subcellular localization, we crossed
310 pDex:L6MHV-3myc/*eds1-2* line with transgenic lines expressing EDS1-YFP or mislocalized EDS1-
311 YFP variants (fused to NLS, NES, nls, or nes sequence under the control of the EDS1 native promoter
312 (pEDS1:EDS1-YFP) in Col-0 *eds1-2* background (García et al., 2010; Stuttmann et al., 2016). For
313 each cross, F3 segregating seedling populations were selected for the presence of the *pDex:L6MHV-*
314 *3myc* transgene and PCR screened to select individuals that contained the EDS1 variants or not. When
315 transferred to non-inducing (DMSO) media, seedlings from all genotypes showed normal
316 development (Figure S8). Upon transfer to Dex-containing media, seedlings that were not
317 complemented with EDS1-YFP variants also developed normally, similarly to wild-type Col-0 plants
318 (Figure 5A). At 4 dai, seedlings that were complemented with any EDS1-YFP variant showed similar
319 symptoms to the pDex:L6MHV:3myc line that contains the native allele of EDS1, i.e, stunted growth
320 and chlorosis (Figure 5A). By 8 dai, individuals complemented with EDS1-YFP, and EDS1-YFP-
321 NES variants were fully necrotic, similar to individuals from pDex:L6MHV:3myc line. In contrast,
322 individuals from lines complemented with EDS1-YFP-NLS, nls, or nes remained similar to the
323 phenotype observed at 4 dai, and did not die (Figure 5A). Similar results were obtained when
324 pDex:L6MHV:3myc/*eds1-2* line was crossed with independent transgenic lines expressing
325 pEDS1:EDS1-YFP-NLS (#A5) and pEDS1:EDS1-YFP-NES (#2-11) (García et al., 2010; Stuttmann
326 et al., 2016) (Figure S10).

327 Confocal microscopy of roots of complemented lines (from Figure 5) showed that EDS1-YFP, EDS1-
328 YFP-NLS and EDS1-YFP-NES displayed the expected nucleocytoplasmic, nuclear restricted or nuclear
329 excluded fluorescence labelling, respectively (Figure S11). In contrast, control variants EDS1-YFP-
330 nls and EDS1-YFP-nes mainly labelled the nuclear compartment, and were lowly expressed
331 compared to the other EDS1-YFP variants, which may explain their lack of complementation.

332 Immunoblot analysis revealed that 24h after Dex induction, the L6MHV protein accumulates
333 similarly in the pDex:L6MHV-3myc line and in all complemented lines except the one complemented
334 with EDS1-YFP, in which accumulation of the L6MHV protein is slightly reduced (Figure 5B). By
335 using anti-GFP antibodies, we repeatedly observed an increased accumulation of EDS1-YFP variants
336 in seedlings transferred to Dex-containing media, compared to those transferred to non-inducing
337 media, although not identical in every line. However, the level of protein accumulation of EDS1 upon
338 Dex induction do not seem to correlate with the immune response output. Indeed, upon Dex induction,
339 the EDS1-YFP-NLS variant protein accumulation was similar to EDS1-YFP and stronger than EDS1-
340 YFP-NES, which both induce a stronger immune response compared to EDS1-YFP-NLS. Altogether,

341 it seems that L6MHV signaling leads to seedling death only when EDS1 is present in the cytosolic
342 pool, while nuclear-localised EDS1 is sufficient to mediate the stunting phenotype.

343

344 **RPS4TIR domain preferentially signals cell death via the cytosolic pool of EDS1 in Arabidopsis**

345 EDS1 is essential for RPS4/RRS1-mediated bacterial immunity in Arabidopsis and for RPS4 TIR
346 signaling in *N. tabacum* and *N. benthamiana* (Wirthmueller et al., 2007; Swiderski et al., 2009;
347 Horsefield et al., 2019). In order to test whether AtEDS1 subcellular localization is important for
348 RPS4TIR signaling, we used the same approach as for L6MHV described above. We generated
349 transgenic lines expressing Dex-inducible RPS4¹⁻²³⁵ with a C-terminal FSBP (consisting in a triple
350 Flag tag fused to the streptavidin binding protein(SBP)). When seedlings from three independent lines
351 expressing pDex:RPS4¹⁻²³⁵-FSBP were grown on a non-inducing MS media, the *RPS4*¹⁻²³⁵ transgene
352 was not expressed and seedling development was similar to wild-type Col-0 (Figures S12 and S13).
353 In contrast, when transferred on Dex-containing media, seedlings from all lines died after several
354 days, similarly to pDex:L6MHV-3myc transgenic seedlings (Figure S12). We used RPS4¹⁻²³⁵-FSBP
355 line #F for further characterization. Upon transfer on Dex-inducing media, pDex: RPS4¹⁻²³⁵-
356 containing seedlings showed chlorotic symptoms from 3 to 4 days after induction , and then growth
357 arrest with fully necrotic symptoms by 6 to 7 dai (Figure 6A), demonstrating that induced RPS4¹⁻²³⁵-
358 FSBP is functional and can activate immune responses in Arabidopsis.

359 In order to test whether RPS4¹⁻²³⁵ depends on EDS1 subcellular localization, we introduced the *eds1*-
360 2 mutation in the pDex:RPS4¹⁻²³⁵-FSBP line and complemented it with EDS1 and mislocalized
361 variants as described above. When transferred to non-inducing (DMSO) media, seedlings from all
362 genotypes showed a normal development, similar to wild-type Col-0 seedlings (Figure S13). Upon
363 transfer on Dex-containing media, pDex:RPS4¹⁻²³⁵-FSBP/*eds1*-2 seedlings also showed similar
364 development to Col-0 seedlings (Figure 6A), demonstrating that RPS4¹⁻²³⁵-FSBP requires EDS1 to
365 signal. At 3 dai, seedlings complemented with any of the EDS1-YFP variants started to show chlorotic
366 symptoms, although seedlings complemented with the EDS1-YFP-NES variant showed slightly
367 stronger autoimmune phenotype that was closer to the pDex: RPS4¹⁻²³⁵-FSBP line, i.e. the plantlets
368 were smaller and showed stronger chlorosis symptoms. At 6dai, seedlings from pDex:RPS4¹⁻²³⁵-
369 FSBP line complemented with EDS1-YFP-NES were dead, while seedlings from all other lines
370 remained stunted but survived (Figure 6A). Similar results were obtained when pDex: RPS4¹⁻²³⁵-
371 FSBP/*eds1*-2 line was crossed with independent transgenic lines expressing pEDS1:EDS1-YFP-NLS
372 (#A5) and pEDS1:EDS1-YFP-NES (#2-11) (García et al., 2010; Stuttmann et al., 2016)(Figure S14).
373 Confocal analysis of the complemented lines showed that EDS1 mislocalized variants displayed the
374 expected subcellular localization in these lines, except that EDS1-YFP-nls was again mainly nuclear
375 (Figure S15). Upon Dex induction, RPS4¹⁻²³⁵-FSBP protein accumulated at 24hai in all lines (Figure

376 6B). Similarly to the results obtained with L6MHV, we repeatedly observed an increased
377 accumulation of all EDS1-YFP mislocalized variants upon Dex induction in comparison to non-
378 inducing conditions where RPS4¹⁻²³⁵-FSBP is not expressed. The EDS1-YFP and EDS1-YFP-NLS
379 variants showed the highest protein accumulation, but not the strongest autoimmune phenotype
380 (Figure 6B). Thus, similar to Golgi-localized L6MHV, RPS4TIR induction leads to cell death when
381 EDS1 is in the cytosol, with a stunting phenotype without necrosis when EDS1 is restricted to the
382 nucleus.

383

384 **DISCUSSION**

385

386 In the last few years, important breakthroughs have revealed the complexity of TIR-containing NLR
387 signaling. However, the subcellular requirement for TNL signaling and downstream immune
388 complexes to activate defense responses is not clearly understood. By studying mislocalized variants
389 of signaling TIR domains isolated from different TNLs, we showed that TNLs do not all activate
390 signaling from the same cellular compartment. Flax L6TIR exclusively signal from the cytosol while
391 Arabidopsis RPS4 and SNC1TIR domains can activate immune signaling from both nuclear and
392 cytosolic compartments in *N. benthamiana*.

393 TIR-only constructs isolated from Arabidopsis RPS4, SNC1 or flax L6 are not able to trigger
394 signaling, in contrast to larger TIR constructs containing an N-terminal signal anchor for L6 or a C-
395 terminal extension before the start of the NB domain for RPS4 and SNC1. In the case of L6, this may
396 be a consequence of the N-terminal membrane anchor causing concentration of the protein at the
397 Golgi (or tonoplast in the case of the M signal anchor) membrane, thereby promoting self-association.
398 Similarly, fusion of the L6TIR to AvrM, which forms a stable dimer, also promoted signaling in the
399 absence of the membrane anchor. This is consistent with recent data on the requirement of TNL
400 oligomeric assembly to promote TIR self-association to activate NADase catalytic activity
401 (Horsefield et al., 2019; Duxbury et al., 2020; Ma et al., 2020; Martin et al., 2020). As AvrM forms
402 a stable dimer *in planta* (Ve et al., 2013), it is interesting to note that dimerization of L6TIR seems
403 sufficient to activate immune responses.

404 In contrast, a fusion to AvrM did not induce RPS4 and SNC1 TIR-only signaling. Hence, it is possible
405 that AvrM cannot dimerize or fold properly when fused to RPS4 and SNC1 TIRs, and hence does not
406 promote induced proximity of the TIRs. Interestingly, confinement of RPS4 or SNC1 TIR-alone in
407 the nucleus induced signaling, showing that increasing RPS4 or SNC1 TIR concentration in the
408 nucleus may be sufficient to promote oligomerisation. This is consistent with earlier data showing
409 that increased accumulation of RPS4 or SNC1 full-length protein in the nucleus is sufficient to induce
410 AvrRps4-independent cell death in tobacco or enhanced disease resistance in Arabidopsis,

411 respectively (Wirthmueller et al., 2007; Xu et al., 2014). While interaction with transcriptional
412 corepressor or with DNA in the nucleus suggest that native full length SNC1 and RPS4/RRS1 have
413 nuclear functions (Zhu et al., 2010; Le Roux et al., 2015), our results brings new insights in TNL
414 early signaling as we showed individual TIR domains may have specific subcellular localisation
415 requirements to induce signaling. RPS4 and SNC1 TIR-mediated cell-death signaling can be
416 activated from both nuclear and cytosolic compartments while L6TIR signals in the cytosol but not
417 in the nucleus, highlighting some specificities in TIR domain function and trigger downstream
418 signaling (Sup figure 16).

419 EDS1 is a central regulator of plant immunity and functions downstream of TNL activation. Two
420 recent studies have filled a major gap in how TIR signaling connects to EDS1. The by-products of
421 TIR NADase enzymatic activity act as signaling molecules by binding to and activating EDS1/PAD4
422 or EDS1/SAG101 heterodimers, leading to the association of EDS1 complex to downstream RNL-
423 type helper NLRs and the activation of immune responses (Huang et al., 2022; Jia et al., 2022). By
424 using Arabidopsis transgenic lines conditionally expressing L6MHV, and RPS4TIR¹⁻²³⁵ in an *eds1*
425 mutant background, we found that autoimmune responses triggered by these two constructs rely on
426 AtEDS1. We also found that L6MHV and RPS4TIR signaling induction enhance AtEDS1 protein
427 accumulation. Increased EDS1 protein accumulation was also observed in the *snc1* autoimmune
428 mutant compared to wild-type plants (García et al., 2010), supporting a direct connection between
429 TIR signaling and EDS1 accumulation. Indeed, TIR-catalysed small molecules may stabilize the
430 EDS1 protein and/or upregulate *EDS1* transcription. Here, we showed that only the cytosolic pool of
431 AtEDS1 can translate signaling from nucleocytoplasmic RPS4TIR¹⁻²³⁵ and from Golgi membrane-
432 anchored flax L6MHV to a cell death response. However, plants expressing nuclear-restricted EDS1
433 still showed a stunting phenotype but remained alive, indicating that some aspects of immune
434 signaling can also be mediated by the nuclear fraction of EDS1, even when the inducing TIR is
435 confined to the cytosolic space in the case of the Golgi-localised L6MHV (Sup figure 16). This result
436 is consistent with earlier observations that nuclear EDS1 is essential for TNL-mediated resistance
437 and transcriptional reprogramming while the cytosolic pool is involved in cell death activation
438 (García et al., 2010; Bhandari et al., 2019; Lapin et al., 2019). Interestingly, restricting EDS1 to the
439 nuclear compartment do not prevent TNL RPP4-mediated hypersensitive response (localized cell
440 death) upon infection with avirulent *Hyaloperonospora arabidopsidis* in Arabidopsis (Stuttman et
441 al., 2016). It is possible that the activation of effector-independent TIR signaling (inducible
442 expression in transgenic lines) may differ from pathogen-delivered effector-mediated TNL activation.
443 Given evidence of cross-talk between PTI and ETI this may explain the discrepancy (Bernoux et al.,
444 2022).

445 Previous studies suggest that the EDS1/PAD4/ADR1 module controls pathogen restriction whereas
446 EDS1/SAG101/NRG1 activate cell death (Lapin et al., 2019).

447 Interestingly, AtSAG101 was found to be nuclear localized upon transient expression in *N.*
448 *benthamiana* leaves Arabidopsis (Lapin et al. 2019). However, this may not reflect its normal
449 distribution as part of an EDS1-SAG101 complex as the Arabidopsis SAG101 does not function with
450 the *N. benthamiana* EDS1 protein.

451 . A recent study reported the formation of a complex between the EDS1-SAG101 module and NRG1
452 in both nuclei and at the plasma membrane, nevertheless the stoichiometry of these complexes
453 seemed different in the nucleus and may explain functional differences (Feehan et al., 2022). Nuclear
454 complexes seem to form heterotrimers while larger resistosome-like structures associate with the
455 plasma membrane. Hence, nuclear restricted EDS1 may be able to associate with SAG101 and NRG1
456 to activate an immune response in the nucleus, but is not able to further induce the formation of a
457 pore-forming resistosome to induce cell death in the cytosol.

458 Nuclear export signal leads to enhanced nuclear export but does not fully prevent nuclear import.
459 Hence, it is possible that brief nuclear import of EDS1-YFP^{NES} to the nucleus is sufficient to interact
460 with nuclear SAG101 and activate NRG1 to trigger cell death at the plasma membrane. The
461 contribution of the EDS1/PAD4/ADR1 to transcriptional reprogramming vs cell death execution
462 remains unclear as EDS1 and PAD4 both shuttles to the nucleus and the cytosol while ADR1s are
463 mainly targeted to the plasma membrane (Saile et al., 2021). Studying the subcellular dynamics of all
464 signaling partners upon TNL activation needs to be thoroughly addressed to fully understand the roles
465 of EDS1/SAG101/NRG1 vs EDS1/PAD4/ADR1 modules and how they differentially engage into
466 cell death or resistance/gene reprogramming pathways.

467

468 How nuclear-restricted EDS1 senses cytosolic TIR signaling activity such as from Golgi-localized
469 L6MHV is unclear but may be explained by the diffusion of TIR-catalysed molecules through the
470 nuclear envelope to activate EDS1. On the other hand, it is possible that EDS1-YFP-NLS is weakly
471 expressed in the cytosol or encounters Golgi localised L6MHV during protein translation and that
472 this is sufficient to activate signaling in the early stages after Dex induction, explaining why cell death
473 symptoms slow down after a few days after Dex induction. NLR-mediated immune responses lead to
474 core-immunity gene reprogramming, including upregulation of *TNLs* (Bernoux et al., 2022). Hence,
475 it is also possible that L6MHV induces rapid expression of *TNLs*, some of which proteins may be
476 targeted to the nucleus and activate nuclear restricted EDS1. Lastly, we cannot exclude that the other
477 members of the EDS1 family (PAD4 and SAG101) can sense cytosolic TIR-catalysed signaling
478 molecule and shuttle between the cytoplasm and nucleus to translate the signal to nuclear-restricted
479 EDS1. Indeed, we recently obtained evidence that TIR proteins can interact with all members of the

480 EDS1 family in *N. benthamiana* and in yeast, independently or as heterodimers (Chen et al., in
481 preparation).

482 Interestingly, the nuclear pool of SIEDS1 and SISAG101 is required and sufficient for effector-
483 triggered Roq1-mediated cell death in *N. benthamiana* (Zönnchen et al., 2022). In contrast, the
484 cytosolic pool of AtEDS1 and AtSAG101 can activate Roq1-mediated cell death (Wang et al. 2023),
485 suggesting that NLR signaling and subcellular requirements may not be conserved across species. .
486 Investigating the specificities and commonalities of NLR activation and signaling in other plant
487 species will be crucial for improving disease resistance in crops.

488

489 **EXPERIMENTAL PROCEDURES**

490

491 **Vectors and plasmid construction**

492 All DNA plasmids and primers used in this study are listed in Supplemental table 1 and 2. Plasmids
493 for *in planta* transient expression were constructed by Gateway cloning (GWY; Invitrogen). PCR
494 products flanked by the attB sites were recombined into pDONR207 (Invitrogen) and then into
495 corresponding destination vectors: pAM-PAT 35S:GWY-YFPv (Bernoux et al., 2008), pAM-PAT
496 35S:GWY-AvrM, pAM-PAT 35S:GWY-YFPv-NLS/nls (Cesari et al., 2016), pAM-PAT 35S:GWY-
497 YFPv-NES/nes, pAM-PAT 35S:GWY-AvrM-YFPv-NLS/NES series, pDex:GWY-3myc or
498 pDex:GWY-FSBP.

499 To generate pAM-PAT-35S:GWY-YFPv-NES/nes, *SmaI/XbaI* PCR fragments containing the YFPv
500 sequence with a functional NES sequence from HIV Rev (LQLPPLERLTL) or a non-functional nes
501 (LQAPPAERATL)(Wen et al., 1995) at the C-terminal end of the YFP, were ligated into pAM-PAT
502 35S:GWY-YFPv digested with *SmaI* and *XbaI* to excise and replace the original YFPv fragment. The
503 pAM-PAT 35S:GWY-AvrM was generated in a 2-step process by fusing two PCR fragments with
504 overlapping sequence and ligation into pAM-PAT 35S:GWY-YFPv digested with *PstI* and *XbaI* to
505 dislodge and replace the original YFPv. PCR fragment 1 was generated to contain the 3' end of the
506 GWY cassette sequence of the PAM-PAT-GWY-YFP (with a 5' *PstI* restriction site), up to the AttR2
507 sequence. PCR fragment 2 was generated to amplify AvrM-A sequence with a 3' *XbaI* site and
508 overlapping sequence with PCR fragment 1 in its 5' end. The two PCR fragments containing
509 overlapping sequence in their 3' and 5', respectively, were assembled by PCR and then ligated into
510 pAM-PAT 35S:GWY-YFPv digested with *PstI* and *XbaI* to dislodge and replace the original YFPv
511 fragment. pAM-PAT 35S:GWY-AvrM-YFP-NLS/NES plasmid series were also generated in a 2-
512 step PCR process. Two PCR fragments containing AvrM-A with a 5' *PstI* restriction site and another
513 one containing YFPv-NLS/NES/nls/nes with a 3' *XbaI* restriction site and an overlapping linker
514 sequence (GGSGG) in their 3' and 5' end, respectively were assembled by PCR and then ligated into

515 pAM-PAT 35S:GWY-YFPv digested with *PstI* and *XbaI* to dislodge and replace the original YFPv.
516 The L6 signal anchor (L6s, residues 1-58) was inserted into the *XhoI* and *HindIII* sites of the pAM-
517 PAT 35s:GWY-YFP vector upstream the AttR1 site to generate the pAM-PAT 35s: L6s-GWY-YFP.
518 pDex:GWY-3Myc and pDex:GWY-FSBP were modified from pOpOff2(Hyg) -derived vectors
519 (Craft et al., 2005; Wielopolska et al., 2005). pOpOff2(Hyg) GWY-derived vector, was kindly
520 provided by Chris Helliwell (CSIRO, Canberra). It was lacking the hairpin GWY cassette (as
521 described in (Wielopolska et al., 2005)) and instead contained a simple GWY cassette but no
522 terminator. Hence, a PCR fragment containing either a triple myc or the FSBP (3xFLAG-streptavidin
523 binding protein) epitopes followed by the 35s terminator sequence flanked with *KpnI* restriction sites,
524 were cloned into pOpOff2(Hyg)-derived vector digested with *KpnI*. All plasmids and constructs were
525 verified by sequencing. Details of constructs and primers used in this study are listed in Supplemental
526 table 1 and 2.

527

528 **Plant material**

529 *Nicotiana benthamiana* and *N. tabacum* W38 plants were grown in growth chambers at 21°C under
530 9h photoperiod. *A. thaliana* seeds were sown on MS (Murashige and Skoog) media, vernalized for
531 24 h and transferred in growth chambers for ten days under 22°C and 16h photoperiod conditions.

532

533 **Arabidopsis crosses and PCR screening**

534 Transgenic lines expressing pDex:L6MHV-3myc (line #2) or pDex:RPS4¹⁻²³⁵-FSBP (line#F) were
535 selected on MS media supplemented with hygromycin 15 µg/ml, and crossed with Col-0 *eds1-2*
536 deletion mutant. F2 segregating seedling population were selected for the presence of the transgene
537 (hygromycin selection) and individually PCR screened to check for the presence of the *EDS1* deletion
538 by using Thermo Scientific™ Phire plant direct PCR kit. In brief, the first emerging leaf of 10 day-
539 old of individual seedling was sampled and ground in 10µl extraction buffer provided by the kit. 0.5µl
540 of this extract was then used to run a PCR, following the manufacturer recommendations, with
541 primers located on each side of the deletion in *EDS1* (*EDS1* F563/ *EDS1* R1806) (Supplemental table
542 2). Individuals carrying pDex transgene and homozygous *eds1-2* deletion (pDex:L6MHV-3myc x
543 *eds1-2* line #4.5 and pDex:RPS4¹⁻²³⁵-FSBP x *eds1-2* line #8.1) were crossed with lines expressing
544 mislocalized variants of *EDS1* (pEDS1:EDS1-YFP fused to nuclear localization signal (NLS),
545 nuclear exclusion signal (NES) or corresponding mutants (nls, nes), pEDS1:EDS1-YFP-NLS#B2,
546 pEDS1:EDS1-YFP-NLS#A5, pEDS1:EDS1-YFP-NES#3-4, pEDS1:EDS1-YFP-NES#2-11,
547 pEDS1:EDS1-YFP-nls#B5, pEDS1:EDS1-YFP-nes#1-2 (García et al., 2010; Stuttmann et al., 2016).
548 Arabidopsis F3 segregating populations were individually PCR screened to select individual

549 seedlings that were homozygous for *eds1-2* mutation (*eds1/eds1*) or contained the EDS1 mislocalized
550 variants for cell death assays, confocal microscopy and western-blots analyses.

551
552 **Agrobacterium-mediated transient expression in flax and tobacco**

553 *Agrobacterium tumefaciens* cells were grown for 24h at 28°C in LB medium containing appropriate
554 antibiotics selection. Cells were pelleted, resuspended in the infiltration medium (10 mM MgCl₂,
555 10mM MES PH5,6, 150 μM acetosyringone), adjusted to OD₆₀₀ = 1, 0.5 or 0.1 before infiltration into
556 leaves of 4 week-old flax (Hoshangabad) or OD₆₀₀ = 0.5 or 0.1 into leaves of 4 week-old *Nicotiana*
557 *benthamiana*.

558
559 **Dexamethasone induction and cell death assay in *Arabidopsis thaliana***

560 Ten day-old seedlings were transferred on MS media supplemented with dexamethasone (10μM) or
561 DMSO (equivalent volume as dexamethasone) and left in growth chambers under 22°C and 16 h
562 photoperiod conditions. The apparition of cell death symptoms were monitored every day. For
563 immunoblot analysis, five seedlings per sample were collected 24h after induction.

564
565 **Immunoblot analysis**

566 Total proteins were extracted by grinding two flax leaves or two *N. benthamiana* leaf discs (1cm
567 diameter), three days and two days after agroinfiltration, respectively. Plant material was resuspended
568 in 100 μL of loading Laemmli buffer. Total proteins of *Arabidopsis* were extracted by grinding five
569 ten day-old seedlings 24h after Dex-induction, in 100μl of protein extraction buffer (150mM Tris-
570 HCl pH 7.5, 150mMNaCl, 1mM EDTA, 1%NP40, 1% plant protease inhibitor cocktail
571 (SIGMA),10mM DTT, 10μM MG132). Protein lysates were centrifuged to remove cell debris and
572 50μl of supernatant was combined with 50μl of loading Laemmli buffer. All protein samples were
573 denatured at 95°C for three minutes before loading on SDS-PAGE and transfer to nitrocellulose
574 membrane. Membranes were blocked in 5% skim milk and probed with anti-Flag-HRP (Sigma,
575 A8592), anti-c-myc-peroxidase (clone 9E10, Roche) or anti-GFP mouse monoclonal antibodies
576 (clones 7.1 and 13.1, Roche, Ref#11814460001, Lot 13921700), followed by goat anti-mouse HRP
577 conjugate (Bio-Rad, Cat#170-5047). Proteins were revealed using BioRad Chemidoc™ Imaging
578 system with the Bio-Rad Western ECL Substrate. Membranes were stained with Red Ponceau to
579 verify protein loading.

580
581 **Measurement of ion leakage from leaf discs**

582 Ion leakage measurements were performed as in (Zhang et al., 2017b). Briefly, for each construct,
583 four leaf discs (0.9 cm diameter) of *N. benthamiana* leaves were sampled 48h after agroinfiltration,

584 quickly rinsed with distilled water and incubated for 24h with 1.8ml of fresh MilliQ water.
585 Conductivity of the bathing solution was measured before (C1) and after (C2) boiling for 10 minutes
586 to release the total ion content of the leaf discs, by using a Horiba B-173 conductivity meter. Ion
587 leakage was expressed as C1/C2 ratio.

588

589 **Confocal microscopy**

590 Confocal fluorescence images were recorded on a Leica SP8 confocal system, using 514-nm laser
591 line to image YFP signal. In *A. thaliana*, roots of 10 week-old Arabidopsis seedlings were analysed
592 24h after Dexamethasone induction. In *N. benthamiana*, leaf discs were analysed 48h after infiltration
593 with *A. tumefaciens*.

594

595 **DATA AVAILABILITY STATEMENT**

596 All relevant data can be found within the manuscript and its supporting materials.

597

598 **ACKNOWLEDGEMENTS**

599 Dexamethasone-inducible pOpOff2-derived vectors used to generate pDex:GWY-3myc and
600 pDex:GWY-FSBP were kindly provided by Dr Chris Helliwell (CSIRO Canberra, Australia).

601 Col-0 *eds1-2* and Col-0 *eds1-2* lines complemented with EDS1-YFP mislocalized variants were
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608

609 **AUTHOR CONTRIBUTIONS**

610 MB and PD initiated the project and designed the experiments, MB, JC, XZ, KN and JH performed
611 experiments. MB, JC, XZ, and PD analysed the data. MB wrote the manuscript with the contributions
612 of PD, JC, XZ and LD.

613

614 **CONFLICT OF INTERESTS**

615 The authors declare there are no conflict of interests.

616

617

618

619 **SUPPORTING INFORMATION**

620

621 **Figure S1.** Signalling activity, and protein stability of mislocalized variants of P2/L6TIR.

622 **Figure S2.** Signalling activity of membrane-tethered L6 TIR is not affected by a fusion to AvrM
623 and a nuclear localization or nuclear exclusion signal.

624 **Figure S3.** Subcellular localization of RPS4 and SNC1 TIR constructs.

625 **Figure S4.** Subcellular localization of mislocalized variants of RPS4 and SNC1 TIR-only.

626 **Figure S5.** Subcellular localization of mislocalized variants of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ TIR
627 constructs.

628 **Figure S6.** Signaling activity, subcellular localization and protein accumulation of RPS4¹⁻²³⁵ and
629 SNC1¹⁻²²⁶ TIR constructs fused to L6 signal anchor.

630 **Figure S7.** Phenotype of independent transgenic Arabidopsis seedlings expressing Dexamethasone
631 (Dex)-inducible L6MHV-3myc (pDex:L6MHV-3myc).

632 **Figure S8.** Phenotype of transgenic Arabidopsis seedlings expressing pDex:L6MHV on a non-
633 inducing media.

634 **Figure S9.** Flax L6 signaling in Arabidopsis depends on AtEDS1.

635 **Figure S10.** Phenotype of transgenic Arabidopsis seedlings from crosses of pDex:L6MHV-
636 3myc/*eds1-2* line with independent mislocalized EDS1 variants lines.

637 **Figure S11.** Subcellular localization of EDS1-YFP variants in Dex-inducible transgenic Arabidopsis
638 seedlings (10 day-old) expressing pDex:L6MHV in *eds1-2* mutant background complemented with
639 mislocalized EDS1-YFP variants.

640 **Figure S12.** Phenotype of independent transgenic Arabidopsis seedlings expressing Dexamethasone
641 (Dex)-inducible RPS4¹⁻²³⁵-FSBP.

642 **Figure S13.** Phenotype of transgenic Arabidopsis seedlings expressing pDex:RPS4¹⁻²³⁵ on a non-
643 inducing media.

644 **Figure S14.** Phenotype of transgenic Arabidopsis seedlings from crosses of pDex: RPS4¹⁻²³⁵-FSBP
645 /*eds1-2* line #8.1 with independent mislocalized EDS1 variants lines.

646 **Figure S15.** Subcellular localization of EDS1-YFP variants in Dex-inducible transgenic Arabidopsis
647 seedlings (10 day-old) expressing pDex:RPS4¹⁻²³⁵ in *eds1-2* mutant background complemented with
648 mislocalized EDS1-YFP variants.

649 **Figure S16.** TIR signaling subcellular localization requirement model.

650

651

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862

863 **FIGURES LEGENDS**

864

865 **Figure 1: Membrane attachment is required for flax L6TIR signaling activity.**

866 (A-D) Subcellular localization and (E-H) signaling activity (cell death/chlorosis) of yellow
867 fluorescent protein (YFP) fusion of L6TIR and recombinants upon agro-mediated transient
868 expression in flax leaves. Confocal images show YFP (yellow) labelling in Golgi bodies for L6TIR-
869 YFP (A), in the nucleocytoplasm for P2/L6TIR-YFP (B), in the tonoplast for M/L6TIR-YFP (C) and
870 mostly in the nucleocytoplasm for M*/L6TIR-YFP, overlaid with chloroplast autofluorescence (pink)
871 and bright field images. Confocal images were taken 3 days after agro-infiltration (dai), in flax leaves.
872 White bar scale represents 25µm. Photos of flax leaves (E-H) were taken 12dai. This experiment was
873 repeated at least three times with similar results. (I) Immunoblot analysis of YFP fusion of L6TIR
874 and recombinants upon agro-mediated transient expression in flax leaves. Samples were taken 3 days
875 after agro-infiltration and detected with anti-GFP antibodies. Equal loading of proteins is indicated
876 by red Ponceau staining

877

878

879 **Figure 2: Fusion of signaling inactive P2/L6TIR to the flax rust effector AvrM leads to signaling
880 activation in the cytosolic compartment only.**

881 (A-B) Signaling activity of P2/L6 TIR-YFP and P2/L6 TIR-AvrM-YFP fusion series with a nuclear
882 localization (NLS) or nuclear exclusion signal (NES) and corresponding mutants (nls and nes,
883 respectively) upon agro-mediated transient expression in flax (A) or in *N. benthamiana* (B) leaves.
884 Photos were taken 12 dai for flax and 5 dai for *N. benthamiana*. Infiltrated areas are surrounded by
885 dotted circles (black dotted line circles correspond to positive controls). This experiment was repeated
886 at least three times with similar results. (C) Ion leakage measurement of *N. benthamiana* leaves 48h
887 after agro-mediated expression of mislocalized variants of P2/L6TIR fused to YFP or AvrM-YFP with
888 a nuclear localization (NLS) or nuclear exclusion signal (NES) and corresponding mutants (nls and
889 nes, respectively). Leaf disk samples were collected 2 days after infiltration and incubated in Milli-
890 Q water. C1 corresponds to the ions released in solution 24 h after sampling (µs/cm). C2 corresponds
891 to the total ion contents in the sample (µs/cm). Ion leakage was calculated as C1/C2 ratio
892 (see methods for more details). *N. benthamiana* leaves infiltrated with empty agro strain (GV3103)
893 were used as control. Statistical differences were calculated by one-way ANOVA followed by
894 multiple comparison test (Tukey's HSD). Constructs sharing letters above boxplots are not
895 significantly different ($\alpha = 0.05$, $n > 8$). (D-E) Immunoblot analysis of mislocalized variants of
896 P2/L6TIR fused to YFP or AvrM-YFP fusion series with a nuclear localization (NLS) or nuclear
897 exclusion signal (NES) and corresponding mutants (nls and nes, respectively) upon transient

898 expression in flax (D) or *N. benthamiana* (E) leaves. Samples were taken 3 days or 48h after agro-
899 infiltration in flax and *N. benthamiana*, respectively, and detected using anti-GFP antibodies. Equal
900 loading of proteins is indicated by red Ponceau staining. Arrows point at the band size corresponding
901 to P2/L6TIR fusion to AvrM-YFP (a), YFP (b) or to cleaved YFP (c). (F) Confocal images show YFP
902 (yellow) labelling of mislocalized variants of P2/L6 TIR-YFP (left panel) or P2/L6 TIR-AvrM-YFP
903 (right panel) constructs series fused to a nuclear localisation (NLS) or exclusion (NES) signal and
904 corresponding mutants (nls and nes, respectively), overlaid with chloroplast autofluorescence (pink)
905 and bright field images. Images were taken 48h after agroinfiltration in *N. benthamiana* leaves. White
906 bar scale represents 25µm.

907

908 **Figure 3: Forcing inactive RPS4TIR and SNC1TIR-only in the nucleus is sufficient to induce**
909 **signaling.**

910 (A-B) Signaling activity of YFP fusions of TIR-only SNC1¹⁻¹⁷⁹, RPS4¹⁻¹⁹³ and P2/L6TIR (left part
911 of the leaf) and signaling active SNC1¹⁻²²⁶, RPS4¹⁻²³⁵ and L6TIR, upon agro-mediated transient
912 expression in leaves of *N. benthamiana* (A) and *N. tabacum* (B). (C) Immunoblot analysis of TIR-
913 only (RPS4¹⁻¹⁹³ and SNC1¹⁻¹⁷⁹) or larger TIR constructs (RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶) upon transient
914 expression in *N. benthamiana* leaves. Samples were taken 48h after agro-infiltration and detected
915 with anti-GFP antibodies. Equal loading of proteins is indicated by red Ponceau staining. (D-G)
916 Signaling activity of mislocalized variants of RPS4¹⁻¹⁹³ and SNC1¹⁻¹⁷⁹ fused to YFP (D,F) or AvrM-
917 YFP (E,G) with a nuclear localization (NLS) or nuclear exclusion signal (NES) and corresponding
918 mutants (nls and nes, respectively) upon agro-mediated transient expression in *N. benthamiana*.
919 Photos were taken 5 dai. Infiltrated areas are surrounded by dotted circles (black dotted line circles
920 correspond to positive controls). These experiments were repeated at least three times with similar
921 results. (H-I) Ion leakage measurement of *N. benthamiana* leaves 48h after agro-mediated expression
922 of mislocalized variants of RPS4¹⁻¹⁹³ (H) or SNC1¹⁻¹⁷⁹ (I) fused to YFP or AvrM-YFP with a nuclear
923 localization (NLS) or nuclear exclusion signal (NES) and corresponding mutants (nls and nes,
924 respectively). Leaf disk samples were collected 2 days after infiltration and incubated in Milli-Q
925 water. C1 corresponds to the ions released in solution 24 h after sampling. C2 corresponds to the total
926 ion contents in the sample. Ion leakage was calculated as C1/C2 ratio (see methods for more details).
927 *N. benthamiana* leaves infiltrated with signaling active RPS4¹⁻²³⁵ or SNC1¹⁻²²⁶ were used as positive
928 controls, respectively. Statistical differences was calculated by one-way ANOVA followed by
929 multiple comparison test (Tukey's HSD). Constructs sharing letters above boxplots are not
930 significantly different ($\alpha = 0.05$, $n=4$ for RPS4TIR serie, $n>8$ for SNC1TIR serie). (J-K) Immunoblot
931 analysis of RPS4¹⁻¹⁹³ and SNC1¹⁻¹⁷⁹ fused to YFP or AvrM-YFP with a nuclear localization (NLS)
932 or nuclear exclusion signal (NES) and corresponding mutants (nls and nes, respectively) upon

933 transient expression in *N. benthamiana* leaves. Samples were taken 48h after agro-infiltration and
934 detected with anti-GFP antibodies. Equal loading of proteins is indicated by red Ponceau staining.

935

936 **Figure 4: Altered subcellular localization of signaling active RPS4 and SNC1 TIR domains do**
937 **not affect their ability to induce immune responses.**

938 (A-B) Signaling activity of mislocalized variants of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ fused to YFP with a
939 nuclear localization (NLS) or nuclear exclusion signal (NES) and corresponding mutants (nls and
940 nes, respectively) upon agro-mediated transient expression in *N. benthamiana*. (C-D) Immunoblot
941 analyses of RPS4¹⁻²³⁵ (C) and SNC1¹⁻²²⁶ (D) fused to YFP with a nuclear localization (NLS) or
942 nuclear exclusion signal (NES) and corresponding mutants (nls and nes, respectively) upon transient
943 expression in *N. benthamiana* leaves. Samples were taken 48h after agroinfiltration. Proteins were
944 detected with anti-GFP antibodies. Equal loading of proteins is indicated by red Ponceau staining.
945 (E-F) Signaling activity of RPS4¹⁻²³⁵-YFP and SNC1TIR¹⁻²²⁶-YFP fused to the flax L6 Golgi
946 membrane signal anchor in their N-terminus, upon agro-mediated transient expression at OD₆₀₀=0,1
947 in *N. benthamiana*. (E) Photos of cell death symptoms were taken 5 dai. Infiltrated areas are
948 surrounded by dotted circles (black dotted line circles correspond to positive controls). These
949 experiments were repeated at least three times with similar results. (F) Ion leakage measurement of
950 *N. benthamiana* leaves 48h after agro-mediated expression of mislocalized variants of RPS4¹⁻²³⁵ and
951 SNC1¹⁻²²⁶ fused to YFP. Leaf disk samples were collected 2 days after infiltration and incubated in
952 Milli-Q water. C1 corresponds to the ions released in solution 24 h after sampling ($\mu\text{s/cm}$). C2
953 corresponds to the total ion contents in the sample ($\mu\text{s/cm}$). Ion leakage was calculated as C1/C2 ratio
954 (see methods for more details). Statistical differences were calculated by one-way ANOVA followed
955 by multiple comparison test (Tukey's HSD). Constructs sharing letters above boxplots are not
956 significantly different ($\alpha = 0.05, n > 6$).

957

958 **Figure 5: Subcellular requirement of AtEDS1 for flax L6 signaling in Arabidopsis.**

959 (A) Signaling activity of flax L6 in transgenic Arabidopsis seedlings (10 day-old) expressing
960 Dexamethasone (Dex)-inducible L6 MHV autoimmune variant in *eds1-2* mutant background
961 complemented with mislocalized EDS1-YFP variants. F3 individuals from each cross
962 (pDex:L6MHV-3myc x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants (pEDS1:EDS1-YFP-
963 NLS (#B2) pEDS1:EDS1-YFP-NES (#3-4), pEDS1:EDS1-YFP-nls (#B5), pEDS1:EDS1-YFP-nes
964 (#1-2)) were PCR screened to verify the presence or absence (-) of EDS1-YFP variant transgene and
965 transferred to 10 μM Dex-containing media. Photos were taken on the day plants were transferred
966 (t0), and four (4dai) and eight (8dai) days after transfer (dai: days after Dex induction). Transgenic
967 pDex:L6MHV-3myc and wild-type Col-0 seedlings were used as positive and negative controls,

968 respectively. This assay was repeated three times with similar results. (B) Immunoblot analysis of
969 EDS1-YFP variants and L6MHV-3myc 24h after Dex induction, using anti-GFP and anti-myc
970 antibodies, respectively. Protein loading is indicated by red Ponceau staining.

971

972 **Figure 6: Subcellular requirement of AtEDS1 for flax RPS4TIR signaling in Arabidopsis.**

973 (A) Signaling activity of Arabidopsis RPS4 TIR in transgenic Arabidopsis seedlings (10 day-old)
974 expressing Dexamethasone (Dex)-inducible RPS4¹⁻²³⁵ in *eds1-2* mutant background complemented
975 with mislocalized EDS1-YFP variants. F3 individuals from each cross (pDex: RPS4¹⁻²³⁵-Flag-SBP
976 x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants (pEDS1:EDS1-YFP-NLS (#B2) pEDS1:EDS1-
977 YFP-NES (#3-4), pEDS1:EDS1-YFP-nls (#B5), pEDS1:EDS1-YFP-nes (#1-2)) were PCR screened
978 to verify the presence or absence of EDS1-YFP variant transgene and transferred to 10μM Dex-
979 containing media. Photos were taken on the day plants were transferred (t0), three (3dai) and six
980 (6dai) days after transfer (dai:days after Dex induction). Transgenic pDex: RPS4¹⁻²³⁵-FSBP and wild-
981 type Col-0 seedlings were used as positive and negative controls, respectively. This assay was
982 performed twice with similar results. pDex:EDS1-YFP-NES (line#2) is a sibling line of pDex:EDS1-
983 YFP-NES (line#1) but in which the EDS1-YFP-NES transgene is homozygous unlike line#1 in which
984 the transgene still segregates. (B) Immunoblot analysis of EDS1-YFP variants and RPS4¹⁻²³⁵-flag-
985 SBP 24h after Dex induction, using anti-GFP and anti-flag antibodies, respectively. Protein loading
986 is indicated by red Ponceau staining.

Figure 1: Membrane attachment is required for flax L6TIR signaling activity

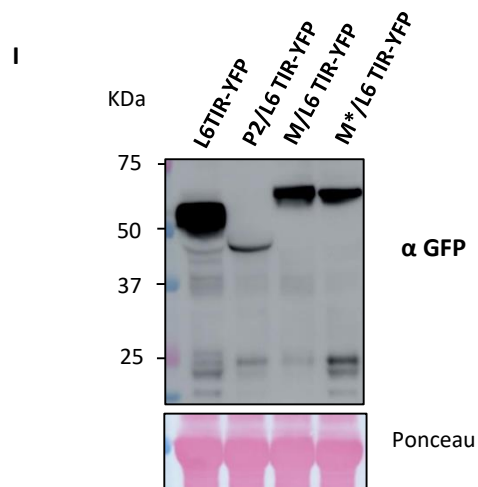
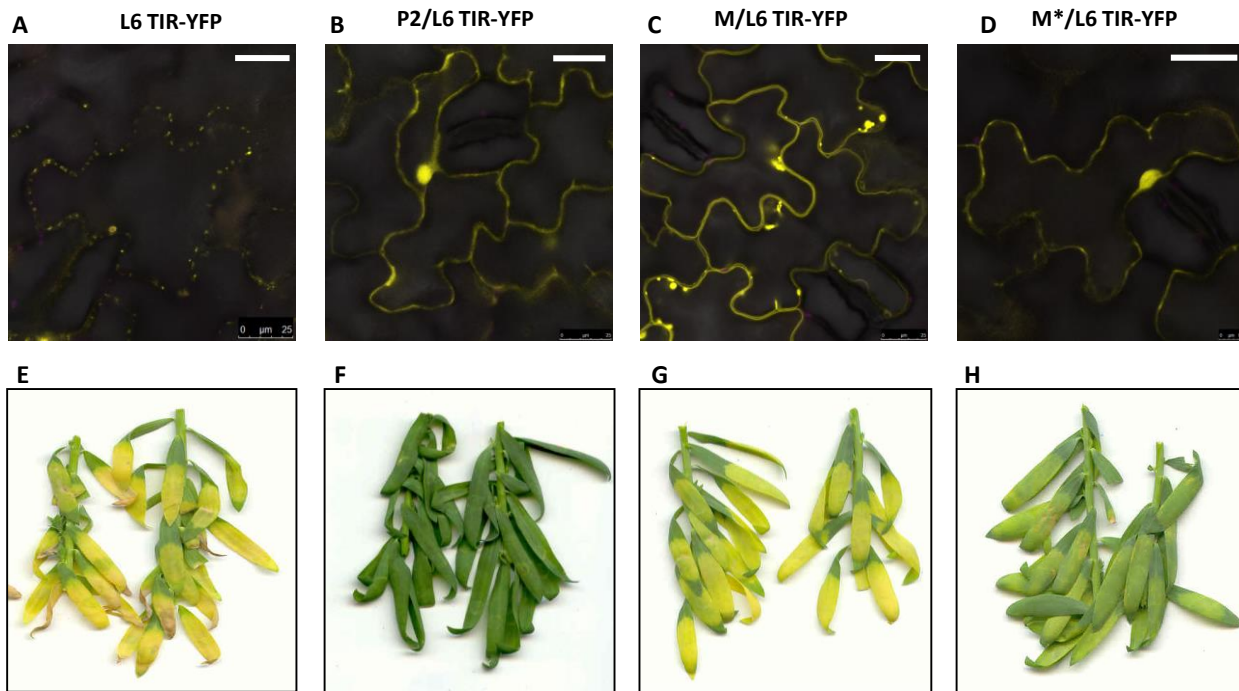
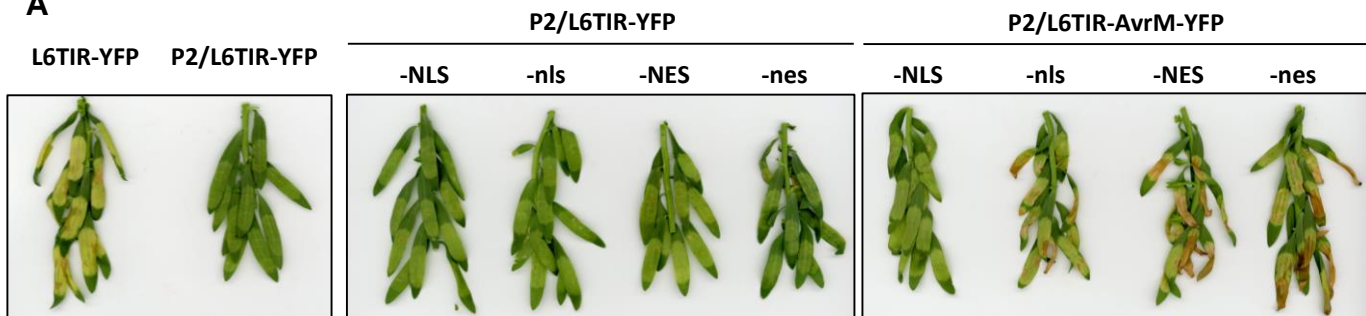
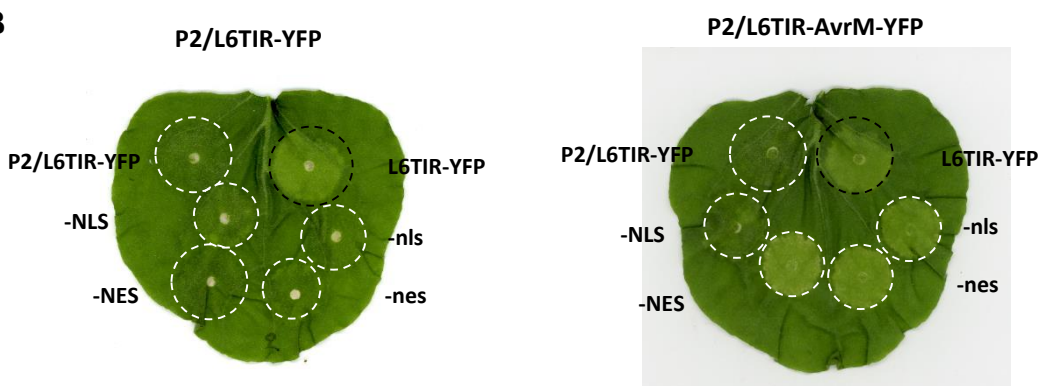


Figure 2: Fusion of signaling inactive P2/L6TIR to the flax rust effector AvrM leads to TIR signaling activation in the cytosolic compartment only

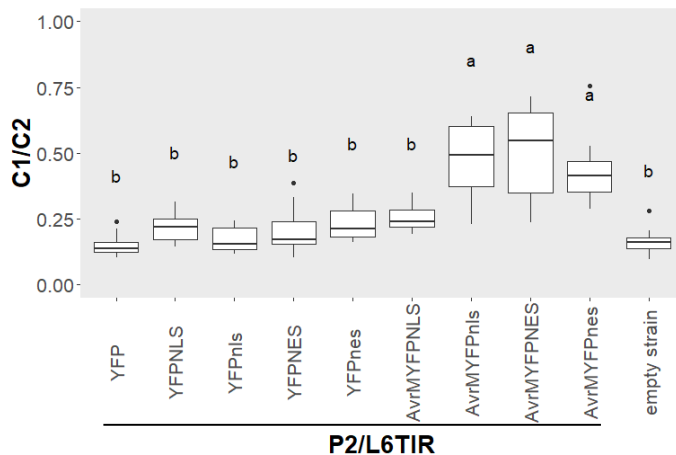
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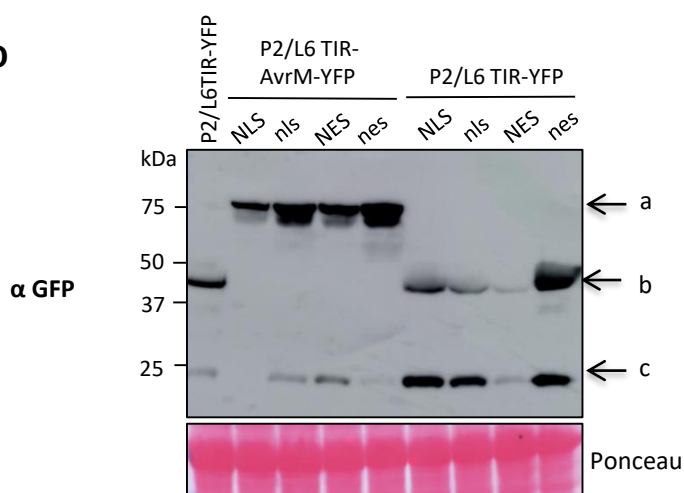
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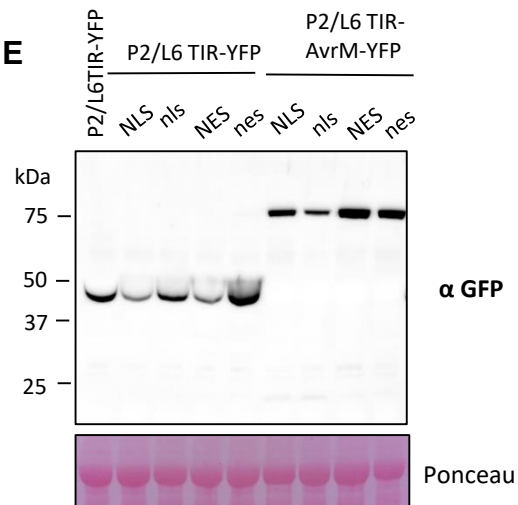
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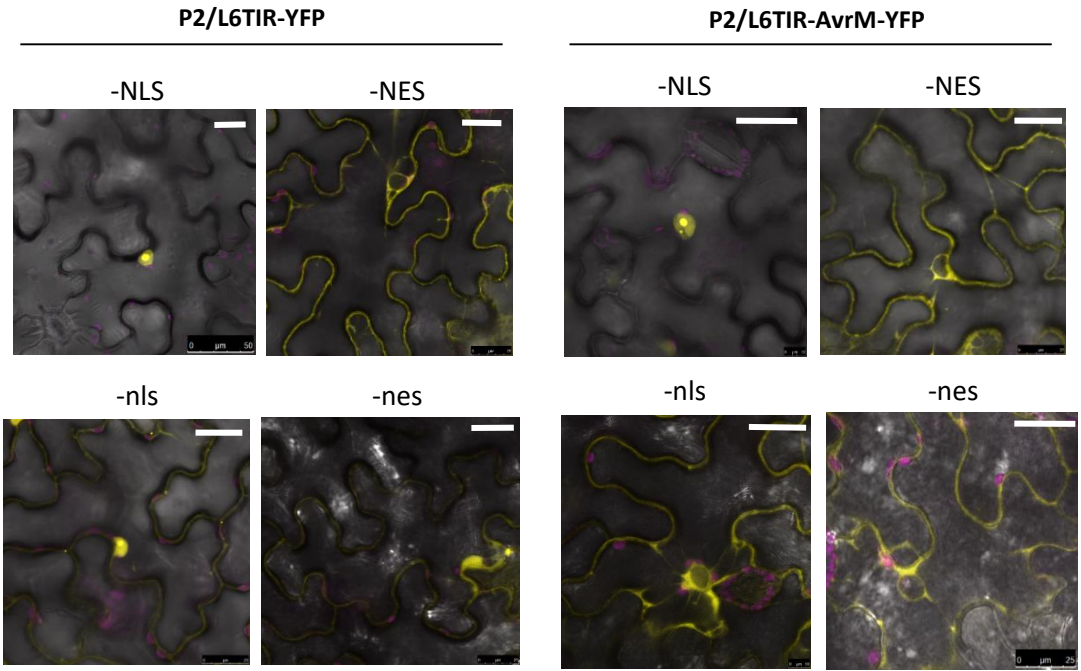
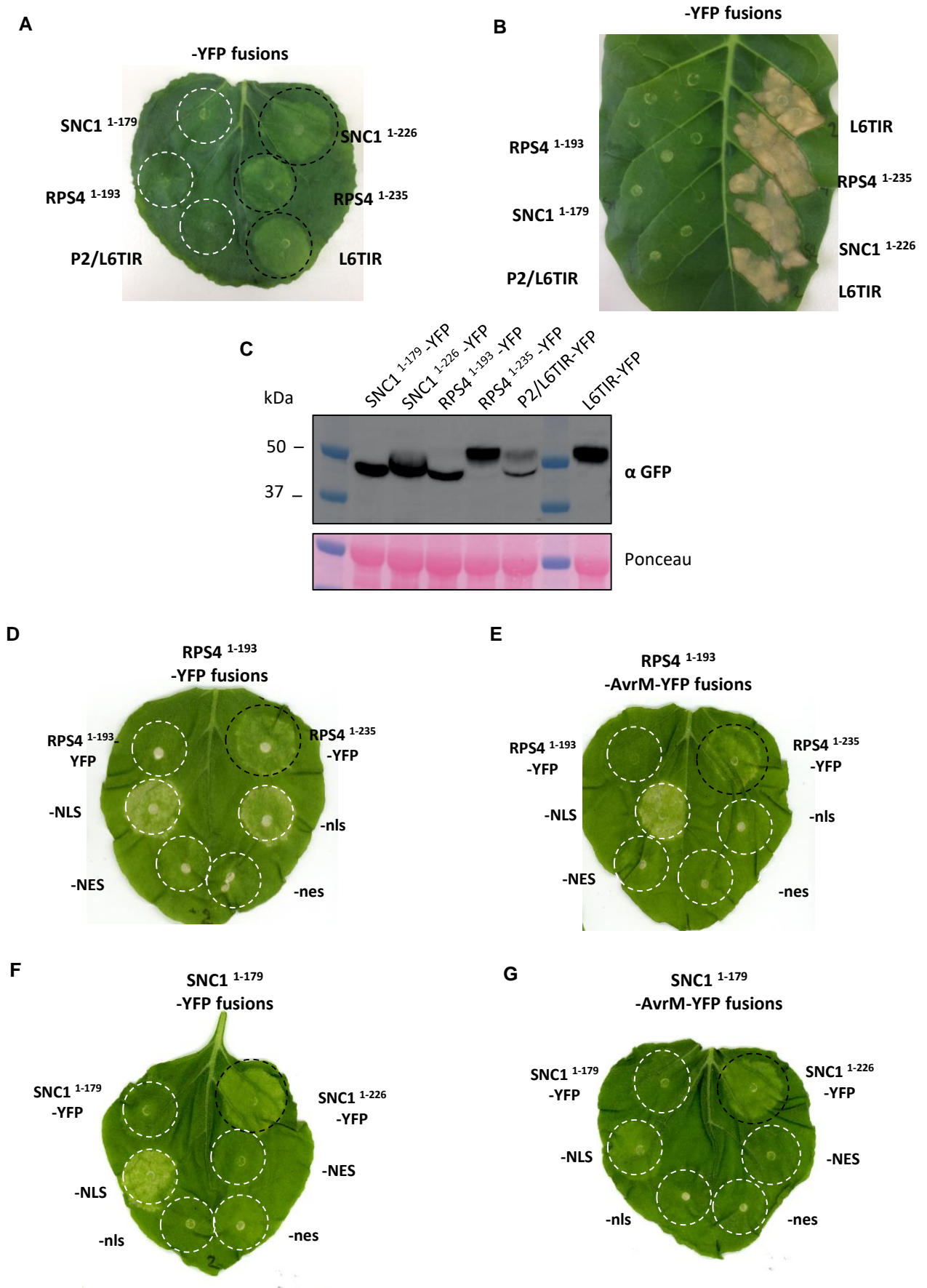
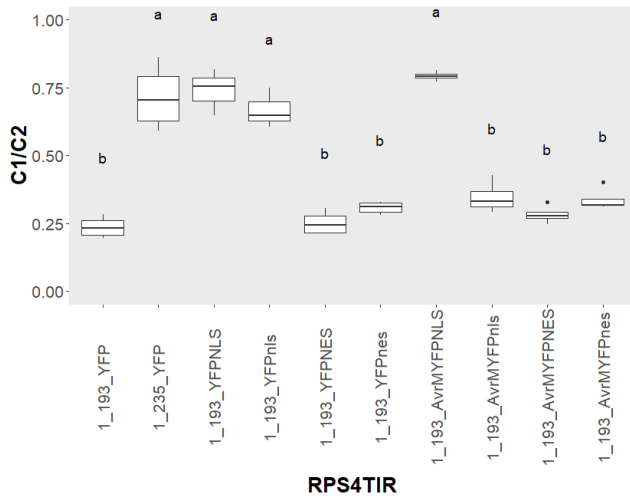


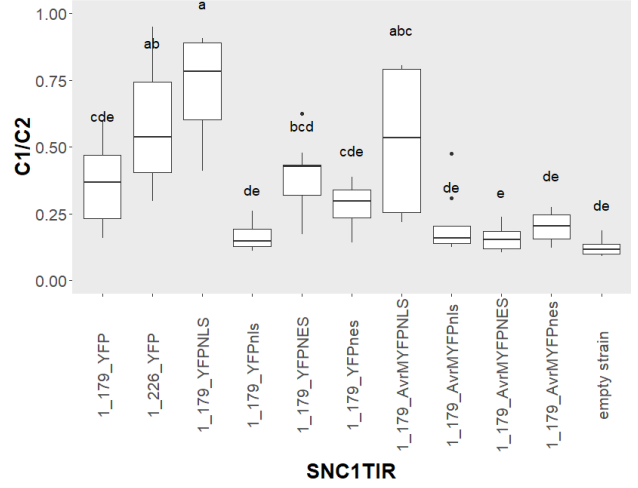
Figure 3: Forcing inactive RPS4TIR and SNC1TIR-only in the nucleus is sufficient to activate TIR signaling



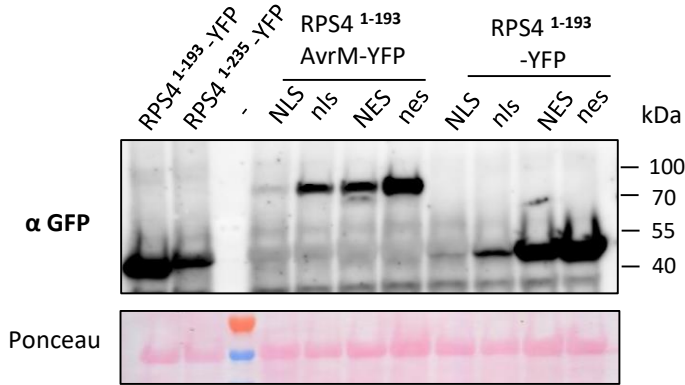
H



I



J



K

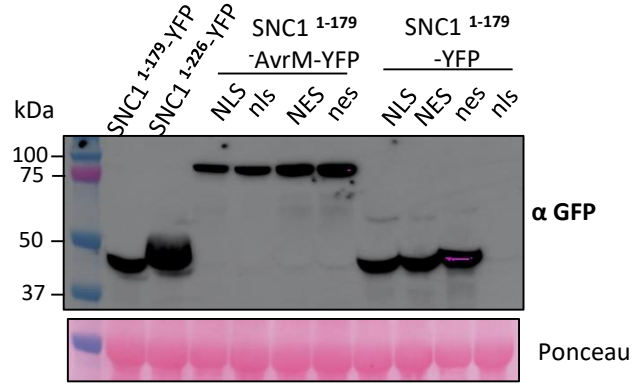


Figure 4: Altered subcellular localization of signaling active RPS4 and SNC1 TIR domains do not affect their ability to induce immune responses

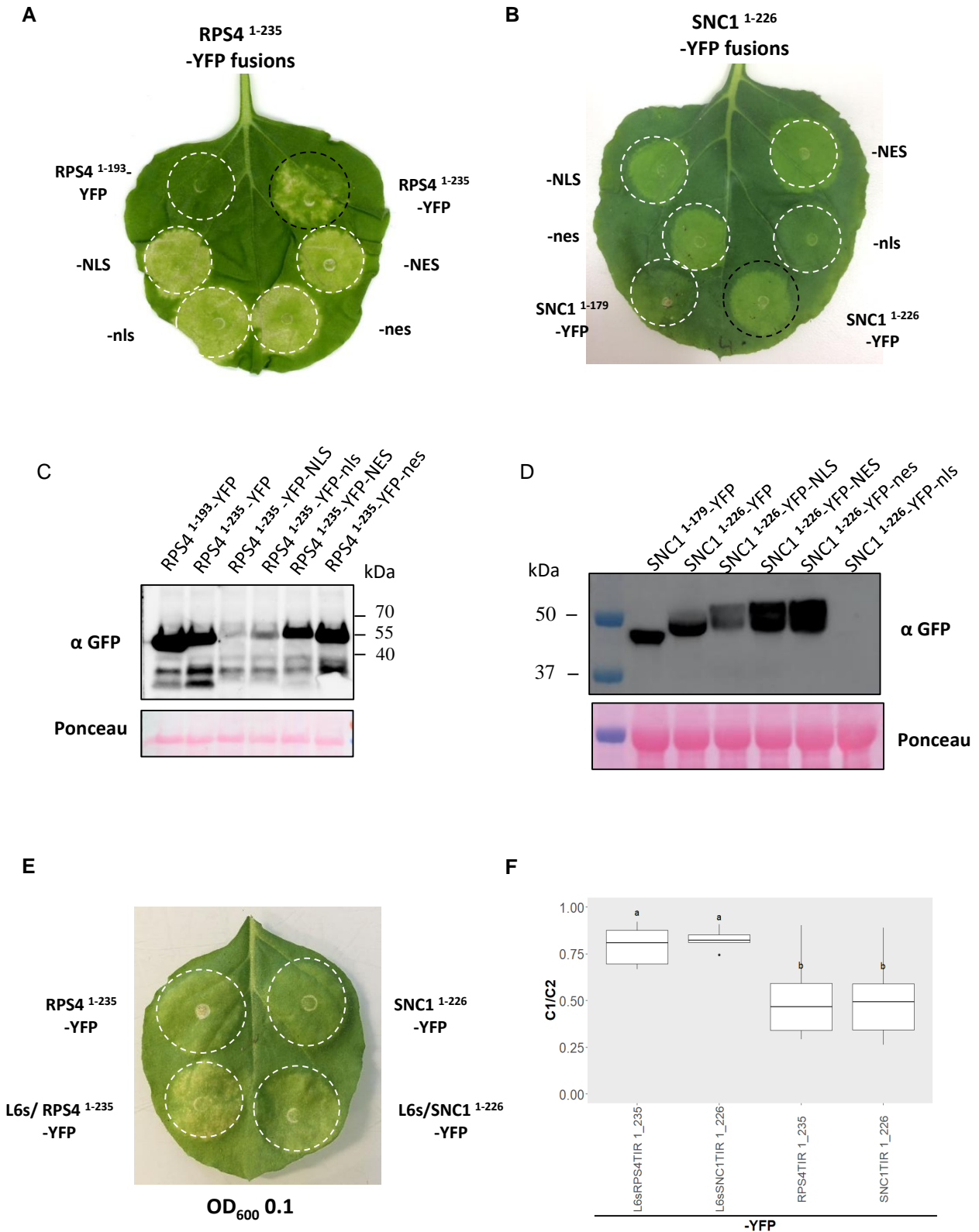
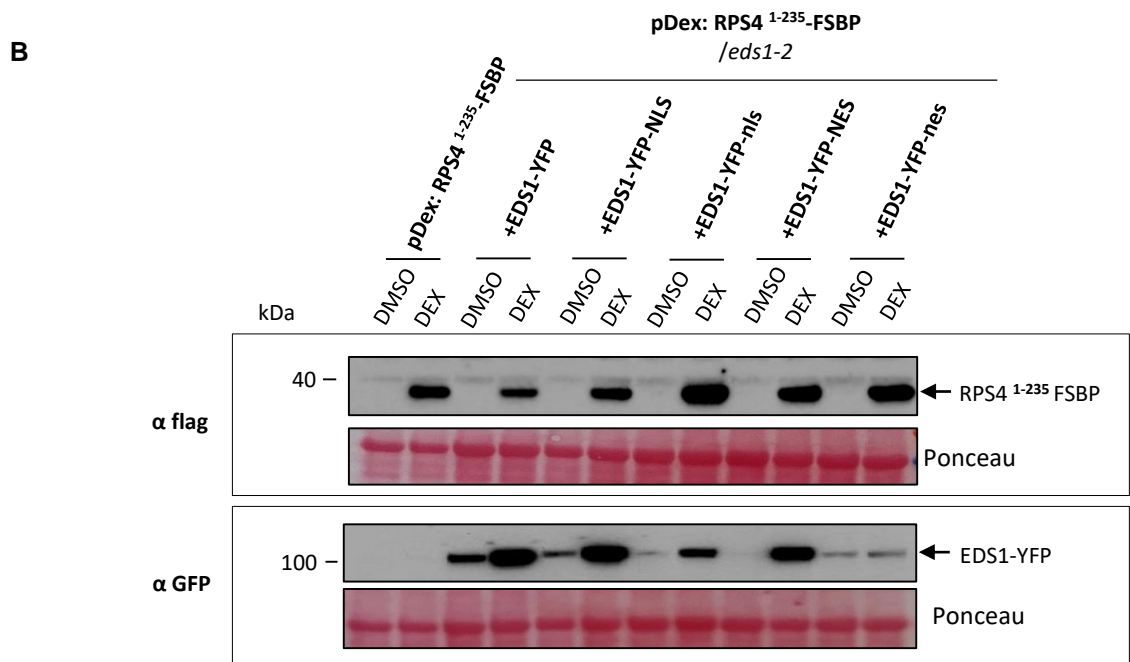
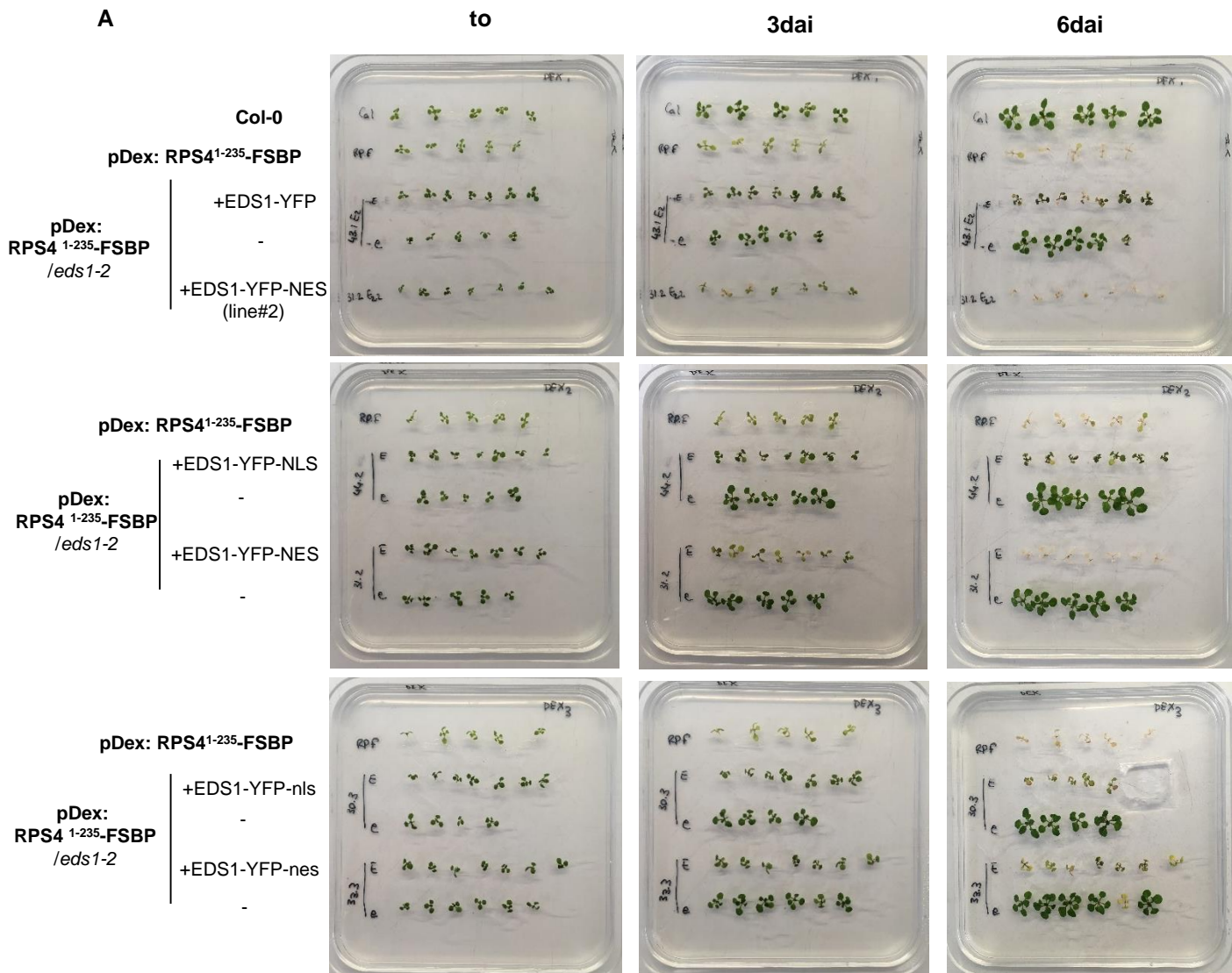


Figure 6: Subcellular requirement of AtEDS1 for flax RPS4TIR signaling in Arabidopsis



Supplemental figures

Figure S1: Signalling activity, and protein stability of mislocalized variants of P2/L6TIR.

(A) Signaling activity of L6TIR and P2/L6 TIR fused to -YFP or flax rust effector –AvrM upon agro-mediated transient expression in flax . Photos were taken 12 dai. (B) Live cell fluorescence image show YFP (yellow) cytosolic labelling of AvrM-YFP, overlaid with chloroplast autofluorescence (pink). Confocal images were taken 48h after agro-infiltration, in *N. benthamiana* leaves. White bar scale represents 25µm.

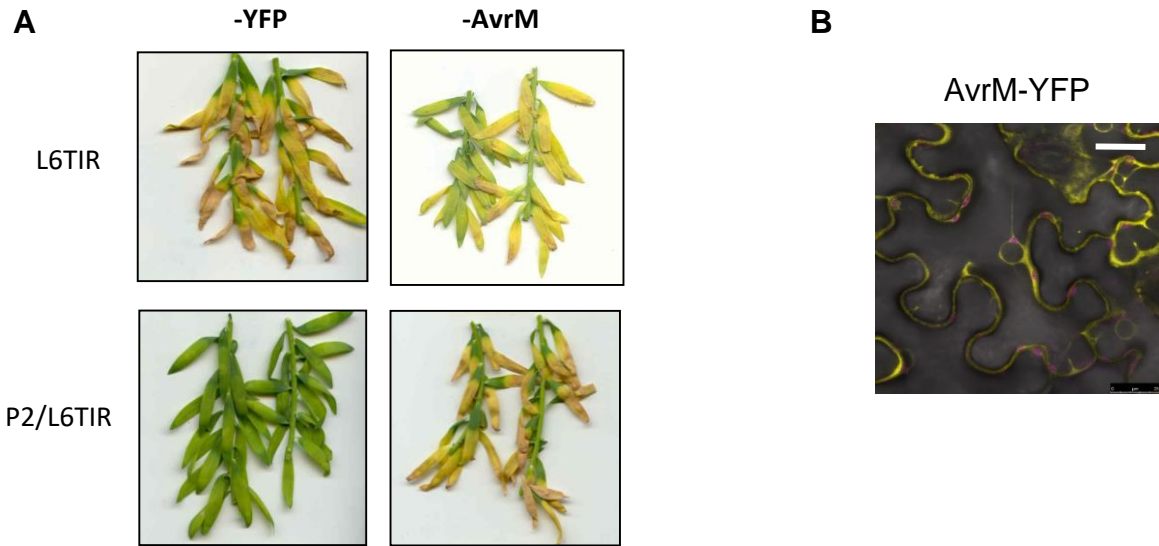


Figure S3: Subcellular localization of RPS4 and SNC1 TIR constructs.

Live cell fluorescence images show YFP (yellow) labelling of TIR-only (RPS4¹⁻¹⁹³ and SNC1¹⁻¹⁷⁹) or larger TIR constructs (RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶), overlaid with chloroplast autofluorescence (pink). Confocal images were taken 48h after agro-infiltration, in *N. benthamiana* leaves. White bar scale represents 25 μ m.

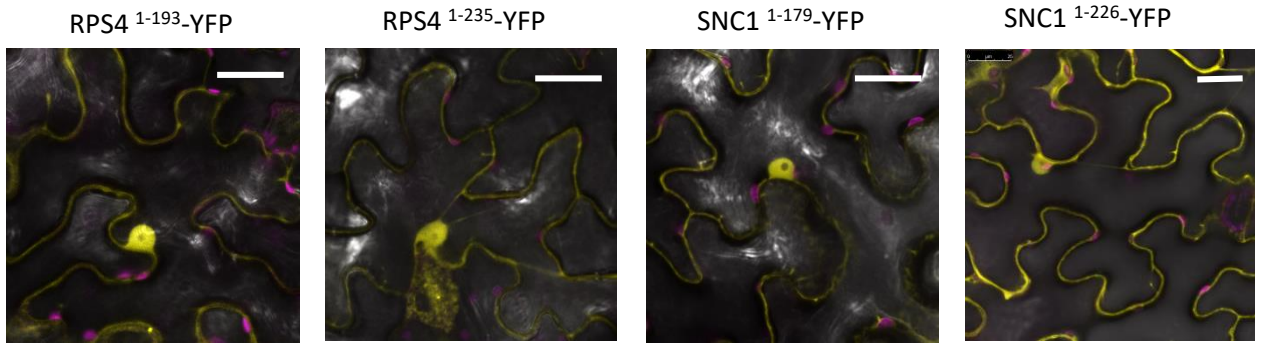
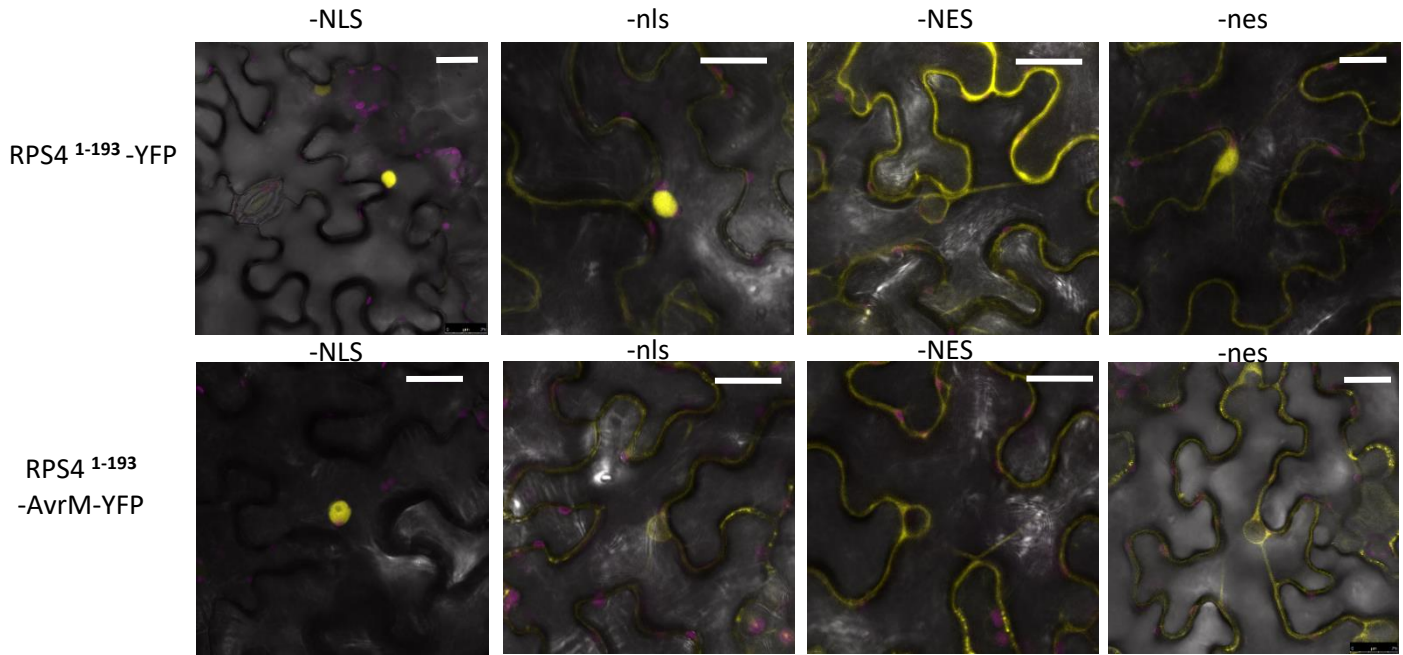


Figure S4: Subcellular localization of mislocalized variants of RPS4 and SNC1 TIR-only.

Live cell fluorescence images show YFP (yellow) labelling of RPS4¹⁻¹⁹³ (A) and SNC1¹⁻¹⁷⁹ (B) fused to YFP or AvrM-YFP with a nuclear localization (NLS) or nuclear exclusion signal (NES) and corresponding mutants (nls and nes, respectively), overlaid with chloroplast autofluorescence (pink). Confocal images were taken 48h after agro-infiltration, in *N. benthamiana* leaves. White bar scale represents 25µm.

A



B

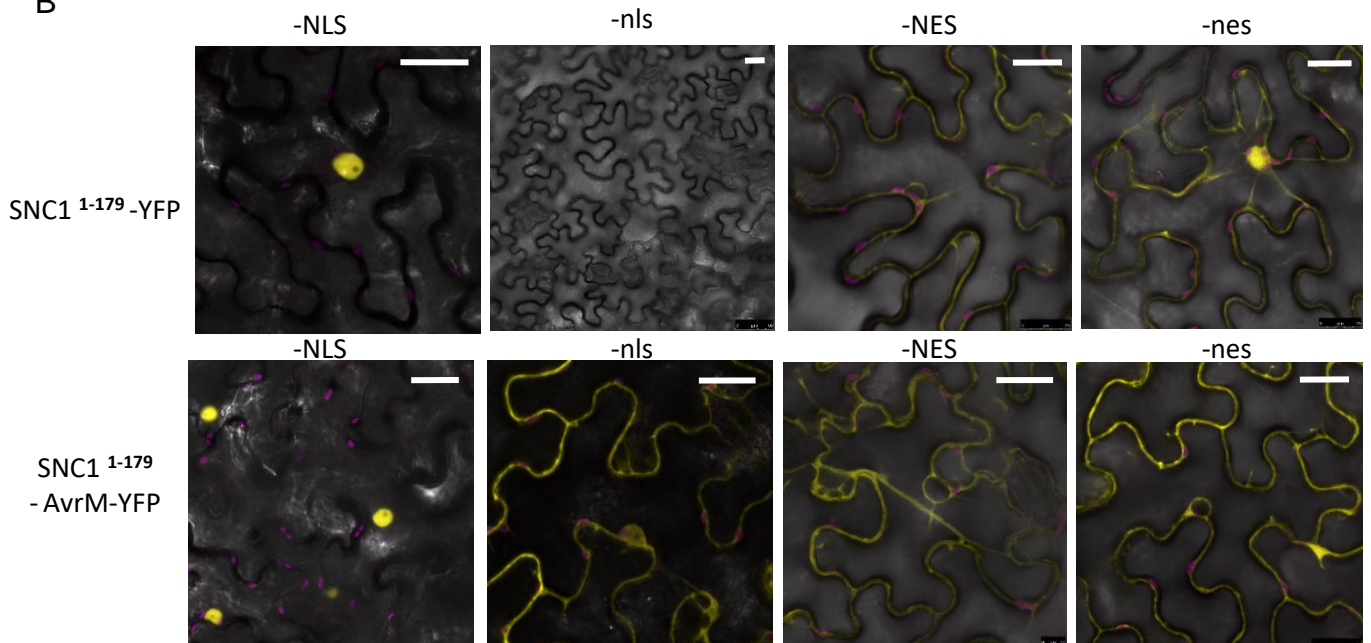
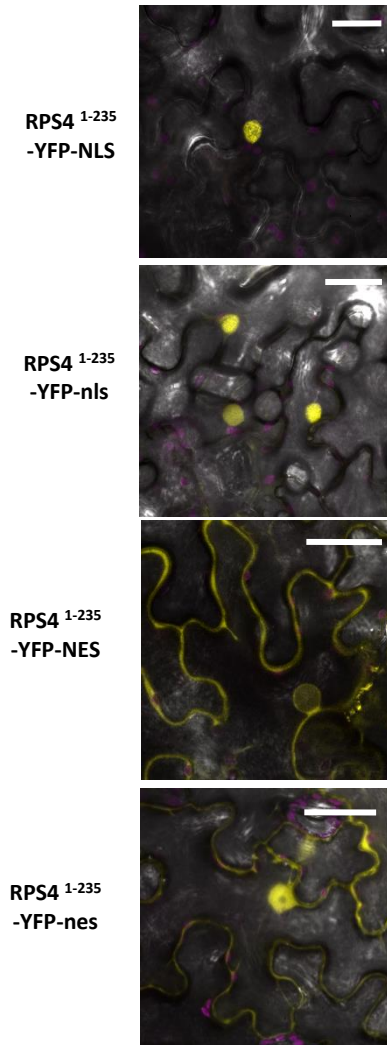


Figure S5: Subcellular localization of mislocalized variants of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ TIR constructs.

Immunoblot (A) and confocal analyses (B) of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ fused to YFP with a nuclear localization (NLS) or nuclear exclusion signal (NES) and corresponding mutants (nls and nes, respectively) upon transient expression in *N. benthamiana* leaves. (A) Samples were taken 48h after agroinfiltration. Proteins were detected with anti-GFP antibodies. Equal loading of proteins is indicated by red Ponceau staining. (B) Live cell fluorescence images show YFP (yellow) labelling overlaid with chloroplast autofluorescence (pink). Confocal images were taken 48h after agro-infiltration, in *N. benthamiana* leaves. White bar scale represents 25µm.

A



B

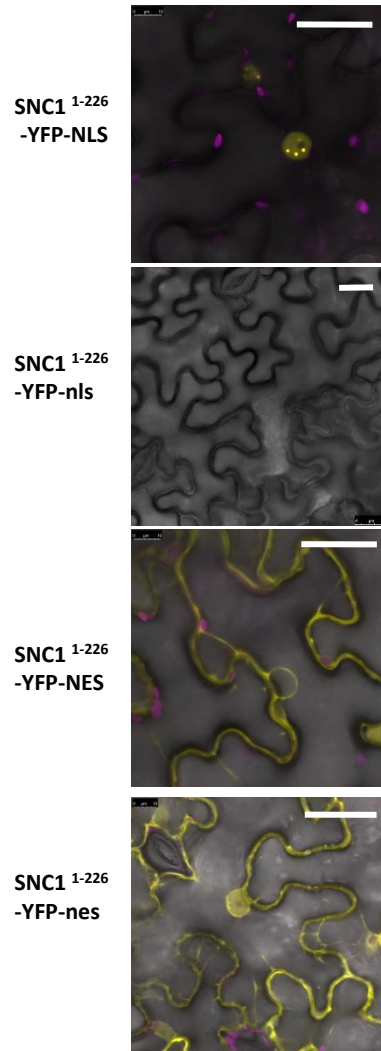


Figure S6: Signaling activity, subcellular localization and protein accumulation of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ TIR constructs fused to L6 signal anchor. (See legends next slide)

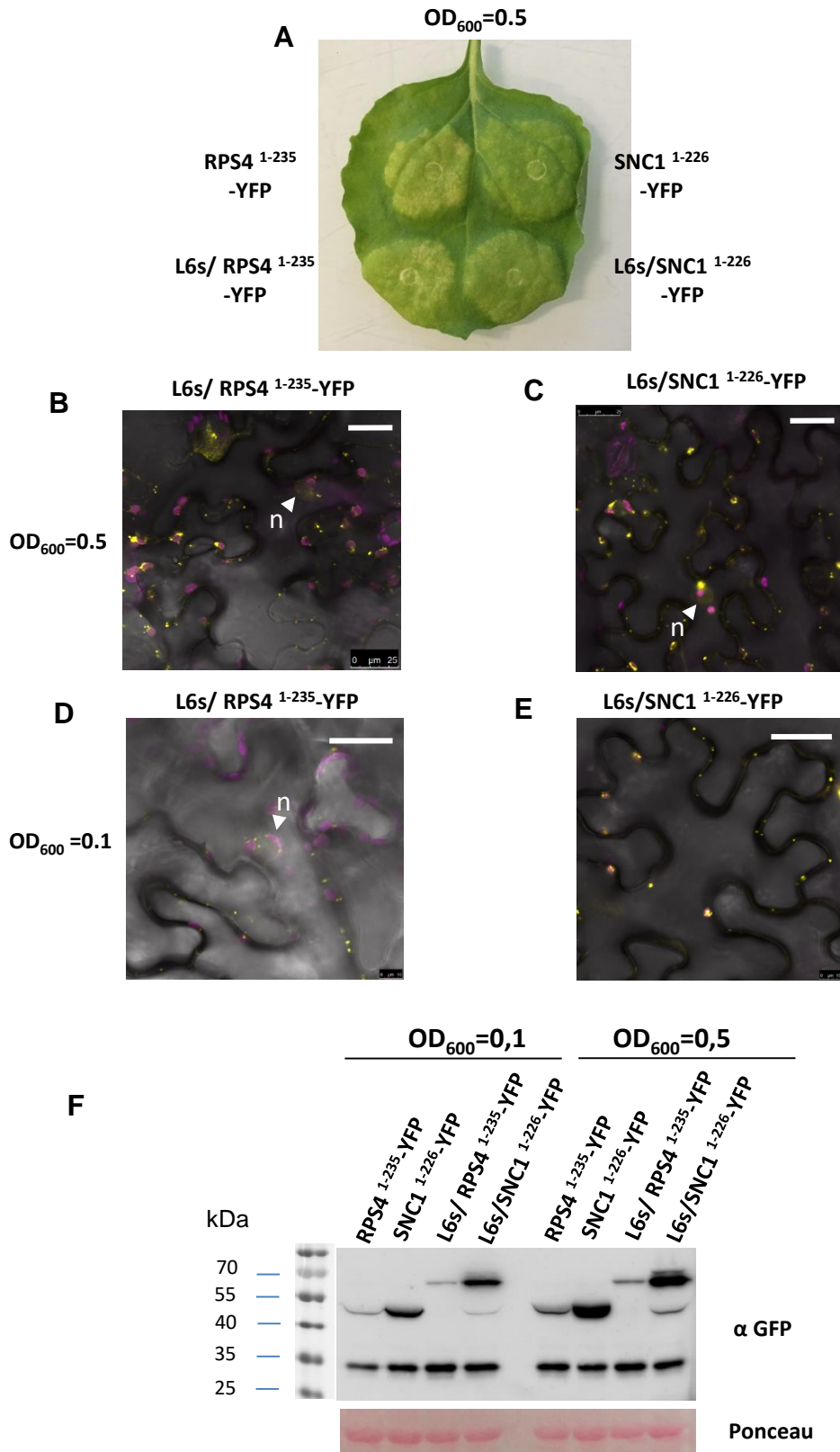


Figure S6: Signaling activity, subcellular localization and protein accumulation of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ TIR constructs fused to L6 signal anchor.

Signalling activity (A), subcellular localization (B-E) and protein accumulation (F) of RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ TIR constructs fused to L6 signal anchor upon transient expression in *N. benthamiana*.

(A) *N. benthamiana* leaves photos were taken 5 days after agro-infiltration at OD₆₀₀ 0.5 in *N. benthamiana*. (B-E) Live cell fluorescence images show YFP (yellow) labelling of Golgi membrane-tethered RPS4¹⁻²³⁵ and SNC1¹⁻²²⁶ TIR constructs fused to L6 signal anchor, overlaid with chloroplast autofluorescence (pink). Confocal images were taken 48h after agro-infiltration at OD₆₀₀ 0.5 or OD₆₀₀ 0.1, in *N. benthamiana* leaves. The arrow head points at a nucleus (n). White bar scale represents 25µm. (F) Immunoblot analysis. Proteins were detected using anti-GFP antibodies. Equal loading of proteins is indicated by red Ponceau staining.

Figure S7: Phenotype of independent transgenic Arabidopsis seedlings expressing Dexamethasone (Dex)-inducible L6MHV-3myc (pDex:L6MHV-3myc).

F2 individuals (10 day-old) were transferred to non-inducing (DMSO) or 10 μ M Dex-containing media. Photos were taken 14 days after transfer.

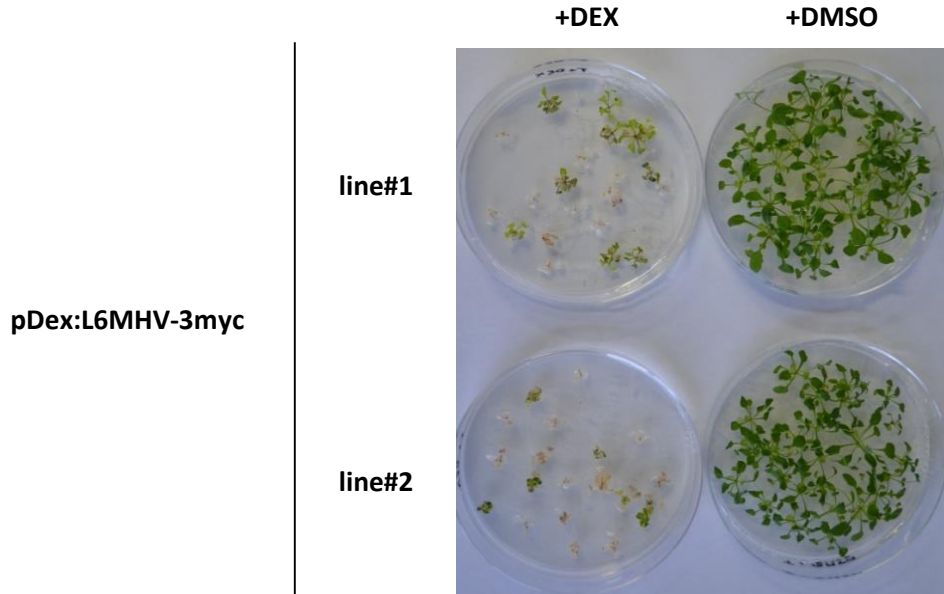


Figure S8: Phenotype of transgenic Arabidopsis seedlings expressing pDex:L6MHV on a non-inducing media.

F3 individuals of transgenic Arabidopsis seedlings (10 day-old) expressing Dexamethasone (Dex)-inducible L6MHV-3myc in wild-type Col-0 or in *eds1-2* mutant background complemented with mislocalized EDS1-YFP variants (pDex:L6MHV-3myc x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants. Each individuals were PCR screened to verify the presence or absence of EDS1-YFP variant transgene and transferred to non-inducing (DMSO containing) MS media. Photos were taken on the day plants were transferred (t0), four (3dai) and eight (6dai) days after transfer (dai: days after induction). This assay was performed two times with similar results.

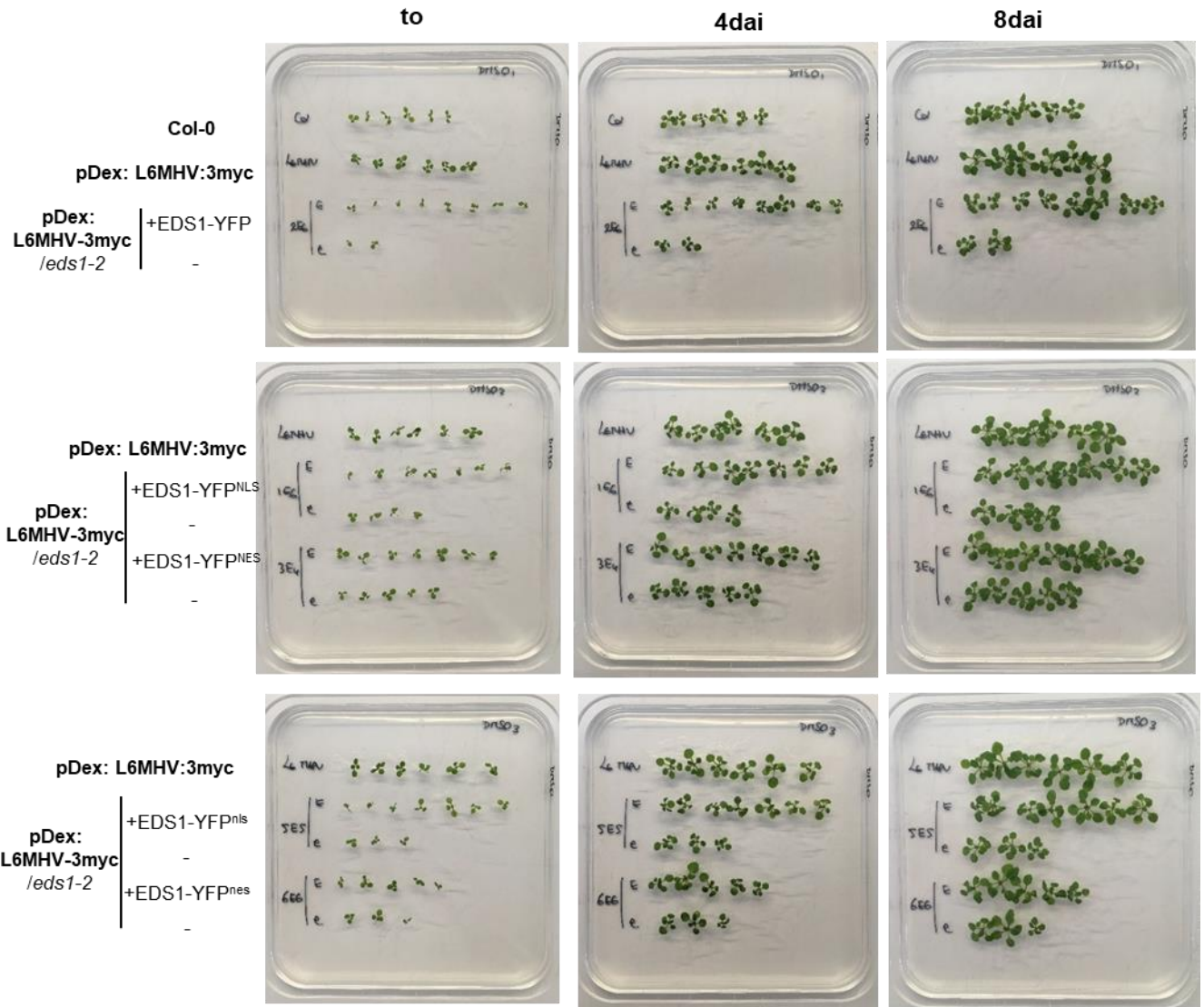


Figure S9: Flax L6 signaling in Arabidopsis depends on AtEDS1.

Cell death phenotype of transgenic Arabidopsis seedlings (10 day-old) expressing Dexamethasone (Dex)-inducible L6MHV-3myc in *eds1-2* mutant background. F2 individuals from pDex:L6MHV-3myc x *eds1-2* (line #2) were PCR screened to select seedlings homozygous for *EDS1* mutation (*eds1/eds1*) or displaying heterozygous or homozygous wild-type *EDS1* (*EDS1/eds1* or *EDS1/EDS1*). Seedlings were transferred to 10 μ M Dex-containing media. Photos were taken 6 days after Dex induction.

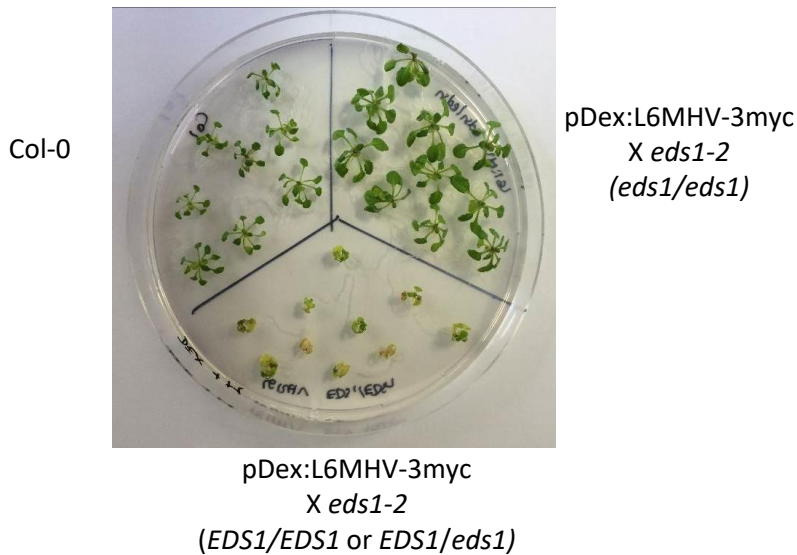


Figure S10: Phenotype of transgenic Arabidopsis seedlings from crosses of pDex:L6MHV-3myc/*eds1-2* line with independent mislocalized EDS1 variants lines. (See legends next slide).

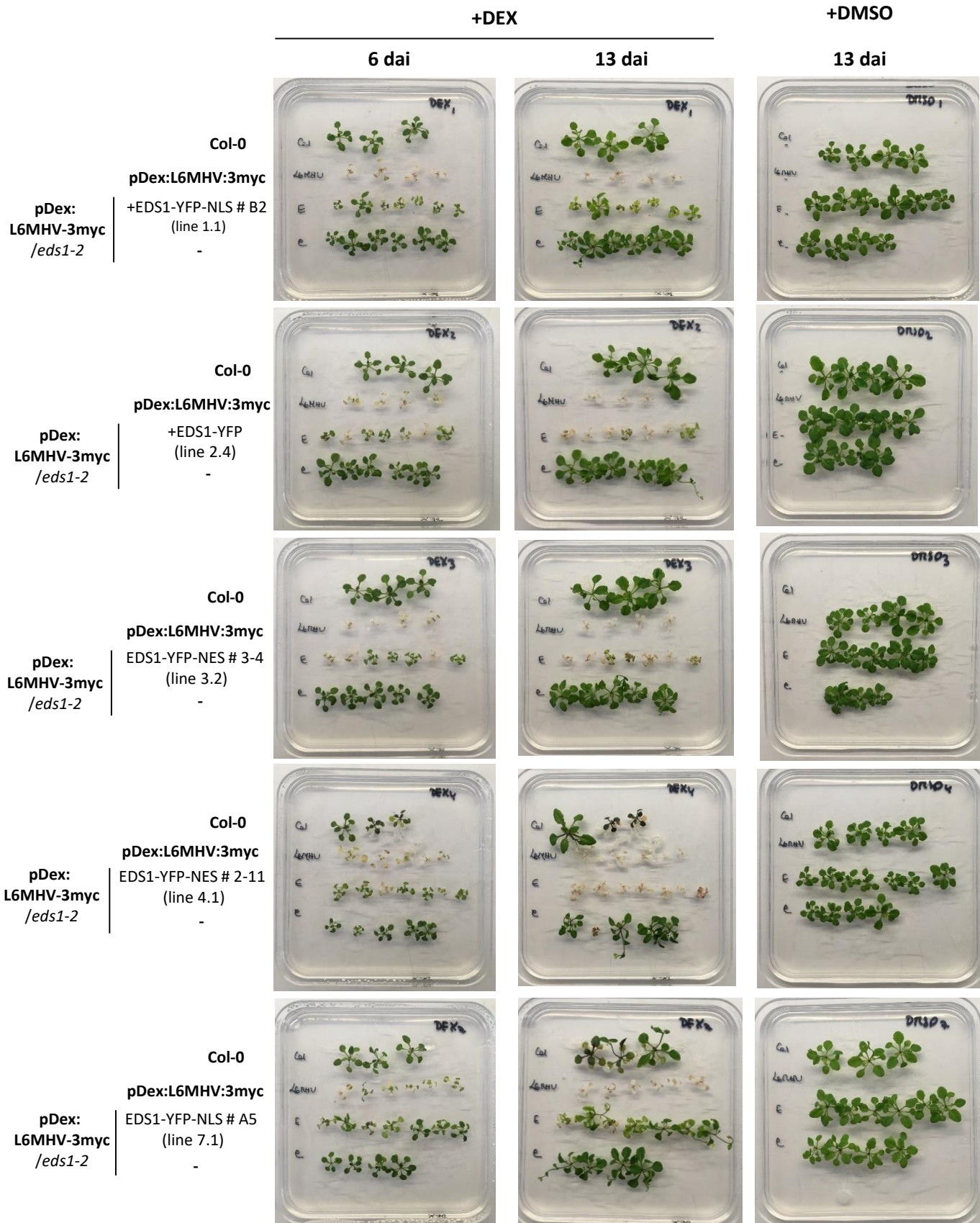


Figure S10: Phenotype of transgenic Arabidopsis seedlings from crosses of pDex:L6MHV-3myc/*eds1-2* line with independent mislocalized EDS1 variants lines. F3 individuals (12 day-old seedlings) from each cross (pDex:L6MHV-3myc x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants (pEDS1:EDS1-YFP-NLS (#B2), pEDS1:EDS1-YFP-NLS (#A5), pEDS1:EDS1-YFP-NES (#2-11), pEDS1:EDS1-YFP-NES (#3-4), pEDS1:EDS1-YFP-nls (#B5), pEDS1:EDS1-YFP-nes (#1-2)) were PCR screened to verify the presence or absence (-) of EDS1-YFP variant transgene and transferred to 10µM Dex-containing media. Photos were taken at six (6 dai) and thirteen (13 dai) days after transfer on Dex inducing media (DEX), and at 13 dai on non-inducing media (DMSO). On Dex-inducing media, transgenic pDex:L6MHV-3myc and wild-type Col-0 seedlings were used as positive and negative controls, respectively. On non-inducing media wild-type Col-0 seedlings were used as controls.

Figure S11: Subcellular localization of EDS1-YFP variants in Dex-inducible transgenic Arabidopsis seedlings (10 day-old) expressing pDex:L6MHV in *eds1-2* mutant background complemented with mislocalized EDS1-YFP variants. F3 individuals from each crosses (pDex:L6MHV x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants were PCR screened for the presence of EDS1-YFP variant (and transferred to inducing (Dex)-containing MS media. The roots of at least two seedlings were analysed by confocal microscopy. Images were taken 24h after Dex induction. Left: Yellow Fluorescence images, right: Overlay of brightfield and yellow fluorescence images. White scale represents 50µm.

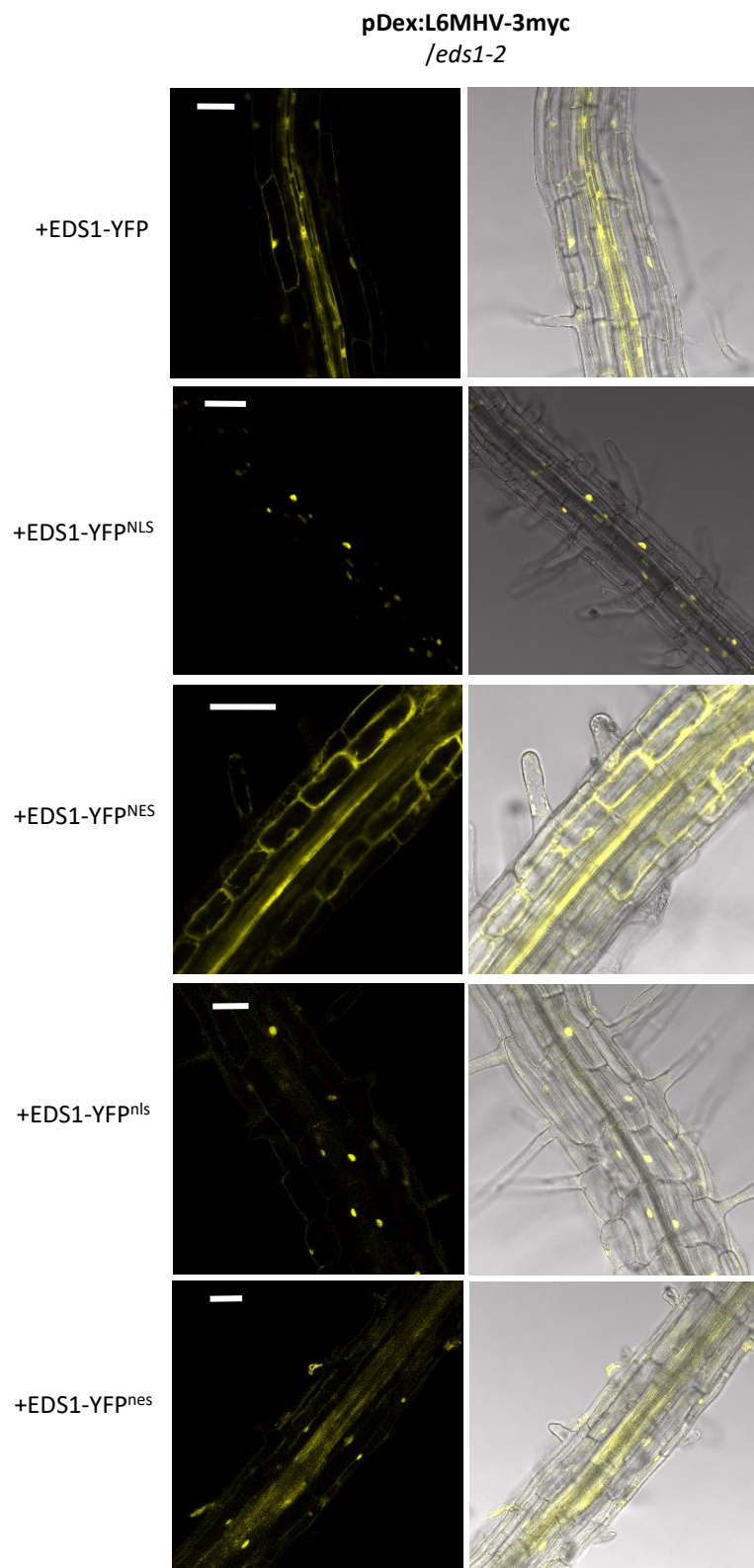


Figure S12: Phenotype of independent transgenic Arabidopsis seedlings expressing Dexamethasone (Dex)-inducible RPS4¹⁻²³⁵-FSBP.

F2 individuals (10-day old) were transferred to non-inducing (DMSO) or 10 μ M Dex-containing media (DEX). Photos were taken 8 days after transfer.

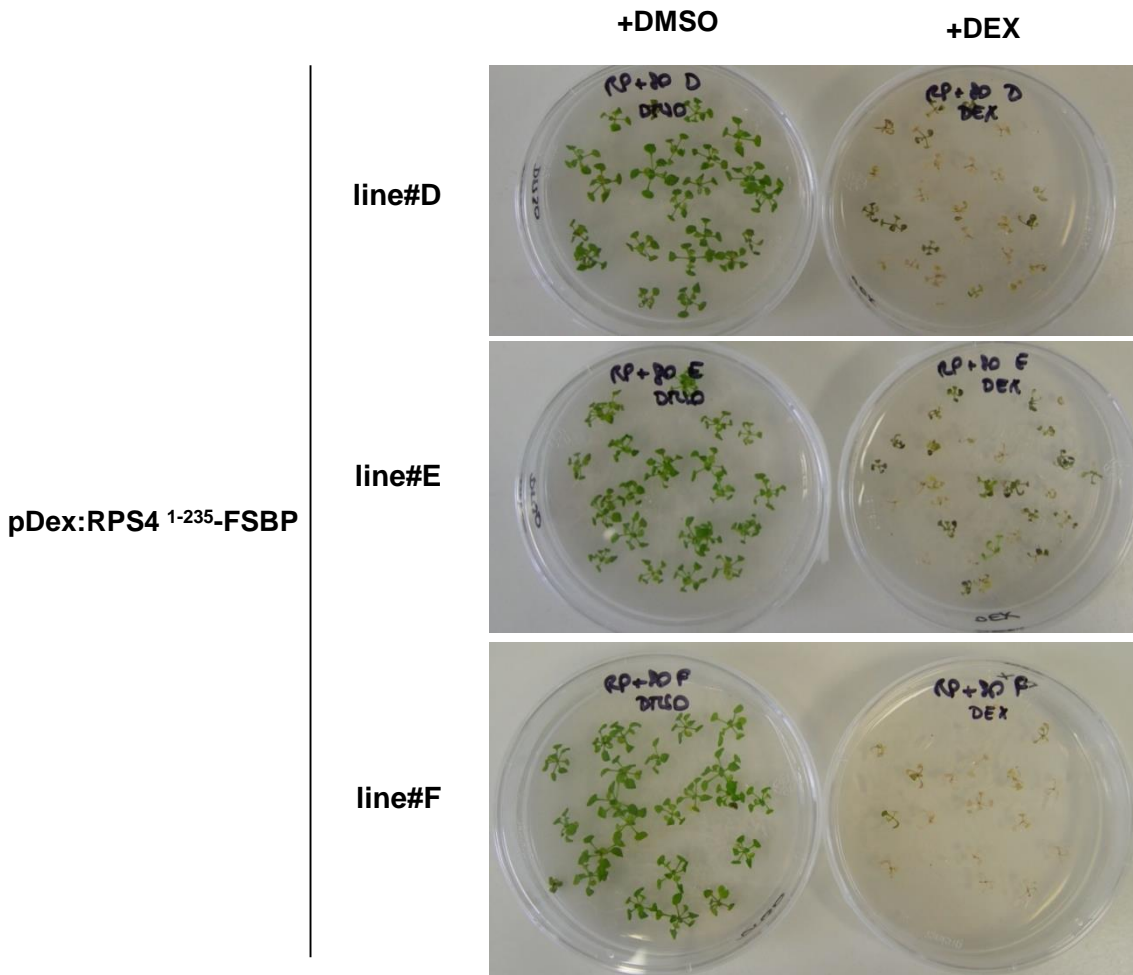


Figure S13: Phenotype of transgenic Arabidopsis seedlings expressing pDex:RPS4¹⁻²³⁵ on a non-inducing media.

F3 individuals of Arabidopsis seedlings (10 day-old) expressing Dexamethasone (Dex)-inducible RPS4¹⁻²³⁵ in wild-type Col-0 or in *eds1-2* mutant background complemented with mislocalized EDS1-YFP variants (pDex: RPS4¹⁻²³⁵-FSBP x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants. Each individuals were PCR screened to verify the presence or absence of EDS1-YFP variant transgene and transferred to non-inducing (DMSO containing) MS media. Photos were taken on the day plants were transferred (t0), four (3dai) and eight (6dai) days after transfer (dai: days after induction). This assay was performed two times with similar results.

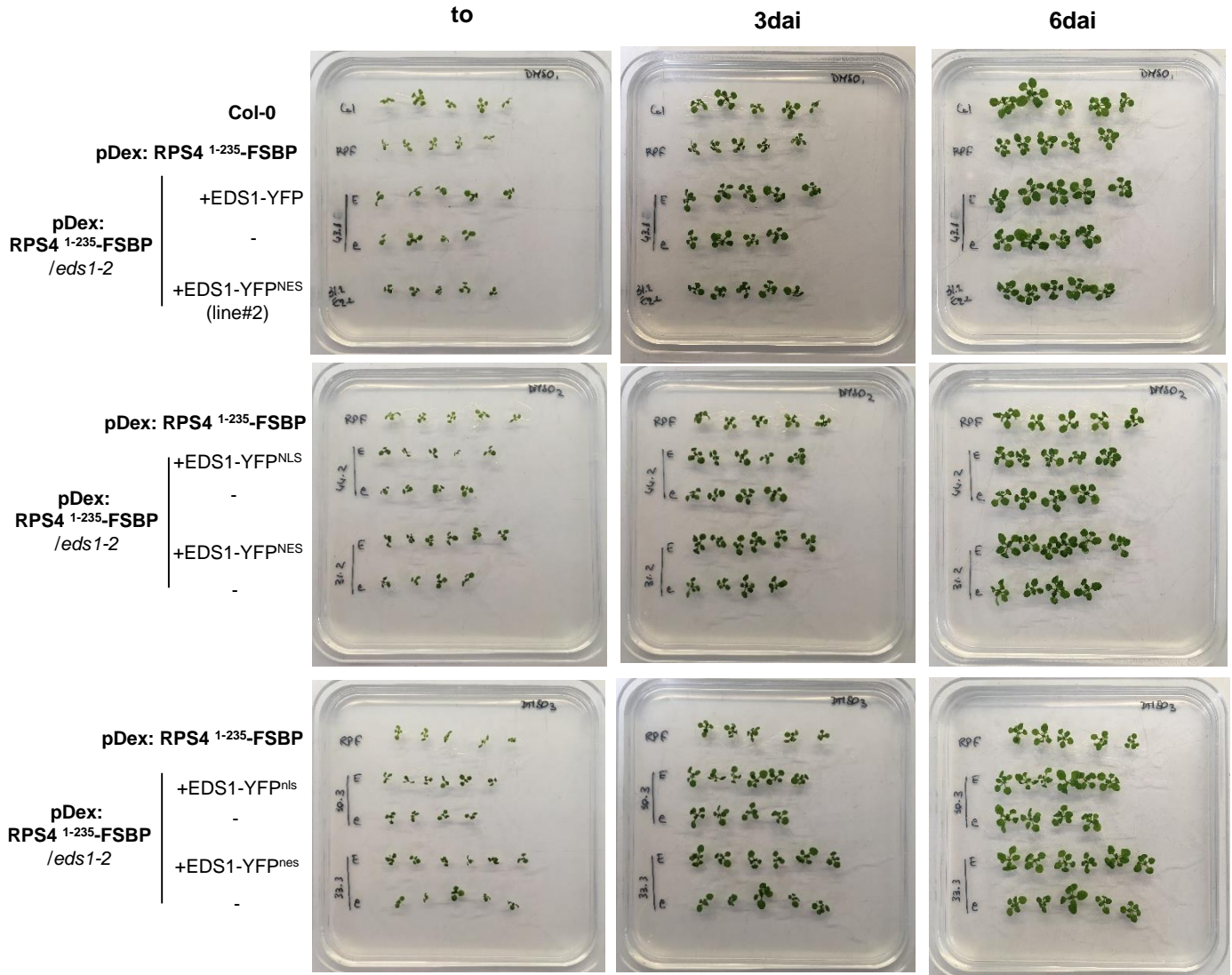


Figure S14: Phenotype of transgenic Arabidopsis seedlings from crosses of pDex: RPS4¹⁻²³⁵-FSBP /eds1-2 line #8.1 with independent mislocalized EDS1 variants lines. F2 individuals (12 day-old seedlings) from each cross (pDex: (L6MHV-3myc x eds1-2) x pEDS1:EDS1-YFP mislocalized variants (pEDS1:EDS1-YFP-NLS (#B2), pEDS1:EDS1-YFP-NLS (#A5), pEDS1:EDS1-YFP-NES (#2-11), pEDS1:EDS1-YFP-NES (#3-4), pEDS1:EDS1-YFP-nls (#B5))), were PCR screened to verify the presence or absence (-) of EDS1-YFP variant transgene and transferred to 10μM Dex-containing media. Photos were taken at six days after transfer on Dex inducing media (DEX), or non-inducing media (DMSO). Wild-type Col-0 seedlings were used as negative controls.

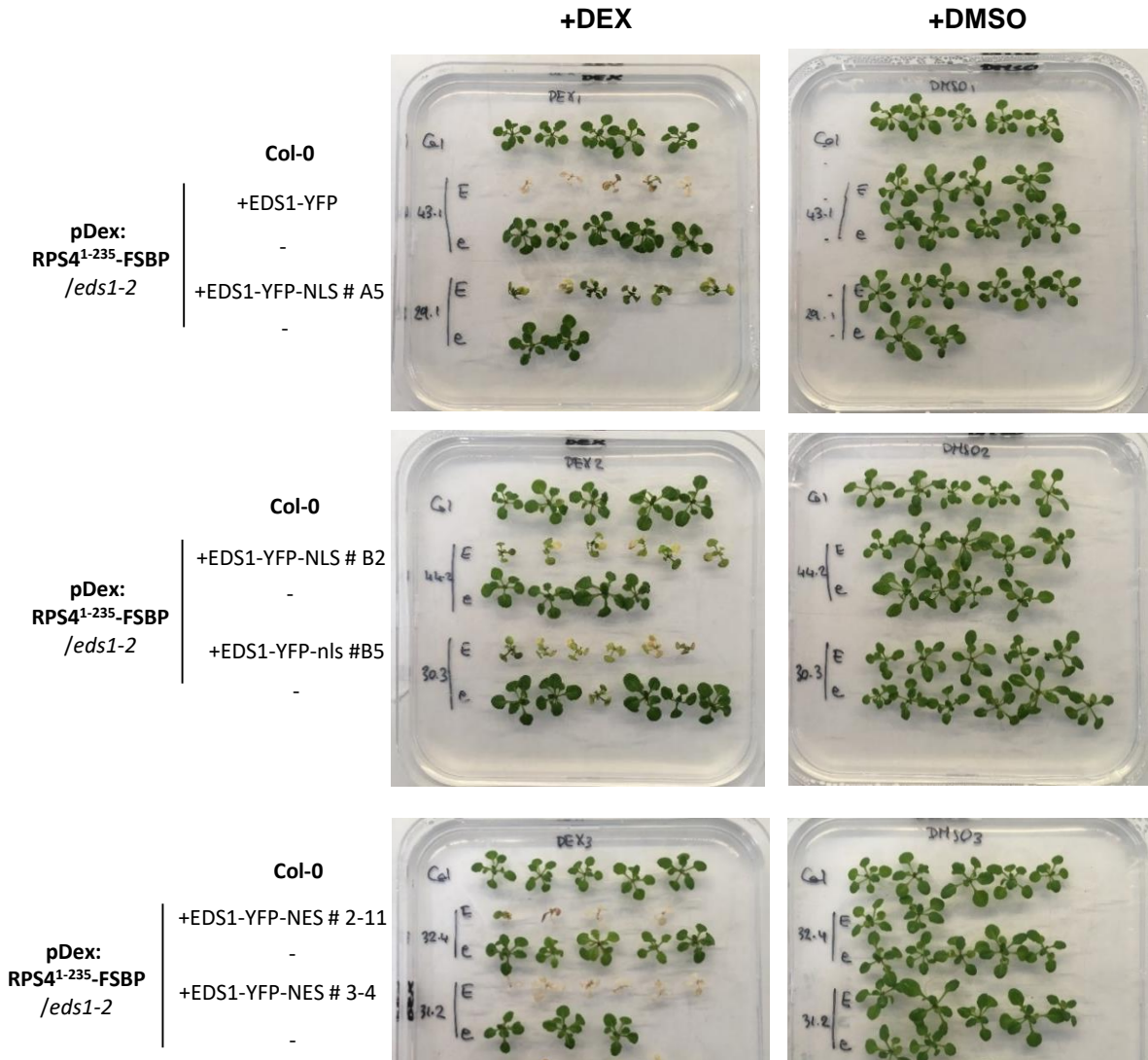


Figure S15: Subcellular localization of EDS1-YFP variants in Dex-inducible transgenic Arabidopsis seedlings (10 day-old) expressing pDex:RPS4¹⁻²³⁵ in *eds1-2* mutant background complemented with mislocalized EDS1-YFP variants. F3 individuals from each crosses (pDex: RPS4¹⁻²³⁵-FSBP x *eds1-2*) x pEDS1:EDS1-YFP mislocalized variants were PCR screened for the presence of EDS1-YFP variant (and transferred to inducing (Dex)-containing MS media. The roots of at least two seedlings were analysed by confocal microscopy. Images were taken 24h after Dex induction. Left: Fluorescence images, right: Overlay of brightfield and yellow fluorescence images. White scale represents 40µm.

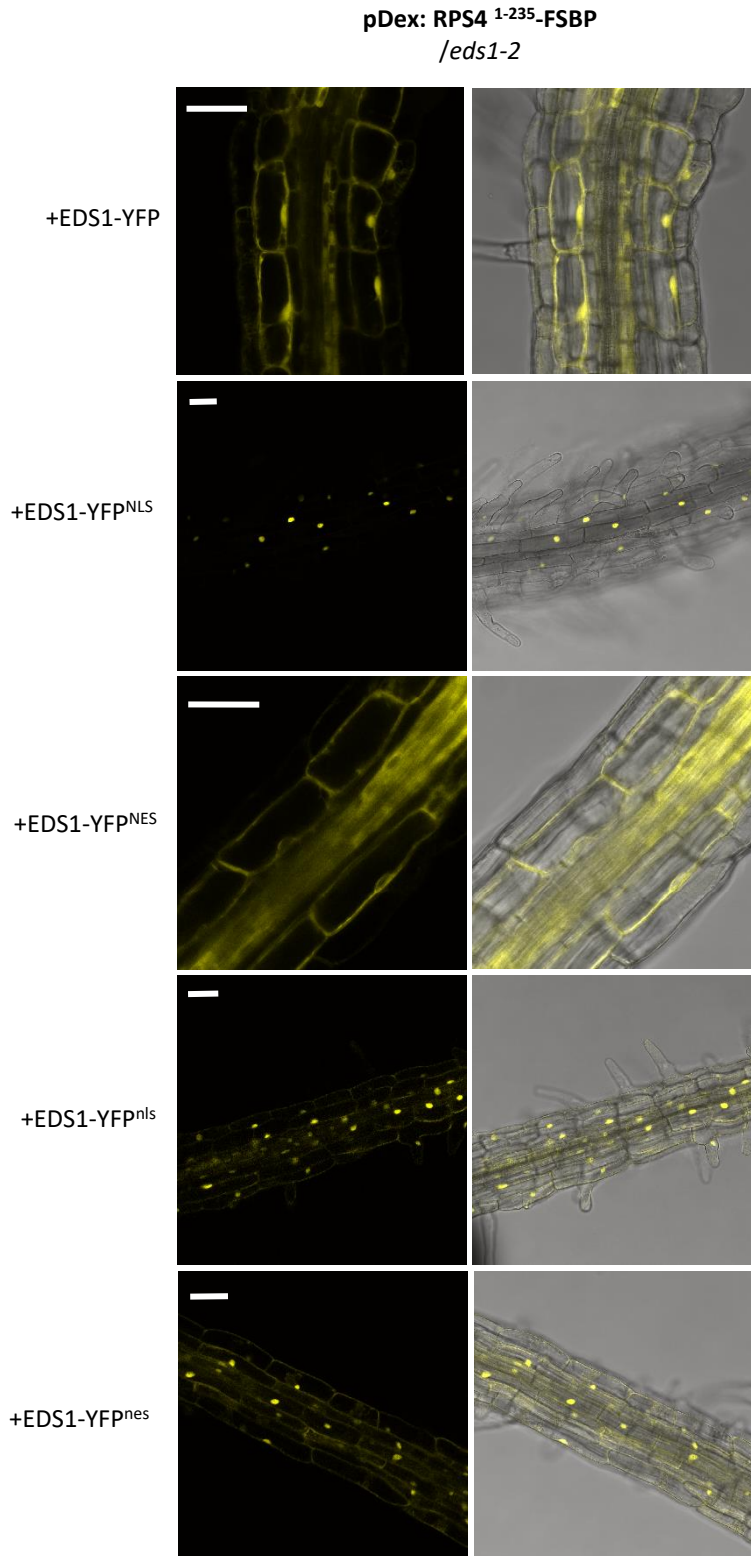


Figure S16: TIR signaling subcellular localization requirement model.

TIR domain (purple ovals) self-association leads to the production of TIR-catalysed signaling molecules (orange star), which is sensed by EDS1 family heterodimer (EDS1/PAD4 or EDS1/SAG101), which shuffle between the cytosolic and nuclear compartments. In turn, EDS1 heterodimer activation promotes interaction with RNL-type helper NLRs to activate transcriptional reprogramming in the nucleus and cell death in the cytosol via the formation of Ca²⁺ permeable channels at the plasma membrane (RNL resistosome). How Ca²⁺ signals is perceived and transduced by the cell to trigger cell death and/or immune reprogramming remains unknown.

Golgi-membrane anchored L6 can only trigger signaling from the cytosolic compartment while nucleocytoplasmic RPS4 and SNC1 TIRs can activate signaling from both cytosolic and nuclear compartments. Both type of TIRs can activate cell death and autoimmune responses when EDS1 is restricted to the cytosol but can activate immune responses but not cell death when EDS1 is sequestered in the nucleus.

Protein-protein interaction between EDS1 heterodimer and RNLs is represented as black double sensed arrows. EDS1 is depicted as filled blue half-circle and SAG101/PAD4 partner as clear blue half-circle. RNLs resistosome CC domains are depicted as green cylinders.

