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Immune signaling induced by plant Toll/interleukin-1 receptor (TIR) domains is thermostable

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Summary

Plant disease is a major threat in agriculture and climate change is predicted to intensify it. Above the optimal plant's growth range, plant immunity and in particular immune responses induced by nucleotide-binding leucine rich repeat receptors (NLRs) are dampened, but the underlying molecular mechanisms remains elusive. NLRs usually contain an N-terminal signaling domain, such as Toll/interleukin-1 receptor (TIR) domain, which is self-sufficient to trigger immune signaling. By using inducible Arabidopsis transgenic lines expressing TIR-containing NLRs (TNLs) or corresponding isolated TIR domains from Arabidopsis RPS4 and flax L6 NLRs, we showed that immune signaling induced downstream of TNL activation is not affected by an elevation of temperature. Conditional activation of TNL- and isolated TIR-mediated immune responses follow the same signaling route at permissive temperature (EDS1/RNLs requirement and activation of the salicylic acid sector). Yet, this signaling pathway is maintained under elevated temperature (30°C) when induced by isolated TIRs, but not full-length TNLs. This work underlines the need to further study how NLRs are impacted by an increase of temperature, which is particularly important to improve the resilience of plant disease resistance in a warming climate.

Keywords

Plant immunity, TIR signaling, temperature stress, NLR, RNL

1 Introduction

2

3 Climate change intensifies extreme weather conditions such as increased variation of temperature,
4 soil salinity and flooding. Such stresses make plants more vulnerable and threaten ecosystems and
5 agricultural production, which has already been impacted (1). Notably, plant disease is currently a
6 major threat for agriculture (2), and climate change is predicted to intensify crop losses due to plant
7 pathogens (3). The average global temperature is expected to keep rising over the years. Yet, plant
8 immunity is negatively impacted above the optimal temperature plant growth range (4, 5).

9 The plant immune system relies on two major types of immune receptors to fight off pathogens
10 efficiently (6). At the cell surface, pathogen-recognition receptors (PRRs) recognize microbial- or
11 damaged-associated molecular patterns (MAMPs/DAMPs). Upon ligand binding, PRRs induce a
12 response called pattern-triggered immunity (PTI). Intracellular receptors from the nucleotide-binding
13 leucine-rich repeats receptors (NLRs) family recognize microbial virulence protein effectors that are
14 secreted inside the cell, which induces effector-triggered immunity (ETI). PTI and ETI are closely
15 interconnected and mutually potentiate each other to mount a robust immune response that includes
16 calcium influxes, Reactive Oxygen Species (ROS) burst, Mitogen-activated protein kinases (MAPK)
17 activation, and a massive transcriptional reprogramming, which sometimes culminates into cell death
18 -the so-called hypersensitive response (HR)-, to locally prevent pathogens progression (7). However,
19 this sophisticated immune network can be modulated by temperature changes, by impacting global
20 immune transcriptomic remodeling (8), PRR-induced defense responses (9, 10) but also NLR-mediated
21 immune outputs, which are often dampened upon an increase of temperature (11-16). Given the
22 importance of NLRs in crop breeding programs, the negative impact of temperature elevation on NLR-
23 mediated immunity is a matter of concern in the current context of global warming. However, the
24 molecular mechanisms involved are poorly understood.

25 NLRs are modular proteins that are commonly composed of three domains. The C-terminal domain
26 has a leucine-rich repeat structure that can be involved in effector sensing. The central nucleotide-
27 binding domain can control the activation state of NLRs, depending on the nucleotide that is bound to
28 it (ADP: ON state, ATP: OFF state), (17-19). The N-terminal domain often acts as a signaling unit, which
29 is mainly found as a Toll/interleukin-1 receptor (TIR) or a coiled-coil (CC) domain (20-23). When TIR-
30 and CC-containing NLRs (termed TNLs and CNLs, respectively) sense, directly or indirectly, the presence
31 of pathogen effectors, they are qualified as sensor NLRs. A small subclade of NLRs act downstream of
32 sensor NLRs to transduce immune signaling and are therefore called helper NLRs (24). Helper NLRs
33 that display a RPW8-like N-terminal CC_R domain (RNLs) play a central role in the plant immune system

34 as they are required for immune signaling induced by multiple sensor NLRs and PRRs (25-27)(Collier
35 2011, Saile 2020, Pruitt et al. 2021).

36 In the recent years, major discoveries have shed light on NLRs structural information and TIR enzymatic
37 functions (28). Upon effector recognition, activation of Arabidopsis CNL ZAR1 and wheat CNL Sr35
38 leads to the formation of a pentameric structure called a resistosome. CNL oligomerization drives close
39 proximity of the CC domain of each protomer, creating a funnel-like structure that function as Ca²⁺
40 permeable channel at the plasma membrane, which is necessary for immune signaling activation (29-
41 31). Similarly, TNLs such as the Arabidopsis RPP1 and tobacco ROQ1 also form resistosomes upon
42 effector recognition, but with a tetrameric stoichiometry. TNL oligomerization promotes TIR enzymatic
43 NADase activity, which in turn activates the ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) family and
44 RNLs (32-35). In Arabidopsis, EDS1 forms mutually exclusive heterodimers with the other two members
45 of the family, PHYTOALEXIN DEFICIENT 4 (PAD4) or SENESCENCE-ASSOCIATED GENE101 (SAG101) (36).
46 NAD⁺-derived small molecules specifically bind to either EDS1-PAD4 and EDS1-SAG101 complex, which
47 in turn selectively associate with and activate different RNLs subgroups, ACTIVATED DISEASE
48 RESISTANCE 1 (ADR1s) or N REQUIREMENT GENE 1 (NRG1s), respectively (37-39). Upon activation,
49 RNLs form oligomeric complexes that are targeted to membranes to form cation-permeable channels
50 (40, 41). Although RNLs function redundantly, ADR1s seem to predominantly activate transcriptional
51 reprogramming, including genes involved in the salicylic acid (SA) biosynthesis pathway to induce basal
52 immunity, while NRG1s favor the activation of cell death (26, 42).

53 Interestingly, TIR domains seem to display dual enzymatic function. The nature of the catalytic site can
54 differ by using NAD⁺ as substrate or by hydrolyzing dsRNA/dsDNA to generate various NAD⁺-derived
55 small molecules or 2',3'-cAMP/cGMP, respectively (38, 39, 43). Both enzymatic activities are required
56 to activate immune signaling, although it is not known how 2',3'-cAMP/cGMP contribute to signaling
57 activation. SA plays a crucial role in basal immunity and NLR-mediated immune responses. Notably, SA
58 accumulation is promoted by EDS1 and PAD4 activity (44, 45). However, accumulating evidence
59 feature SA as a potential Achille's heel of plant immunity under elevated temperatures. At 30°C, the
60 transcript level of genes encoding central regulators of the SA biosynthesis pathway, such as
61 transcription factor CBP60g, biosynthetic enzyme isochorismate synthase 1 ICS1 but also EDS1 and
62 PAD4, is dramatically reduced or abolished in plants challenged with virulent pathogens (9, 46). NLR-
63 mediated resistance is also dampened under elevated temperature (above 28°C) (5). However, it is not
64 clear how the heat sensitivity of the SA sector may impact NLR activity or *vice versa*. An increase of
65 temperature seems to affect NLRs subcellular localization, which might disrupt their function (14, 47).
66 A thermotolerant variant of the Arabidopsis TNL SNC1 maintains its subcellular localization and can
67 propagate PAD4-dependent immune signaling, including SA marker PR1, at elevated temperature,

68 suggesting that increased temperature might alter receptor functions upstream of induced signaling
69 (14). However, the underlying mechanisms are not clearly understood.

70 As signaling units, TIR domains isolated from TNLs can induce autoimmune responses when
71 overexpressed *in planta* in absence of pathogens (20, 22, 23, 48-50) and hence, can be used as a tool
72 to study signaling events downstream of the activation of TNLs. In order to investigate the impact of
73 temperature on TNL function and signaling, we used inducible Arabidopsis transgenic lines expressing
74 TIR domains isolated from different thermosensitive TNLs. By comparing lines expressing isolated TIRs
75 and corresponding full-length TNLs at permissive (21°C) and non-permissive (30°C) temperature, we
76 showed that signaling induced by isolated TIRs is maintained under elevated temperature, and this
77 includes the main known actors involved in TNL signaling, such as EDS1, the SA sector and RNLs.
78 Altogether, our data suggest that sensor NLRs are impacted by environmental factors such as
79 temperature, whereas downstream signaling is more resilient.

80

81 **Results**

82 **Cell death induced by isolated TIR domains but not full-length TNLs is maintained under** 83 **elevated temperature**

84 When overexpressed *in planta*, TIR domains isolated from TNLs are self-sufficient to induce an immune
85 response (20, 22, 51). Hence, they represent a useful tool to investigate TNL signaling events
86 independently of effector recognition and NLR activation, by overcoming constraints that can be met
87 with full-length receptors intermolecular interactions and conformational changes. To investigate and
88 compare the impact of temperature on full-length TNL- or TIR-induced signaling, we monitored cell
89 death, a proxy for NLR-induced signaling, upon activation of either autoimmune full-length TNLs or
90 corresponding isolated TIRs, under permissive and non-permissive temperature growth conditions.
91 Under permissive temperature (18-22°C), overexpression of the full-length Arabidopsis TNL RPS4 and
92 the flax TNL L6MHV autoimmune variant (which contains an aspartate to valine substitution in the
93 MHD motif, located in the NB domain) leads to autoimmune responses and cell death in transgenic
94 Arabidopsis lines (11, 52, 53). Hence, we used inducible Arabidopsis transgenic lines expressing RPS4
95 and L6MHV, or their corresponding isolated TIR domains, RPS4^{TIR} and L6^{TIR} ((53), Table S1). All gene
96 constructs are under the control of a Dexamethasone (Dex)-inducible promoter in order to
97 conditionally activate TNL- or isolated TIR-mediated signaling, and monitor subsequent cell death.
98 They also harbor a C-terminal FSBP (3xFlag/streptavidin-binding peptide), or 3xmyc tag fusion, to
99 follow protein accumulation. At permissive temperature (21°C), Arabidopsis seedlings carrying
100 *pDex:L6MHV*, *pDex:L6^{TIR}* and *pDex:RPS4^{TIR}* constructs induced autoimmune responses from 3 to 4 days

101 upon transfer on Dex-containing media (+Dex) to reach seedling death after 7 days (Bernoux et al.
102 2023, Figure 1A). In contrast, seedlings transferred to non-inducing media (-Dex) showed no
103 phenotype and their development were similar to wild-type Col-0 seedlings (Figure 1A). Similar
104 autoimmune responses were observed in at least one independent transgenic line carrying each
105 construct, after Dex treatment (Bernoux 2023, Figure S1A,B).

106 Seedling cell death was less clear in transgenic lines carrying *pDex:RPS4*. Out of two independent lines
107 (lines #14 and #11), line #14 showed clear autoimmune responses upon seedlings Dex induction, yet
108 did not reach cell death (Figure 1A, Figure S1C). Line #11 did not show any visible symptoms upon Dex
109 induction in seedlings, which may be due to low level of protein accumulation in these conditions, even
110 at higher Dex concentration (100 μ M) (Figure S1C,D). Cell death was clearly visible on leaves of four
111 weeks-old plants of all Dex-inducible lines, 2 to 3 days after infiltration with a 20 μ M Dex solution
112 (Figure 1B, Figure S2A), including lines carrying *pDex:RPS4*, even though protein accumulation was still
113 very low for line #11 (Figure S2B). These results demonstrate that cell death signaling induced by L6^{TIR},
114 RPS4^{TIR} and L6MHV can be monitored in both seedling and adult plants, whereas cell death can only
115 be reached in adult plants grown in soil when induced by overexpression of RPS4 at 21°C.
116 Consequently, these data indicate that all generated constructs are competent for activation of
117 immune responses under permissive temperature.

118 As many NLRs, including the RRS1-R/RPS4 NLR pair, are compromised above 28°C (11, 12), we
119 compared the response of our inducible transgenic seedlings at 21°C and 30°C upon Dex induction.
120 When seedlings carrying *pDex:L6MHV* or *pDex:RPS4* were transferred at 30°C after Dex induction,
121 immune responses were strongly reduced and only displayed slight stunting phenotype, in comparison
122 to induced seedlings that remained at 21°C (Figure 1A, Figure S1B-C), supporting that both L6 and
123 RPS4-mediated immunity are compromised at elevated temperature in our assay. In contrast,
124 seedlings expressing corresponding TIR domains alone (L6^{TIR} and RPS4^{TIR}) showed cell death symptoms
125 that were similar or even stronger to what was observed at 21°C (Figure 1A). Immunoblot analyses
126 showed that L6^{TIR}, RPS4^{TIR} and corresponding full-length proteins L6MHV and RPS4 can be detected
127 from 8 hours post Dex induction (hpdi) and that protein accumulation increases progressively over the
128 time monitored (24 and 48hpdi) (Figure 1C). Protein accumulation kinetics upon Dex induction were
129 unchanged or stronger at 30°C compared to 21°C for all constructs (Figure 1C), demonstrating that the
130 inhibition of cell death phenotype in seedlings carrying *pDex:L6MHV* and *pDex:RPS4* at 30°C is not due
131 to a lack of protein accumulation. Similar phenotypes were observed in adult plant leaves infiltrated
132 with Dex at 21°C or 30°C. While leaves of lines carrying *pDex:L6^{TIR}* and *pDex:RPS4^{TIR}* showed clear cell
133 death over the infiltration area at both temperatures, lines carrying *pDex:L6MHV* or *pDex:RPS4* showed
134 no symptoms at 30°C, despite protein accumulation at 24hpdi (Figure 1B, Figure S2B). We obtained

135 similar results with independent lines carrying *pDex:L6MHV* or *pDex:RPS4* (Figure S2A, B). Inducible
136 transgenic lines expressing a TIR domain isolated from another TNL from Arabidopsis (*SNC1*), also
137 showed autoimmune symptoms that were similar or stronger at 30°C compared to 21°C (Figure S3),
138 whereas autoimmune phenotype mediated by a mutation in full-length *SNC1* (*snc1-1*) is *reverted at*
139 *28°C* (14, 54). NLRs activity is tightly controlled, notably via intra-domain interactions, which could be
140 sensitive to temperature changes. Given their simpler architecture, isolated TIRs are not subjected to
141 such negative controls, which could explain their heat stable activity. In order to test this hypothesis,
142 we generated inducible transgenic lines to express naturally occurring TIR-containing truncated TNLs
143 that were previously described to trigger an immune response when overexpressed (55), such as TN2
144 (TIR-NB with no LRR domain) and RBA1 (TIR-only protein with a C-terminal unknown domain).
145 Interestingly, induction of TN2 or RBA1 induced clear autoimmune responses at 30° C when expressed
146 in seedlings or adult plant leaves (Figure S4). Even though the function of canonical TNLs is affected by
147 an elevation of temperature, our results demonstrate that signaling induced by TIR domains is
148 thermostable.

149

150 **Enhanced expression of *EDS1* and genes involved in the salicylic acid (SA) sector is** 151 **maintained under elevated temperature when induced by isolated TIRs but not full-length** 152 **TNLs**

153 To decipher and compare the impact of temperature on TNL- and isolated TIR-induced signaling, we
154 monitored the expression of marker genes involved in TNL signaling in our inducible transgenic lines
155 expressing full-length TNLs or isolated TIRs at permissive and elevated temperature.

156 *EDS1* is a major node of plant immunity and most TNLs including *RPS4*, *SNC1* and *L6* depend on *EDS1*
157 to activate immune signaling ((36, 53, 56). *RPS4*^{TIR} and *L6*^{TIR} -induced cell death phenotype is abrogated
158 in Arabidopsis *eds1-2* mutant background, suggesting that isolated TIR-induced immune responses
159 depend on *EDS1* and follow similar signaling routes as sensor TNLs in Arabidopsis ((50, 53), Figure S5).
160 By using anti-*EDS1* antibodies, we monitored *EDS1* protein accumulation in transgenic Arabidopsis
161 seedlings upon Dex induction of full-length TNLs *L6MHV* and *RPS4* or their corresponding isolated TIR
162 domains. Interestingly, at 21°C, native *EDS1* protein accumulation was dramatically higher at 24hpd
163 in all lines, in comparison to non-induced seedlings (0hpd), and this correlated with the accumulation
164 of inducible protein constructs (full-length *L6MHV* and *RPS4* or isolated TIR domains) (Figure 1C).
165 Interestingly, at 30°C, *EDS1* increased accumulation was maintained upon *L6*^{TIR} and *RPS4*^{TIR} induction
166 in contrast to lines expressing *L6MHV* and *RPS4*, despite similar or even higher protein accumulation
167 at 30°C compared to 21°C (Figure 1C). To decipher whether lack of increased *EDS1* protein
168 accumulation at 30°C upon induction of *L6MHV* and *RPS4* was due to transcriptional or translational

169 inhibition, we analyzed by RT-qPCR the effect of elevated temperature on *EDS1* expression at 24hpd
170 in seedlings carrying full-length *pDex:L6MHV* or *pDex:RPS4*, and *pDex:L6^{TIR}* or *pDex:RPS4^{TIR}* at 21°C and
171 30°C. Consistent with the immunoblot data (Figure 1C), *EDS1* expression was upregulated at 21°C in all
172 transgenic lines in comparison to wild-type seedlings, which is in line with previous studies showing
173 that *EDS1* expression is induced in lines constitutively overexpressing RPS4 (Heidrich et al. 2013).
174 However, at 30°C, *EDS1* increased expression was abolished or significantly reduced in seedlings
175 expressing full-length TNLs L6MHV and RPS4, while it was maintained in those expressing L6^{TIR} and
176 RPS4^{TIR} (Figure 2A). These results demonstrate that *EDS1* gene expression and protein accumulation
177 are induced during both TNL- and TIR-only-mediated early immune responses, but that an increase of
178 temperature prevents *EDS1* induced expression mediated by TNLs but not by isolated TIRs. These
179 results further support that temperature elevation disrupts the function and/or properties of full-
180 length TNL receptors rather than the induced downstream signaling.

181 *EDS1* is a regulator of SA biosynthesis, which is crucial for plants to mount a robust defense (44, 45). A
182 recent study highlighted the vulnerability of the SA sector during temperature stress (46). Expression
183 of genes encoding major components of the SA biosynthetic pathway, such as *CBP60g* and *ICS1*, is
184 dramatically reduced at 28°C in Arabidopsis plants challenged with a virulent *Pseudomonas syringae*
185 bacterial strain or treated with the SA analog BTH, compared to 21°C, leading to decreased disease
186 resistance (46). These findings prompted us to test whether the expression of *CBP60g* and *ICS1* was
187 also impacted by an increase of temperature in our Dex-inducible seedling assay upon TNL or TIR-only
188 activation. RT-qPCR analyses revealed that the expression of both *CBP60g* and *ICS1* was upregulated
189 at 21°C in all transgenic lines expressing full-length TNLs or isolated TIRs, in comparison to wild-type
190 Col-0 seedlings (Figure 2B,C). Strikingly, increased expression of *CBP60g* and *ICS1* was maintained at
191 30°C in plants expressing L6^{TIR} and RPS4^{TIR}, whereas it was reduced to basal levels as in wild-type plants
192 in lines expressing full-length L6MHV or significantly reduced in lines expressing full-length RPS4, which
193 is in line with *EDS1* expression profile (Figure 2). These results show that both TNLs and isolated TIR
194 domains induce the expression of genes involved in SA biosynthesis but in contrast to full-length NLRs,
195 this expression is maintained at elevated temperature when induced by isolated TIR domains.
196 Altogether, our results further support that elevated temperature affects NLRs function but not
197 downstream signaling pathways.

198

199 **RNLs are required for signaling induced by both isolated TIR domains and full-length TNLs**

200 TNLs require RNLs to induce immune responses (57). Arabidopsis bares five RNLs that are grouped into
201 two subfamilies, ADR1s and NRG1s. ADR1 subfamily is composed of three members, ADR1, ADR1-L1

202 and ADR1-L2 (58), while NRG1 subfamily consists of two members, NRG1.1 and NRG1.2 (42, 59). In
203 Arabidopsis, ADR1s and NRG1s function downstream of effector-activated NLRs in an unequally
204 redundant manner, with some functional specificities (26, 42). For example, ADR1s mainly contribute
205 to disease resistance induced by the NLR pair RRS1/RPS4, whereas NRG1s seem to translate effector-
206 mediated cell death (26). Autoimmune phenotype induced by the *suppressor of npr1-1, constitutive 1*
207 (*snc1*) mutant depends on ADR1s, although NRG1s contribute to this phenotype to a lesser extent (42).
208 To test whether thermotolerant signaling induced by isolated TIR domains also requires helper NLRs,
209 as for full-length thermosensitive TNLs, we introduced Dex-inducible RPS4^{TIR}, SNC1^{TIR}, L6^{TIR} and L6MHV
210 constructs into RNL subgroup mutants (*adr1 triple (adr1, adr1-L1, adr1-L2)*, *nrg1 double (nrg1.1,*
211 *nrg1.2)* and the *helperless* mutant lacking the five RNLs (Saile 2020)). We next monitored cell death
212 symptoms in seedlings from at least two independent transgenic lines for each construct in each
213 genetic background upon Dex induction (Figure 3A, Figures S6-9). In lines carrying *pDex: RPS4^{TIR}*,
214 seedlings showed strong cell death symptoms in wild-type and *nrg1 double* mutant background, seven
215 days after Dex induction (Figure 3A, Figure S6A). In the *adr1 triple* mutant, autoimmune phenotype
216 was clear but attenuated compared to wild-type or *nrg1 double* mutant backgrounds, while these
217 symptoms were fully abolished in the *helperless* mutant (Figure 3A, Figure S6A). Immunoblot analyses
218 revealed that RPS4^{TIR} generally accumulated at slightly lower levels in *adr1 triple* and *helperless*
219 mutants compared to as in wild-type or *nrg1 double* mutant (Figure 3B). Because TIR-induced
220 autoimmune responses can be dose-dependent, we performed this seedling cell death assay with
221 increased Dex concentration to increase RPS4^{TIR} protein accumulation in *adr1 triple* and *helperless*
222 transgenic lines. At 50 μ M Dex, RPS4^{TIR} protein levels in *adr1 triple* and *helperless* mutants were
223 comparable to levels observed in seedlings induced at 10 μ M Dex in *nrg1 double* mutants (Figure S6B).
224 Consequently, seedlings showed a stronger autoimmune phenotype leading to cell death compared to
225 seedlings induced with 10 μ M Dex in the *adr1 triple* mutant background (line #332-6)(Figure 3A, Figure
226 S6A), suggesting a dose-dependent effect of RPS4^{TIR} -induced cell death. Overall, these results indicate
227 that both NRG1 and ADR1 families contribute to RPS4^{TIR}-mediated immunity.

228 In lines carrying *pDex:SNC1^{TIR}*, *pDex:L6^{TIR}* or *pDex:L6MHV*, seedlings showed autoimmune phenotype
229 that were similar or slightly attenuated in *nrg1 double* compared to wild-type background 7 days upon
230 Dex induction (Figure 3A). At the same time point, no visible or very mild symptoms were visible in the
231 *adr1 triple* mutant background while transgenic seedlings in the *helperless* mutant showed normal
232 development, similarly to non-transgenic wild-type seedlings (Figure 3). Similar trends were observed
233 in at least two independent transgenic lines for each construct and each mutant background (Figures
234 S7-9). Although we attempted to select transgenic lines with similar levels of TIR protein accumulation,
235 we again generally observed a lower level of TIR protein accumulation in *adr1 triple* and *helperless*

236 mutants, compared to *nrg1 double* and wild-type backgrounds, especially for L6^{TIR} (Figure 3B, Figures
237 S7,8). To boost L6^{TIR} protein production, we increased Dex concentration in our induction assay for
238 lines carrying *pDex:L6^{TIR}* in *adr1 triple* and *helperless* backgrounds. At 50 μ M Dex concentration, L6^{TIR}
239 protein accumulated at more comparable levels in *adr1 triple* (line #251-3) and *helperless* (line #312-
240 5) mutants relative to *pDex:L6^{TIR}* carrying lines in wild-type and *nrg1 double* mutant induced at 10 μ M
241 Dex. Yet, seedlings from lines in *adr1 triple* and the *helperless* mutant backgrounds still showed no
242 autoimmune phenotype 7 days after 50 μ M Dex induction, demonstrating that the lack of cell death
243 phenotype in these genetic backgrounds is not due to lower TIR protein accumulation (Figure S8). In
244 lines carrying *pDex:L6MHV*, Dex-induced expression of *L6MHV* transcripts was analyzed by RT-qPCR
245 because protein accumulation was too low for immunodetection. We also observed some differences
246 for *L6MHV* transcript levels in the different mutant backgrounds, with lower expression of *L6MHV* in
247 *adr1 triple* and *nrg1 double* compared to wild-type and *helperless* backgrounds (Figure 3C, Figure S9).
248 Yet, induction of *pDex:L6MHV* led to strong stunting phenotype in the *nrg1 double* mutant, which
249 phenotype was comparable to seedlings carrying *pDex:L6MHV* in wild-type background. In contrast,
250 no autoimmune phenotype was observed in lines carrying *pDex:L6MHV* in *adr1 triple* and *helperless*
251 mutants, as for lines expressing L6^{TIR} and SNC1^{TIR} (Figure 3A).

252 Taken together, these results show that L6^{TIR}, SNC1^{TIR}, RPS4^{TIR} and L6MHV depend on both ADR1 and
253 NRG1 RNL subgroups to induce signaling, but lack of NRG1 family has a lesser or no impact compared
254 to the ADR1 family on autoimmune and cell death symptoms induced by SNC1^{TIR}, L6^{TIR} and L6MHV.
255 Most importantly, our data show that thermotolerant isolated L6^{TIR} and thermosensitive L6MHV
256 behave similarly in the different mutant backgrounds, suggesting they share the same genetic
257 requirements, such as RNLs, to induce signaling.

258

259 **Differential temperature sensitivity of ADR1s-mediated signaling**

260 RNLs function downstream to sensor TNLs when activated by effectors (26). We showed here that the
261 ADR1 family mainly contribute to signaling induced by thermosensitive autoimmune TNL L6MHV as
262 well as thermotolerant L6^{TIR} (Figure 3), suggesting that ADR1s must be resilient to an elevation of
263 temperature, unlike sensor TNLs. To test this hypothesis, we looked at the autoimmune responses
264 induced by seedlings carrying *pDex:L6^{TIR}* in *nrg1 double* mutant background, which carry wild-type
265 *ADR1s* and show clear cell death at 21°C, under elevated temperature conditions. Interestingly, seven
266 days post-induction, seedlings carrying *pDex:L6^{TIR}* displayed clear autoimmune phenotype at 21°C and
267 30°C in both wild-type and *nrg1 double* mutant background, but not in *adr1 triple* mutant background,
268 demonstrating that ADR1s-dependent L6^{TIR}-mediated cell death is still effective at 30°C (Figure 4A).

269 In order to test whether ADR1 family members could individually induce thermotolerant immune
270 signaling, we tested whether the autoimmune phenotype of the previously characterized transgenic
271 line carrying *pADR1-L2:ADR1-L2^{DV}-HA* (60) was affected by an elevation of temperature. When grown
272 at 21°C, *pADR1-L2:ADR1-L2^{DV}-HA* carrying plants exhibit constitutive autoimmune phenotype (dwarf,
273 bushy appearance) with significantly lower dry weight compared to wild-type Col-0, as previously
274 described (60)(Figure 4B,C). Surprisingly, we observed that this phenotype was reverted when this
275 mutant was grown at 30°C, despite similar levels of protein accumulation of ADR1-L2^{DV}-HA at 21°C or
276 30°C (Figure 4B-D). Interestingly, EDS1 protein accumulation was significantly enhanced in this *pADR1-*
277 *L2:ADR1-L2^{DV}* line compared to Col-0 at 21°C (Figure 4D), demonstrating that ADR1-L2^{DV}-mediated
278 autoimmunity triggers the EDS1 pathway at permissive temperature, like TNL and isolated TIR-induced
279 signaling (Figure 1C). In contrast, EDS1 enhanced accumulation was abolished at 30°C, which supports
280 that ADR1-L2^{DV} -mediated autoimmune signaling is inhibited at 30°C and hence is thermosensitive.

281 Together, these results indicate that signaling mediated by ADR1s is thermoresilient when induced by
282 isolated *L6^{TIR}* but not by autoimmune ADR1-L2^{DV}, suggesting a possible differential temperature
283 sensitivity of ADR1 family members.

284

285 **Flg22 treatment enhances pDex:TNLs and pDex:isolated TIRs-mediated immune signaling** 286 **and protein accumulation**

287 Accumulating evidence show that PTI and ETI are tightly interconnected and potentiate each other (7).
288 One piece of evidence was that inducible expression of the bacterial effector AvrRps4 did not trigger
289 any cell death in transgenic Arabidopsis line harboring cognate TNL pair RRS1/RPS4, in absence of
290 pathogens. Instead, RRS1/RPS4-mediated specific HR was restored upon PTI eliciting and AvrRPS4
291 inducing co-treatment (61). Our data show that induction of autoimmune TNLs or isolated TIRs
292 expression is sufficient to activate immune responses and cell death in a PAMP-independent manner
293 (Figure 1). This prompted us to test whether this response could be potentiated by PTI or if the
294 signaling pathways downstream of effector recognition does not rely on PTI. For this, we monitored
295 cell death symptoms induced in leaves of adult Arabidopsis lines carrying *pDex:L6^{TIR}* or *pDex:L6MHV*,
296 in presence or absence of the PTI elicitor flg22 (Figure 5A). Upon treatment of *pDex:L6^{TIR}* and
297 *pDex:L6MHV* lines with mock (DMSO) or flg22 only, infiltrated leaves showed negligible or no
298 symptoms at 2, 3 and 6 days after infiltration, probably due to wounding during infiltration. Dex
299 treatment induced very mild chlorosis two days after infiltration in both *pDex:L6^{TIR}* and *pDex:L6MHV*
300 lines. In contrast, most leaves co-treated with Dex and flg22 (Dex+flg22) developed clear chlorotic
301 symptoms from two days after treatment and the difference between Dex only or Dex+flg22 treatment

302 became even clearer at 3 days post treatment, suggesting that cell death induced by full-length L6MHV
303 and isolated L6^{TIR} is accelerated and could be potentiated by PTI (Figure 5A, Figure S10). At 6 days post-
304 treatment, leaves treated with Dex or Dex+flg22 showed, in average, similar level of cell death in all
305 lines. Similar results were observed on at least one independent inducible line carrying *pDex:L6MHV-*
306 *FSBP (#18)* (Figure S10). Immunoblot analyses showed incremental protein accumulation of both L6^{TIR}
307 and L6MHV at 24h and 48h upon Dex and Dex+flg22 treatments (Figure 5B), as observed in Figure 1C.
308 More surprisingly, we repeatedly observed higher protein accumulation of both L6^{TIR} and L6MHV
309 proteins upon Dex+flg22 co-treatment, compared to Dex-only treatment. However, EDS1 increased
310 accumulation mediated by L6^{TIR} and L6MHV Dex induction remained steady between Dex or Dex+flg22
311 treatments, suggesting that Dex+flg22 co-treatment potentiate L6^{TIR} and L6MHV protein production
312 but not EDS1 increased accumulation. We obtained similar results with *pDex:RPS4^{TIR}* line #F and two
313 independent lines carrying *pDex:RPS4-FSBP* (lines #11 and 14) (Figure S11). Dex+flg22 co-treatment
314 also induced earlier chlorosis/cell death symptoms compared to Dex alone treatment. RPS4^{TIR} and
315 RPS4 full-length protein accumulation was also enhanced in Dex+flg22 compared to Dex alone
316 treatment, while EDS1 accumulation kept steady (Figure S11).

317 Based on these results, we could not conclude whether flg22 treatment potentiates TIR accumulation
318 and hence TIR signaling, or if it has a general impact on the activation of our Dex-inducible system,
319 thus enhancing transcription and protein accumulation and leading to a dose-effect enhanced cell
320 death phenotype. To test this, we used as a control an inducible Arabidopsis line carrying a gene
321 construct that is not involved in immunity (*pDex:GFP-MiniTurbo-flag*). Turbo is a biotin ligase that is
322 used for proximity labelling approaches (62). 24h after treatment, plants carrying *pDex:L6^{TIR}* or
323 *pDex:GFP-MiniTurbo* accumulated increased level of L6^{TIR} and GFP-MiniTurbo protein, respectively, in
324 leaves treated with Dex+flg22 compared to Dex only treatment (Figure 5C). This demonstrates that
325 flg22 boosts gene expression in our Dex-inducible vector but not native gene expression/protein
326 accumulation such as EDS1 (Figure 5B), and raises caution about data interpretation on PTI/ETI
327 potentiation when using Dex+flg22 co-treatment. As TIR signaling seems to be dose dependent, it is
328 likely that the enhanced cell death phenotype observed upon Dex+flg22 treatment compared to Dex
329 only is due to increased protein production by flg22 instead of a potentiation of TIR signaling by PTI.

330

331 Discussion

332 NLR activity can be dampened under elevated temperatures, which is concerning in the current
333 climatic context. This observation was previously reported for several TNLs, including Arabidopsis SNC1
334 or RPS4/RRS1 used in this study (11, 12, 14), but the underlying molecular mechanisms remain poorly
335 understood. We showed that immune responses induced in Arabidopsis by different TIR domains

336 isolated from RPS4, SNC1 and flax L6, are maintained at elevated temperatures, unlike those induced
337 by full-length TNLs, suggesting that a raise of temperature impacts TNL receptor functions but not
338 downstream immune signaling.

339 As signaling units, TIR domains can autonomously induce cell death when overexpressed in absence of
340 pathogens. At 30°C, a non-permissive temperature for most NLRs, we observed that Arabidopsis
341 seedlings expressing RPS4^{TIR}, L6^{TIR} and SNC1^{TIR} displayed clear cell death symptoms, which were similar
342 or even stronger than at permissive temperature (21°C), in contrast to autoimmune transgenic lines
343 expressing full-length RPS4 or autoimmune variant L6MHV. To validate this finding, we showed that
344 thermotolerant signaling induced by isolated TIRs share the same signaling components as
345 thermosensitive full-length TNLs. Both isolated TIRs and full-length TNLs promote the accumulation of
346 EDS1 protein and gene expression of major regulators of the SA pathway, such as *CBP60g* and *ICS1*,
347 and require RNLs to trigger signaling. However, EDS1 accumulation and upregulation of *CBP60g* and
348 *ICS1* gene expression is maintained at 30°C in seedlings expressing isolated TIRs but not full-length
349 NLRs, supporting that immune responses induced downstream of TNLs, including the SA pathway, are
350 not affected by an elevation of temperature. This result contrasts with earlier studies reporting that
351 the expression of *CBP60g*, *ICS1* and other SA-related genes was strongly reduced in immune-triggered
352 plants above 28°C (9, 46). In the study of Kim et al., the authors showed that elevated temperatures
353 inhibit the SA pathway at its onset, by reducing the number of GUANYLATE BINDING PROTEIN-LIKE 3
354 (GBPL3) defence-activated biomolecular condensates (GDACs). Consequently, this impairs the
355 recruitment of GBPL3 and associated transcriptional coactivators to the promotor region of *CBP60g*
356 and *SARD1*, which dramatically reduce their expression at 28°C. *CBP60g* and *SARD1* are master
357 transcription factors that regulate the expression of genes involved in SA biosynthesis, such as *ICS1*,
358 which expression is hence turned off under elevated temperature growth conditions (46).
359 Interestingly, overexpressing *CBP60g* or the EDS1/PAD4 complex is sufficient to recover SA signaling
360 and to maintain resistance to virulent and avirulent bacteria at elevated temperature (45, 46). Hence,
361 overexpression of immune regulators involved in the SA pathway can bypass the negative impact of
362 temperature on GDAC formation and on plant immunity. Given that the expression of *CBP60g* and *ICS1*
363 is dramatically reduced at 30°C upon activation of thermosensitive NLRs, but not when isolated TIR
364 domains are overexpressed, our results demonstrate that activating downstream TIR signaling is also
365 sufficient to bypass the negative impact of temperature on the SA sector. It also supports that the
366 activation status of full-length receptors is negatively impacted by warmer temperature (above 28°C)
367 whereas downstream signaling, including the SA pathway, is thermostable. Hence, it is possible that
368 reduced expression of *CBP60g* and *ICS1* may be controlled by NLR activation status rather than by
369 temperature directly. Alternatively, TNL signaling may activate the expression of *CBP60g* in a GDAC-

370 independent manner. NLRs, and in particular TNLs, widely contribute to different levels of plant
371 immunity. While NLRs were first identified as effector receptors to trigger ETI, they also contribute to
372 amplify PRR-mediated immunity (7, 63, 64). For example, global disruption of NLR homeostasis
373 dramatically reduce PTI (63). Hence, if NLRs function upstream of GDAC formation, temperature
374 vulnerability of the SA pathway may be explained by a collective inactivation of thermosensitive NLRs
375 under elevated temperature. It would be interesting to monitor the formation of GDACs as well as the
376 recruitment of GBPL3 and coactivators to the *CBP60g* promoter upon expression of isolated TIR
377 domains or full-length NLRs to decipher whether TNL-induced expression of *CBP60g* is GDAC-
378 dependent or -independent, especially under elevated temperature.

379 All TNLs characterized so far require RNLs to induce immune signaling. RNLs function redundantly and
380 contribute differentially to cell death or basal defense and gene reprogramming (26, 42, 65). By using
381 Arabidopsis lines expressing isolated TIR domains in different RNLs subgroup mutant backgrounds, we
382 showed that immune signaling induced by isolated TIR domains depends on RNLs, just as effector-
383 dependent or -independent full-length TNL-induced signaling. Here, we showed by monitoring
384 macroscopic cell death, that L6^{TIR} and SNC1^{TIR} signaling mainly depend on the ADR1 family to translate
385 cell death, which is consistent with previously reported requirement of ADR1s for *snc1* autoimmune
386 phenotype (42). For RPS4^{TIR}-mediated immune responses, each RNL subgroup seems to compensate
387 for the absence of the other as RPS4^{TIR} -mediated autoimmunity (severe stunting to cell death) was
388 still visible in both *nrg1 double* and *adr1 triple* mutants, whereas it was fully abolished in the quintuple
389 *helperless* mutant. These results slightly differ from previous studies, in which RPS4/RRS1-mediated
390 cell death requires NRG1s whereas the ADR1 family supports disease resistance and pathogen
391 multiplication restriction (26). Importantly, our results show that thermotolerant flax L6^{TIR} share the
392 same genetic requirements as the corresponding thermosensitive full-length receptor L6MHV, further
393 supporting that this shared pathway is maintained under elevated temperature only when induced by
394 isolated TIR and not by full-length TNL. These data also suggest ADR1s-mediated signaling is
395 thermostable when activated by isolated TIRs. Indeed, we showed that L6^{TIR} can still induce immune
396 signaling in *nrg1 double* mutant at 30°C but not in *adr1 triple* mutant, supporting our primary
397 hypothesis that ADR1s can translate TIR signaling at elevated temperature. However, the autoimmune
398 phenotype mediated by one of the ADR1 family member in the *pADR1:ADR1-L2^{DV}-HA* line at 21°C was
399 reverted at 30°C, suggesting that ADR1-L2^{DV} is thermosensitive. Interestingly, EDS1 enhanced protein
400 accumulation, which correlates with this autoimmune phenotype, is also dramatically reduced at 30°C
401 in this line. This is consistent with previous studies showing that *pADR1:ADR1-L2^{DV}-HA*-mediated
402 autoimmunity genetically requires *EDS1*, possibly to positively regulate SA accumulation via a feedback
403 loop (60, 66), which seems to be inhibited at 30°C (this study). The underlying mechanisms will need

404 to be further investigated to fully understand the differential temperature sensitivity of ADR1s.
405 Another intriguing question is whether ADR1 family members have differential sensitivity to elevated
406 temperature or whether *ADR1-L2^{DV}-HA* autoimmune variant activates a separate pathway that is
407 thermosensitive. ADR1-L2^{DV}-mediated autoimmunity signals through TNL *SADR1* (Suppressor of *ADR1-*
408 *L2 1*), which is dispensable for signaling induced by other TNLs (66). It remains to be determined
409 whether, as for many full-length TNLs, *SADR1* function is inhibited at 30°C too. Alternatively, it is
410 possible that activation of ADR1s in the pDex: L6^{TIR}/*nrg1 double* line may induce a *SADR1*-independent
411 pathway, which is resilient to elevated temperature, explaining why we could still observe cell death
412 at 30°C.

413 Overall, our results point towards the thermoresilience of immune signaling induced by isolated TIR
414 domains, in contrast to corresponding full-length TNLs, which may be explained by a simpler
415 architecture of isolated TIRs. Naturally-occurring truncated NLRs, such as, lack some of the canonical
416 NLR domains (LRR and/or NB domain) but contain a TIR domain. TIR-only or TIR-NB coding genes are
417 widely represented in Arabidopsis genome and massive transcriptional induction of these genes upon
418 immune stimuli indicate they are broadly involved in plant immunity (55, 67-69). Interestingly, we
419 found that two previously described TIR-containing truncated TNLs (*RBA1* and *TN2*) induce
420 thermostable immune signaling. Hence, it will be of great interest to further characterize the function
421 of such non canonical TIR-containing proteins and assess whether they can induce thermotolerant
422 disease resistance. Given that NLRs are broadly used in crop breeding programs for disease resistance,
423 this work underlines the need to further investigate how the function of TNLs or more generally NLRs
424 is impacted by temperature stress. Canonical sensor TNLs are modular proteins which activation is
425 finely regulated via intra- and inter-domain interactions as well as interactions with chaperones
426 proteins (18, 70, 71). Therefore, it is likely that such complex mode of regulation may be affected by
427 environmental changes such as temperature rise, which in turn may turn off TNL-induced enzymatic
428 activities and downstream signaling.

429 Several studies have suggested that NLRs could function at the interface between biotic and abiotic
430 stress responses (72-74), pointing to a role as mediators of growth-defense trade-offs. Hence, it will
431 be important in the future to further investigate how NLRs function is modulated by different abiotic
432 stresses.

433

434 **Limitations of the study**

- 435 • By using inducible transgenic lines expressing autoimmune TNLs or isolated TIRs, this study allowed
436 us to bypass effector recognition to focus on TIR-activated downstream signaling. However, this

437 system revealed some limitations as the signaling output monitored (cell death) is dose-dependent.
438 To deepen our understanding on the impact of temperature stress and the differential requirement
439 of RNL family members for TIR signaling, further RNA-seq analyses would be necessary to obtain a
440 full picture of the plant response in these conditions.

- 441 • Our Dex-inducible vector is sensitized by PTI elicitor flg22. This allowed us to raise attention to the
442 interpretations PTI/ETI interconnections studies when using such system. Hence, it prevented us to
443 conclude on whether PTI may potentiate effector-independent TIR signaling.
- 444 • Our data were obtained upon a single temperature stress in controlled conditions. It will be
445 important in the future to further explore the plant immune response upon combined biotic and
446 abiotic stresses (temperature, drought, CO₂ concentration...), to better mimic predicted climate
447 change conditions.

448

449 **METHODS DETAILS**

450 **Plasmid constructions**

451 All plasmid constructs used for Arabidopsis stable transformation were generated by Gateway cloning
452 (GWY, Invitrogen). PCR products flanked by attB sites were recombined into pDONR207 (Invitrogen)
453 and then into dexamethasone inducible pOpOff2-derived destination vectors pDex:GWY-FSBP,
454 pDex:GWY-YFPv or pDex:GWY-MiniTurbo-3Flag (53, 75). Generation of pDex:GWY-FSBP was
455 previously described in (53). The FSBP tag consist of a triple flag epitope (F) fused with a streptavidin-
456 binding peptide (SBP) (53). pDex:GWY-YFPv or pDex:GWY-MiniTurbo-3Flag were modified from
457 pOpOff2(Kan) GWY vector, which was kindly provided by Chris Helliwell (CSIRO, Canberra). This vector
458 was lacking the hairpin GWY cassette (as described in (76)) and instead contained a simple GWY
459 cassette but no terminator. Hence, a PCR fragment containing either the DNA sequence of the yellow
460 fluorescent protein venus (YFPv)(77) or the MiniTurbo (62) with an in frame triple flag epitope
461 sequence, followed by the 35s terminator sequence, flanked with *KpnI/Pme1* restriction sites, were
462 used to build pDex:GWY-YFP or pDex:GWY-MiniTurbo-3Flag by restriction/ligation using
463 pOpOff2(Kan)-GWY as the vector backbone. All plasmids and constructs were verified by sequencing.
464 All plasmids, constructs and primers used in this study are listed in Tables S2 and S3.

465 **Plant growth conditions**

466 Arabidopsis seeds were sown on MS media supplemented with antibiotics when needed, for
467 transgenic lines selection. After 24h vernalization, MS plates were transferred in growth chambers
468 under 16h photoperiod, 21°C conditions for eight to ten days. For seedling assays, eight to ten day-old
469 seedlings were transferred to regulated climatic chambers (Memmert[®]) set at 21°C or 30°C, under 8h
470 photoperiod, after Dexamethasone treatment (see details in Dex-induction section below). For adult

471 plant assays, eight day-old seedlings were transferred on soil pots (Jiffy®) and grown for two to three
472 weeks in growth chambers under 8h photoperiod conditions at 21°C, and transferred to climatic
473 chambers (Memmert®) set at 21°C or 30°C, under 8h photoperiod, after treatment (dexamethasone
474 or flg22, see details in Dex-induction section below).

475 **Plant material and transgenic lines screening**

476 *Arabidopsis* Col-0, and mutants in Col-0 background (*eds1-2* (78) or *RNL* mutants (26), were
477 transformed by floral-spraying or floral-dipping using *Agrobacterium tumefaciens* strains carrying
478 *pDex:TIRs* or *pDex:TNLs* constructs. Primary transformants were selected on MS media supplemented
479 with hygromycin (50 µg/ml) and were individually PCR genotyped to verify the presence of the
480 transgene or mutations in *RNLs* by using Thermo Scientific™ Phire plant direct PCR kit as previously
481 described (53). Used primers for PCR genotyping are listed in Table S3. Western-blot analyses were
482 further performed on F2 progenies to verify protein accumulation of TIR or TNL constructs, 24h after
483 dexamethasone induction of 8 day-old seedlings. *pADR1:ADR1-L2^{DV}-HA* line (60) was kindly provided
484 by F. El Kasmi. All transgenic lines used in this study are listed in Table S1. For dry weight measurement,
485 plants were individually harvested, dried in paper pockets at 37°C and weighed 3 days after.

486 **Cell death assays upon dexamethasone induction and/or flg22 treatment**

487 For seedling assays, seven to ten day-old seedlings were transferred on MS media plates supplemented
488 with Dexamethasone (10µM) or DMSO (equivalent volume as Dexamethasone) and transferred to
489 regulated climatic chambers (Memmert®) set at 21°C or 30°C, under 8h photoperiod.

490 For adult plants assays, 8 day-old seedlings were transferred on soil pots (Jiffy®) and grown for two to
491 three weeks in a growth chamber under 8h photoperiod conditions at 22°C. Three to five leaves of
492 three to five week-old plants were infiltrated with a needle-less syringe-with solutions containing 20
493 µM Dexamethasone or mock solution (equivalent volume of DMSO as Dexamethasone), a combination
494 of flg22 peptide (100nM) and DMSO; or a combination of 20 µM Dexamethasone and 100nM flg22
495 solutions. For temperature assays, plants were transferred to regulated climatic chambers
496 (Memmert®) set at 21°C or 30°C, under 8h photoperiod upon Dex treatment. The apparition of cell
497 death symptoms was monitored every day.

498 **Immunoblot analyses**

499 For seedling assays, total protein extraction was performed on 10 eight day-old seedlings collected at
500 different time points after Dexamethasone treatment. For adult plant assays, three or five 6mm leaf
501 discs were harvested from three to five different plants per line carrying *pDex:RPS4^{TIR}* or *pDex:L6^{TIR}*, or
502 lines carrying *pDex:RPS4* or *pDex:L6MHV*, respectively, 24h and 48h after treatment. For *ADR1-L2^{DV}*-

503 HA detection, 7 leaf discs (diameter 1cm) were collected from 3 week-old plants grown at 21°C or 30°C.
504 Plant material expressing TIR domains were ground and directly resuspended in 100µl of loading
505 Laemmli buffer. Plant material expressing TNLs or RNLs were ground and resuspended in 100µl
506 extraction buffer [150mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, NP-40 1%, Plant protease inhibitor
507 cocktail (SIGMA) 1%, 10 mM DTT, 10 µM MG132], centrifuged for 5min at 4°C to remove cell debris
508 and 50µl of the supernatant was combined with 50µl loading Laemmli buffer (0.125 M Tris HCl PH7.5,
509 4% SDS, 20% Glycerol, 0.2M DTT, 0.02% Bromophenol blue) for western-blot analysis. After
510 denaturation at 95°C for three minutes, samples were then loaded on sodium dodecyl sulfate
511 polyacrylamide gels for electrophoresis and transferred to nitrocellulose membranes which were
512 blocked with 5% skimmed milk. Membranes were stained using Ponceau red staining to verify total
513 protein loading. Membranes were further probed with anti-flag-peroxidase (HRP) (Sigma,
514 SAB4200119) (dilution ratio: 1/5000), anti-Myc-peroxidase (clone 9E10; Roche) (dilution ratio:
515 1/5000), or rabbit *anti-EDS1* (kindly provided by J. E. Parker) (dilution ratio: 1/500) followed by
516 incubation with a secondary goat anti-rabbit antibodies (Bio-Rad) conjugated to peroxidase (dilution
517 ratio: 1/10000). Protein detection was performed using Bio-Rad Chemidoc™ Imaging system with the
518 Bio-Rad Clarity Western ECL Substrate or Bio-Rad Clarity Max Western ECL Substrate.

519 **RNA extraction and qPCR analyses**

520 Total RNA extraction was performed on 10 eight day-old seedlings or three 6mm leaf discs from three
521 different four week-old plants at 24h and 48h after treatment. Total RNA was extracted using the
522 Macherey Nagel Nucleospin RNA Plus kit following manufacturer's instruction. Total RNA was then
523 subjected to DNase treatment according to the Invitrogen™ Ambion™ TURBO DNA-free Kit
524 instructions. 1µg total RNA was used for reverse transcriptase reactions. RT-qPCR reaction mix was
525 prepared with SYBR green (Takyon™ No ROX SYBR 2X MasterMix blue dTTP) and run on Bio-Rad CFX
526 Opus 384 Real-Time PCR instrument. Used primers are listed in Table S3.

527 **Quantification and statistical analysis**

528 For RT-qPCR data, mean ΔCt were calculated from three technical replicates from two to four
529 biological replicates (as indicated in figure legends). *At1G13320* and *At5G15710* were used as internal
530 control genes. $\log_2(2^{-\Delta Ct})$ values were used to determine the significance of difference in gene
531 expression between the different tested conditions, and were represented as boxplots using R studio.
532 A two-ways ANOVA was used when the distribution of the data fitted assumptions of normality and
533 homogeneity of variances, and was followed by a post-hoc pairwise comparison Tukey test.

534

535

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544

545 Author contributions

546 HD and MB designed and performed experiments. CB generated transgenic plant material. HD, LD,
547 and MB analyzed data. HD and MB wrote the manuscript. HD, LD, and MB edited the manuscript.

548

549 Declaration of interests

550 The authors declare no competing interests.

551

552 Figures titles and legends

553 **Figure 1. Effect of elevated temperature on cell death induced by TNLs or isolated TIRs in**
554 **Arabidopsis.** (A-B) Cell death phenotype observed on seedlings (A) or adult plant leaves (B) of
555 transgenic inducible lines carrying *pDex:L6^{TIR}-FSBP* (line #4), *pDex:L6MHV-myc* (line #2), *pDex:RPS4^{TIR}-*
556 *FSBP* (line #F) and *pDex:RPS4-FSBP* (line #14) after Dex induction. (A) Ten day-old seedling were
557 transferred to 10µM Dex-containing (+Dex) MS media or non-inducing media (DMSO-containing, -Dex)
558 and moved to 21°C or 30°C climatic chambers. Wild-type Col-0 was used as a negative control. Photos
559 were taken seven days after Dex induction. Black scale bars represent 1,5 cm. (B) Leaves of 4 week-old
560 plants were infiltrated with 20 µM Dex (+Dex) or DMSO (-Dex) solutions and moved to 21°C or 30°C
561 climatic chambers. Photos were taken seven days after Dex induction. (C) Immunoblot analysis of L6^{TIR}-
562 FSBP, L6MHV-myc, RPS4^{TIR}-FSBP, RPS4-FSBP and native EDS1 proteins over a 48h time-course post Dex
563 induction (hpdi) of ten day-old seedlings at 21°C or 30°C, using anti-flag, anti-myc and anti-EDS1
564 antibodies detection. Total protein load is indicated by red Ponceau staining. Equal amount of protein
565 extract were loaded for each timepoint to detect transgenic protein construct and EDS1.

566 **Figure 2. Effect of elevated temperature on the expression of genes involved in the SA pathway**
567 **induced by TNLs or isolated TIRs.** (A-C) RT-qPCR analysis of *EDS1* (A), *CBP60g* (B) and *ICS1* (C) gene

568 expression level in wild-type (Col-0) or transgenic Arabidopsis seedlings carrying *pDex:L6^{TIR}-FSBP* (line
569 #4), *pDex:L6MHV-myc* (line #2) and *pDex:RPS4^{TIR}-FSBP* (line #F) or *pDex:RPS4-FSBP* (line #14) at 21 or
570 30°C. Seedlings were collected at time 0 and 24 hours after transfer on 10µM Dex- containing medium
571 (hpdi: hours post Dex induction). Gene expression was normalized relatively to the expression of two
572 housekeeping genes (*At1G13320* and *At5G15710*). Box plots represent values obtained from three
573 independent biological replicates which are represented by black dots. Statistical differences were
574 assessed with two-ways variance analysis (ANOVA) followed by a Tukey's HSD multiple comparison
575 test. Data points with different letters indicate significant differences ($P < 0.05$).

576 **Figure 3. RNLs genetic requirements for signaling induced by TNLs or isolated TIRs.** (A) Cell death
577 phenotype observed on seedlings of transgenic inducible lines carrying *pDex:RPS4^{TIR}-FSBP*,
578 *pDex:SNC1^{TIR}-FSBP*, *pDex:L6^{TIR}-FSBP*, or *pDex:L6MHV-FSBP*, in wild-type (wt) or in *RNLs* mutant
579 backgrounds (*nrg1 double*, *adr1 triple* or *helperless*), after Dex induction. Ten day-old seedling were
580 transferred to 10µM Dex-containing (+Dex) MS media or non-inducing media (DMSO-containing, -
581 Dex). Untransformed wild-type Col-0 was used as a negative control. Photos were taken seven days
582 after transfer. (B) Immunoblot analysis of *RPS4^{TIR}-FSBP*, *SNC1^{TIR}-FSBP* and *L6^{TIR}-FSBP* in wild-type or in
583 *RNLs* mutant backgrounds (*nrg1 double*, *adr1 triple* or *helperless*), 24 hours post Dex induction using
584 anti-flag antibodies detection. Total protein load is indicated by red Ponceau staining. Equal amount
585 of protein extract was loaded for each sample. Background noise is indicated by an asterisk. (C) RT-
586 qPCR analysis of *L6MHV-FSBP* transgene expression in wild-type or *RNLs* mutant backgrounds.
587 Untransformed wild-type Col-0 was used as a negative control. *L6MHV-FSBP* expression was
588 normalized relatively to the expression of two housekeeping genes (*At1G13320* and *At5G15710*). Bar
589 plots represent means +/-SEM obtained from two or three independent biological replicates which are
590 represented by black dots.

591 **Figure 4. Differential sensitivity of ADR1s-induced signaling to elevated temperature.** (A) Cell death
592 phenotype of seedlings carrying *pDex :L6^{TIR}* in wild-type, *nrg1 double* or *adr1 triple* mutants, seven days
593 after transfer on a 10 µM Dex medium at 21°C or 30°C. Untransformed wild-type Col-0 was used as a
594 negative control. *pDex :L6MHV-myc line#2* was used as a thermosensitive control. Photos were taken
595 seven days after Dex induction. Black scale bars represent 1 cm. (B) Representative phenotype of three
596 week-old Arabidopsis plant carrying *pADR1-L2:ADR1-L2^{DV}-HA* compared to wild-type Col-0 at 21° or
597 30°C. After germination on MS media, seven day-old seedlings were acclimated for approximately
598 three days on soil pots at 21°C, then transferred and grown for two weeks in climatic chambers set at
599 21°C or 30°C. (C) Percentage of dry weight ratio of *pADR1-L2:ADR1-L2^{DV}-HA* compared to Col-0 plants
600 at 21°C or 30°C as in panel A. Bar plots represent the percentage of mean dry weight ratios of plants
601 carrying *pADR1-L2:ADR1-L2^{DV}-HA* / wild-type Col-0 +/- SEM. Individual percentage ratios are

602 represented by black dots (n>8). This experiment was performed twice with similar results. (D)
603 Immunoblot analysis of ADR1-L2^{DV}-HA and EDS1 in three week-old transgenics carrying *pADR1-*
604 *L2:ADR1-L2^{DV}-HA* or wild-type Col-0, grown at 21°C or 30°C, using anti-HA and anti-EDS1 antibodies
605 detection, respectively. Total protein load is indicated by Ponceau red staining.

606 **Figure 5. Effect of flg22 treatment on L6^{TIR}- and L6MHV-mediated signaling.** (A) Representative cell
607 death symptoms observed in leaves of four week-old Arabidopsis transgenics carrying *pDex:L6^{TIR}-FSBP*
608 or *pDex:L6MHV-myc*, infiltrated with a solution of DMSO (“mock”), 100 nM flg22 (“+flg22”), 20 μM Dex
609 (“+Dex”) or a co-treatment of both Dex 20 μM and flg22 100 nM (“+Dex + flg22”). Pictures were taken
610 3 and 6 days after treatment. (B) Immunoblot analysis of L6^{TIR}-FSBP, L6MHV-myc and EDS1 in leaves
611 of four week-old Arabidopsis transgenics carrying *pDex:L6^{TIR}-FSBP* or *pDex:L6MHV-myc*, 24h and 48h
612 post treatment (hpt), using anti-flag, anti-myc or anti-EDS1 antibodies detection, respectively. Three
613 discs from three different plants were collected for L6^{TIR}-FSBP detection and five discs from five plants
614 were collected for L6MHV-myc detection. (C) Immunoblot analysis of L6^{TIR}-FSBP and GFP-MiniTurbo-
615 Flag in leaves of four week-old Arabidopsis carrying *pDex:L6^{TIR}-FSBP* or *pDex:GFP-Mini-Flag* 24h post
616 treatment, using anti-flag antibodies detection. Background noise is indicated by an asterisk. (B-C)
617 Total protein amount was verified with Ponceau red staining. Immunoblot analyses were performed
618 at least twice with similar results.

619

620 **Supplementary data**

621 **Supplementary figures titles and legends**

622 Figure S1: Dex-induced seedling cell death phenotype and protein accumulation of independent
623 transgenic lines used in this study.

624 Figure S2. Effect of temperature on Dex-induced cell death phenotype and protein accumulation in
625 leaves of independent transgenic lines used in this study.

626 Figure S3. SNC1^{TIR} induces autoimmune symptoms at 21°C and 30°C.

627 Figure S4. Naturally occurring TIR-containing truncated TNL proteins induce autoimmune symptoms at
628 30°C.

629 Figure S5: L6^{TIR}-mediated immunity depends on EDS1 in Arabidopsis.

630 Figure S6. Seedling phenotype and TIR protein accumulation in independent Arabidopsis lines carrying
631 *pDex:RPS4^{TIR}-FSBP* in different RNL mutant backgrounds (*nrg1 double*, *adr1 triple*, *helperless*).

632 Figure S7. Seedling phenotype and TIR protein accumulation in independent Arabidopsis lines carrying
633 *pDex:SNC1^{TIR}-FSBP* in different RNL mutant backgrounds (*nrg1 double*, *adr1 triple*, *helperless*).

634 Figure S8. Seedling phenotype and TIR protein accumulation in independent Arabidopsis lines carrying
635 *pDex:L6^{TIR}-FSBP* in different RNL mutant backgrounds (*nrg1 double*, *adr1 triple*, *helperless*).

636 Figure S9. Seedling phenotype and *L6MHV* transcript levels in independent Arabidopsis lines carrying
637 *pDex:L6MHV-FSBP* in different RNL mutant backgrounds (*nrg1 double*, *adr1 triple*, *helperless*).

638 Figure S10. Effect of flg22 co-treatment on Dex-induced cell death in independent Arabidopsis lines
639 carrying *pDex:L6MHV-myc* or *pDex:L6MHV-FSBP*.

640 Figure S11. Effect of flg22 co-treatment on Dex-induced cell death in Arabidopsis lines carrying
641 *pDex:RPS4^{TIR}-FSBP* and *pDex:RPS4-FSBP*.

642

643 **Supplemental information**

644 Table S1: List of transgenic lines used in this study

645 Table S2: List of constructs used in this study

646 Table S3: List of primers used in this study

647

648 **References associated to supplementary data**

649

650

651 **RESOURCE AVAILABILITY**

652 **Lead contact**

653 Further information and requests for resources and reagents used in this study should be directed to
654 and will be fulfilled by the lead contact, Maud Bernoux (maud.bernoux@inrae.fr).

655

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831

Figure 1. Effect of elevated temperature on cell death induced by TNLs or isolated TIRs in Arabidopsis.

(A-B) Cell death phenotype observed on seedlings (A) or adult plant leaves (B) of transgenic inducible lines carrying *pDex:L6^{TIR}-FSBP* (line #4), *pDex:L6MHV-myc* (line #2), *pDex:RPS4^{TIR}-FSBP* (line #F) and *pDex:RPS4-FSBP* (line #14) after Dex induction. (A) Ten day-old seedling were transferred to 10 μ M Dex-containing (+Dex) MS media or non-inducing media (DMSO-containing, -Dex) and moved to 21°C or 30°C climatic chambers. Wild-type Col-0 was used as a negative control. Photos were taken seven days after Dex induction. Black scale bars represent 1,5 cm. (B) Leaves of 4 week-old plants were infiltrated with 20 μ M Dex (+Dex) or DMSO (-Dex) solutions and moved to 21°C or 30°C climatic chambers. Photos were taken seven days after Dex induction. (C) Immunoblot analysis of L6^{TIR}-FSBP, L6MHV-myc, RPS4^{TIR}-FSBP, RPS4-FSBP and native EDS1 proteins over a 48h time-course post Dex induction (hpdi) of ten day-old seedlings at 21°C or 30°C, using anti-flag, anti-myc and anti-EDS1 antibodies detection. Total protein load is indicated by red Ponceau staining. Equal amount of protein extract were loaded for each timepoint to detect transgenic protein construct and EDS1.

Figure 2

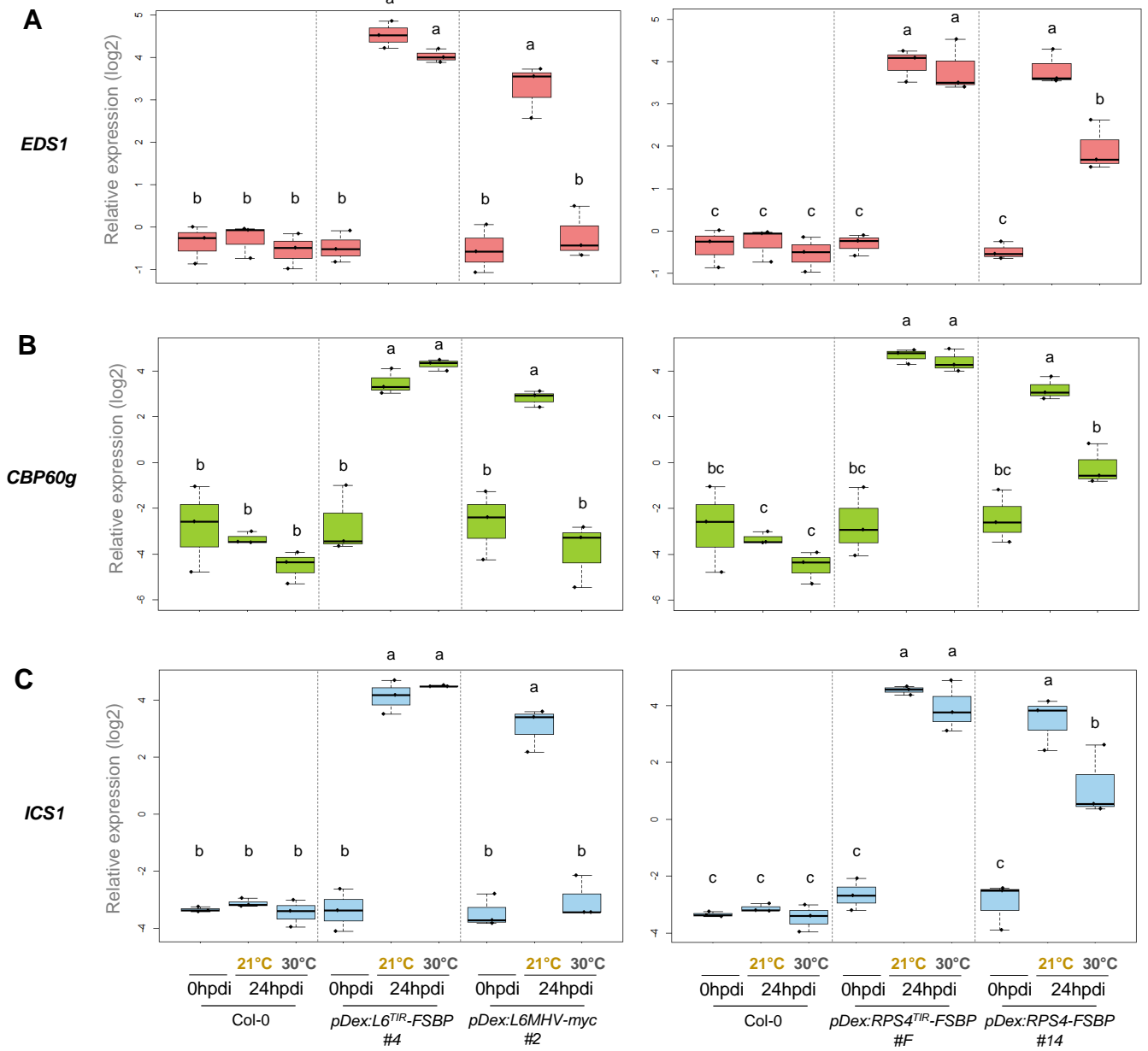


Figure 2. Effect of elevated temperature on the expression of genes involved in the SA pathway induced by TNLs or isolated TIRs. (A-C) RT-qPCR analysis of *EDS1* (A), *CBP60g* (B) and *ICS1* (C) gene expression level in wild-type (Col-0) or transgenic Arabidopsis seedlings carrying *pDex:L6^{TIR}-FSBP* (line #4), *pDex:L6MHV-myc* (line #2) and *pDex:RPS4^{TIR}-FSBP* (line #F) or *pDex:RPS4-FSBP* (line #14) at 21 or 30°C. Seedlings were collected at time 0 and 24 hours after transfer on 10µM Dex- containing medium (hpdi: hours post Dex induction). Gene expression was normalized relatively to the expression of two housekeeping genes (*At1G13320* and *At5G15710*). Box plots represent values obtained from three independent biological replicates which are represented by black dots. Statistical differences were assessed with two-ways variance analysis (ANOVA) followed by a Tukey's HSD multiple comparison test. Data points with different letters indicate significant differences ($P < 0.05$).

Figure 3

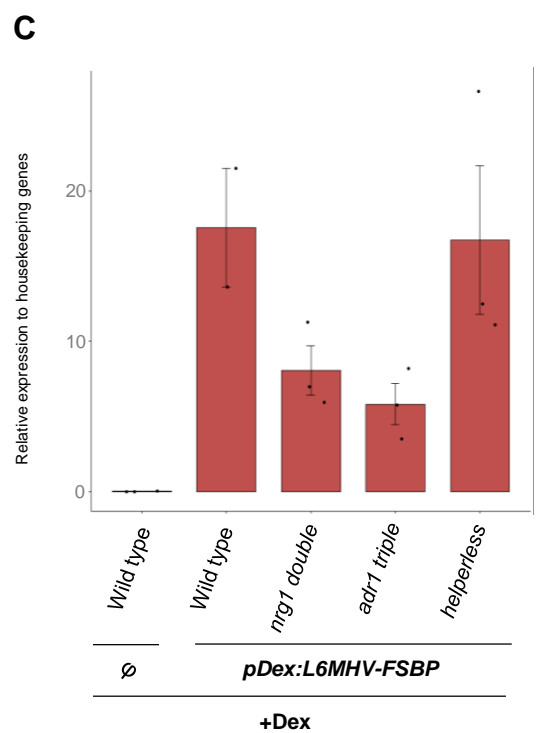
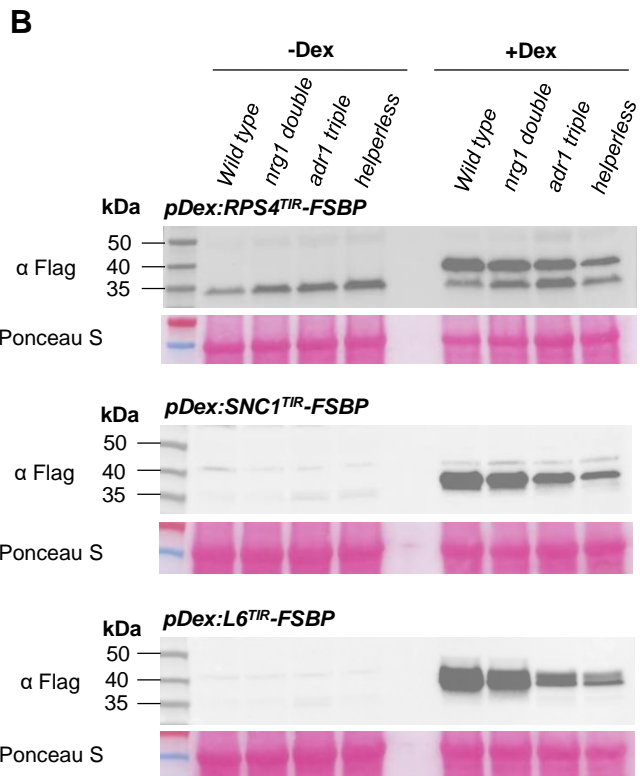
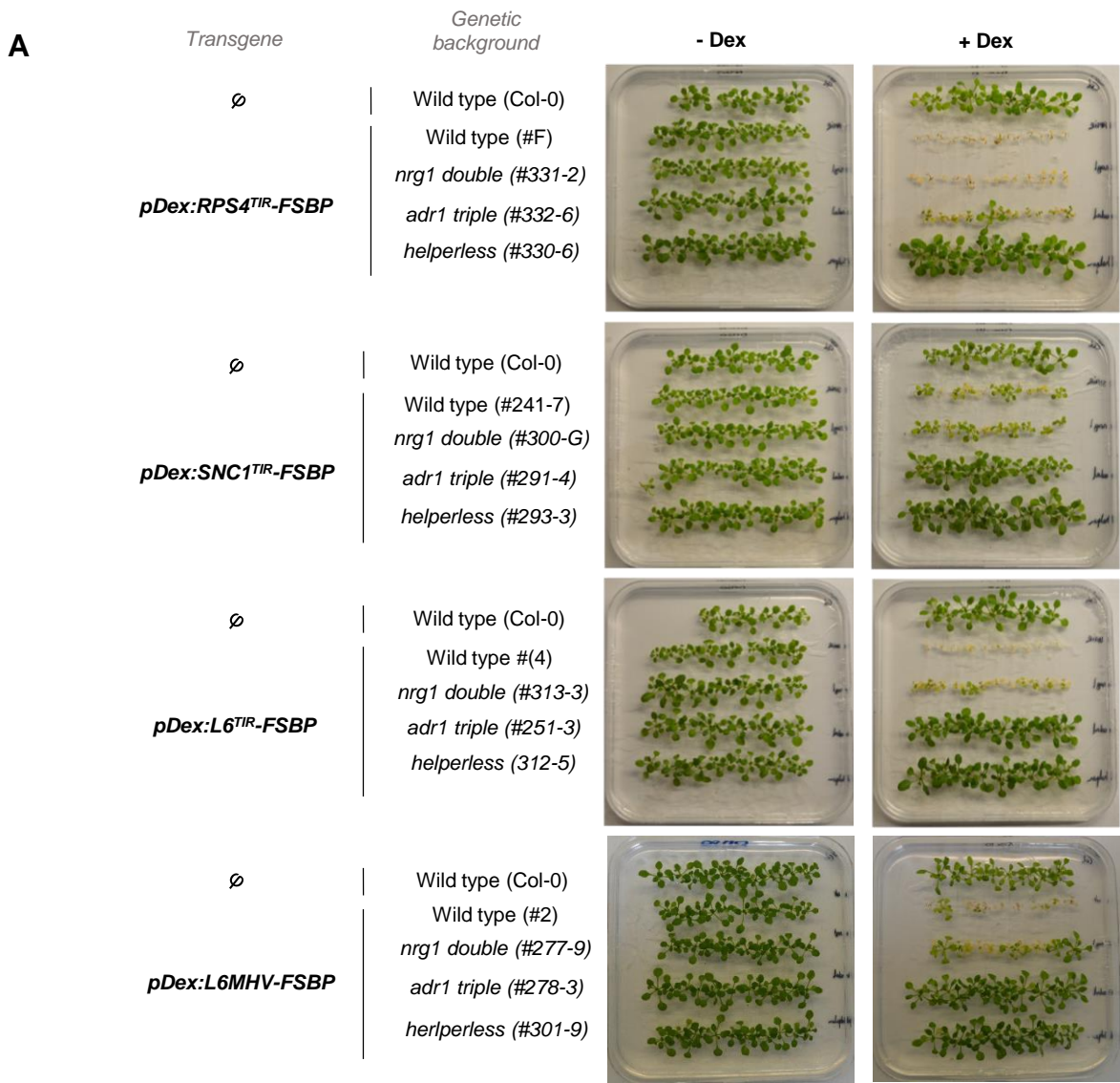


Figure 3. RNLs genetic requirements for signaling induced by TNLs or isolated TIRs. (A) Cell death phenotype observed on seedlings of transgenic inducible lines carrying *pDex:RPS4^{TIR}-FSBP*, *pDex:SNC1^{TIR}-FSBP*, *pDex:L6^{TIR}-FSBP*, or *pDex:L6MHV-FSBP*, in wild-type (wt) or in *RNLs* mutant backgrounds (*nrg1 double*, *adr1 triple* or *helperless*), after Dex induction. Ten day-old seedling were transferred to 10 μ M Dex-containing (+Dex) MS media or non-inducing media (DMSO-containing, -Dex). Untransformed wild-type Col-0 was used as a negative control. Photos were taken seven days after transfer. (B) Immunoblot analysis of RPS4^{TIR}-FSBP, SNC1^{TIR}-FSBP and L6^{TIR}-FSBP in wild-type or in *RNLs* mutant backgrounds (*nrg1 double*, *adr1 triple* or *helperless*), 24 hours post Dex induction using anti-flag antibodies detection. Total protein load is indicated by red Ponceau staining. Equal amount of protein extract was loaded for each sample. Background noise is indicated by an asterisk. (C) RT-qPCR analysis of *L6MHV-FSBP* transgene expression in wild-type or *RNLs* mutant backgrounds. Untransformed wild-type Col-0 was used as a negative control. *L6MHV-FSBP* expression was normalized relatively to the expression of two housekeeping genes (*At1G13320* and *At5G15710*). Bar plots represent means +/-SEM obtained from two or three independent biological replicates which are represented by black dots.

Figure 4

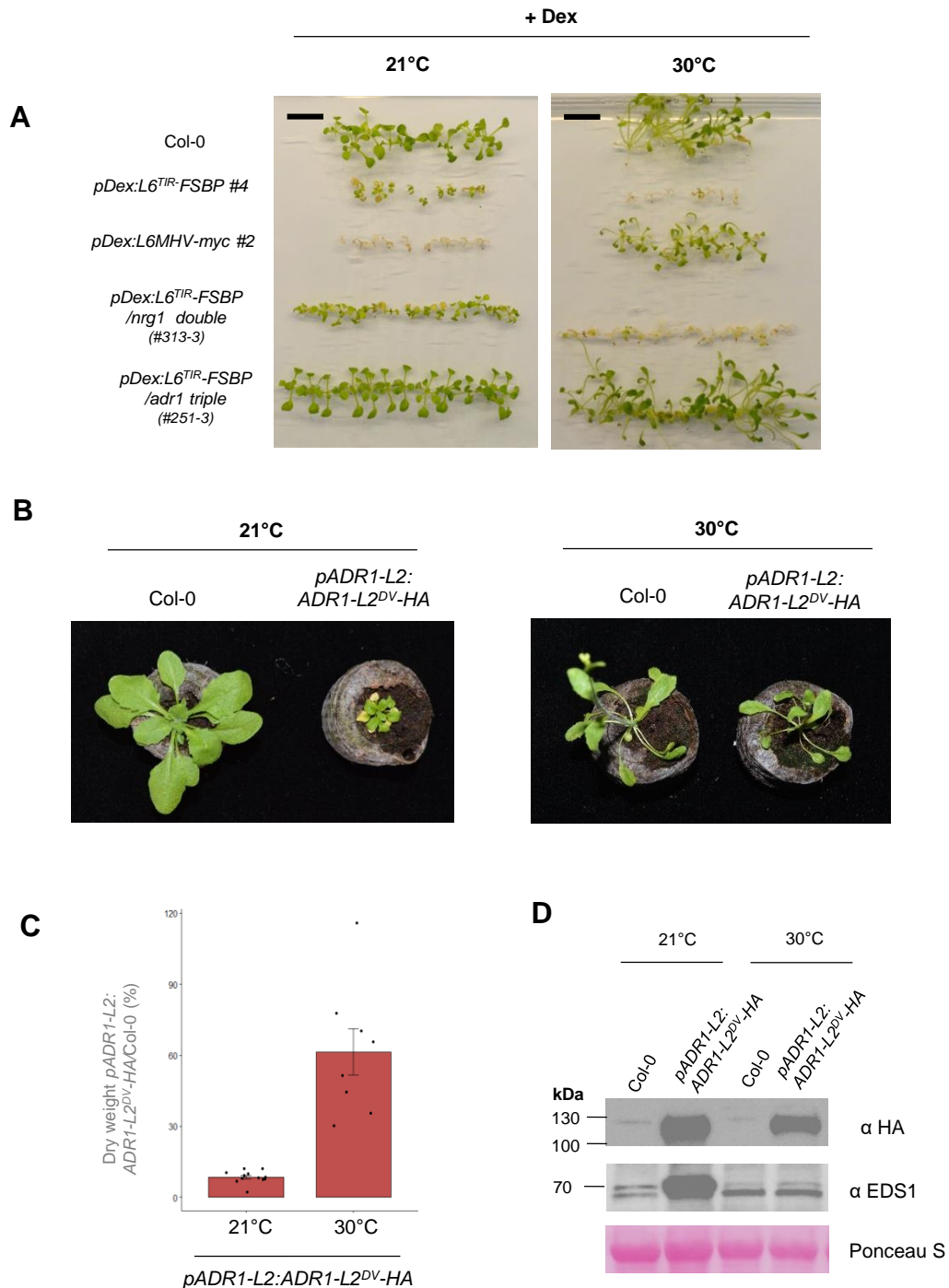
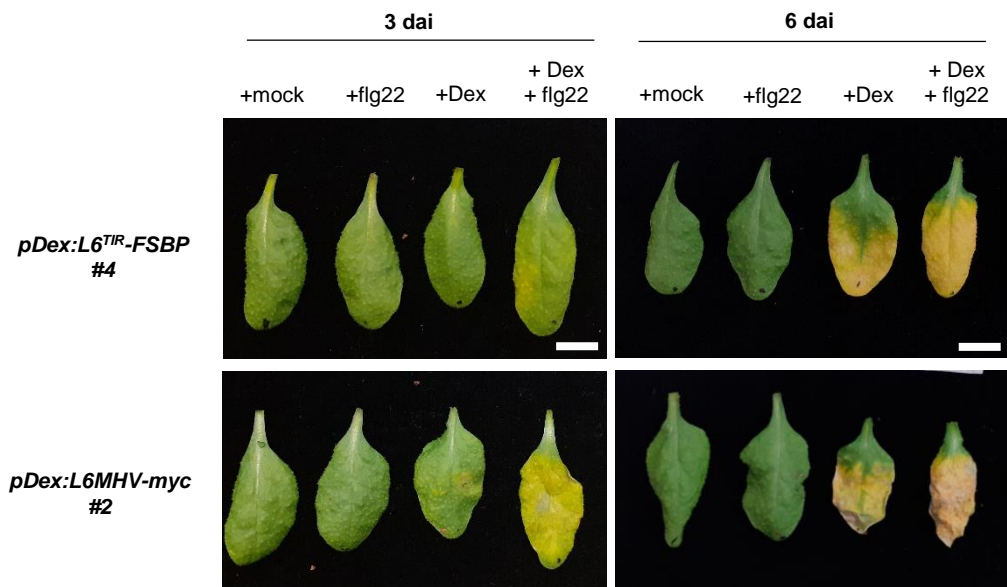


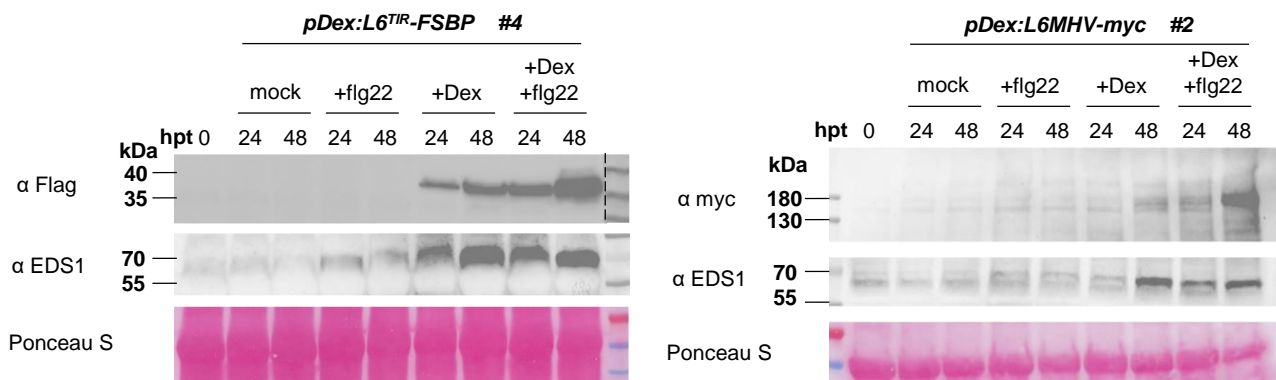
Figure 4. Differential sensitivity of ADR1s-induced signaling to elevated temperature. (A) Cell death phenotype of seedlings carrying *pDex:L6^{TIR}* in wild-type, *nrg1 double* or *adr1 triple* mutants, seven days after transfer on a 10 μ M Dex medium at 21°C or 30°C. Untransformed wild-type Col-0 was used as a negative control. *pDex:L6MHV-myc* #2 was used as a thermosensitive control. Photos were taken seven days after Dex induction. Black scale bars represent 1 cm. (B) Representative phenotype of three week-old Arabidopsis plant carrying *pADR1-L2:ADR1-L2^{DV}-HA* compared to wild-type Col-0 at 21° or 30°C. After germination on MS media, seven day-old seedlings were acclimated for approximately three days on soil pots at 21°C, then transferred and grown for two weeks in climatic chambers set at 21°C or 30°C. (C) Percentage of dry weight ratio of *pADR1-L2:ADR1-L2^{DV}-HA* compared to Col-0 plants at 21°C or 30°C as in panel A. Bar plots represent the percentage of mean dry weight ratios of plants carrying *pADR1-L2:ADR1-L2^{DV}-HA* / wild-type Col-0 +/- SEM. Individual percentage ratios are represented by black dots (n>8). This experiment was performed twice with similar results. (D) Immunoblot analysis of ADR1-L2^{DV}-HA and EDS1 in three week-old transgenics carrying *pADR1-L2:ADR1-L2^{DV}-HA* or wild-type Col-0, grown at 21°C or 30°C, using anti-HA and anti-EDS1 antibodies detection, respectively. Total protein load is indicated by Ponceau red staining.

Figure 5

A



B



C

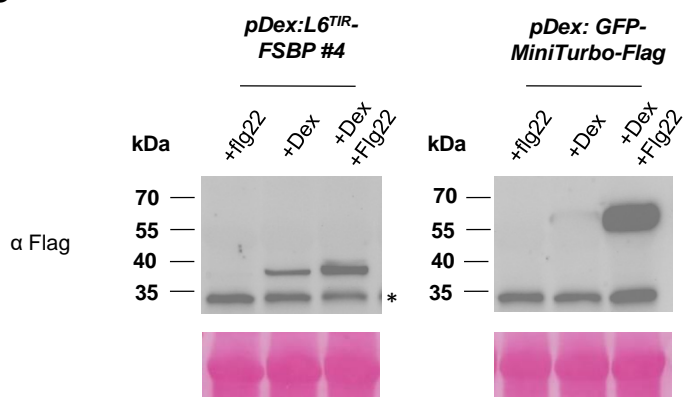


Figure 5. Effect of flg22 treatment on L6TIR- and L6MHV-mediated signaling. (A) Representative cell death symptoms observed in leaves of four week-old Arabidopsis transgenics carrying *pDex:L6^{TIR}-FSBP* or *pDex:L6MHV-myc*, infiltrated with a solution of DMSO ("mock"), 100 nM flg22 ("+flg22"), 20 μM Dex ("+Dex") or a co-treatment of both Dex 20 μM and flg22 100 nM ("+Dex + flg22"). Pictures were taken 3 and 6 days after treatment. (B) Immunoblot analysis of L6^{TIR}-FSBP, L6MHV-myc and EDS1 in leaves of four week-old Arabidopsis transgenics carrying *pDex:L6^{TIR}-FSBP* or *pDex:L6MHV-myc*, 24h and 48h post treatment (hpt), using anti-flag, anti-myc or anti-EDS1 antibodies detection, respectively. Three discs from three different plants were collected for L6^{TIR}-FSBP detection and five discs from five plants were collected for L6MHV-myc detection. (C) Immunoblot analysis of L6^{TIR}-FSBP and GFP-MiniTurbo-Flag in leaves of four week-old Arabidopsis carrying *pDex:L6^{TIR}-FSBP* or *pDex:GFP-Mini-Flag* 24h post treatment, using anti-flag antibodies detection. Background noise is indicated by an asterisk. (B-C) Total protein amount was verified with Ponceau red staining. Immunoblot analyses were performed at least twice with similar results.