1 **An incoherent feed-forward loop underlies robustness and tunability of a plant immune**
2 **network**
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21 **Abstract**

22 Immune signaling networks must be tunable to alleviate fitness costs associated with immunity and,
23 at the same time, robust against pathogen interferences. How these properties mechanistically
24 emerge in plant immune signaling networks is poorly understood. Here, we discovered a molecular
25 mechanism by which the model plant species *Arabidopsis thaliana* achieves robust and tunable
26 immunity triggered by the microbe-associated molecular pattern, flg22. Salicylic acid (SA) is a
27 major plant immune signal molecule. Another signal molecule jasmonate (JA) induced expression of
28 a gene essential for SA accumulation, *EDS5*. Paradoxically, JA inhibited expression of *PAD4*, a
29 positive regulator of *EDS5* expression. This incoherent type-4 feed-forward loop (I4-FFL) enabled
30 JA to mitigate SA accumulation in the intact network but to support it under perturbation of PAD4,
31 thereby minimizing the negative impact of SA on fitness as well as conferring robust SA-mediated
32 immunity. We also present evidence for evolutionary conservation of these gene regulations in the
33 family *Brassicaceae*. Our results highlight an I4-FFL that simultaneously provides the immune
34 network with robustness and tunability in *A. thaliana* and possibly in its relatives.

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38 **Introduction**

39 Proper processing of signals through signaling networks is central for organisms to
40 respond accordingly to the signals. As such, signaling networks are comprised of recurring
41 regulatory sub-network structures called network motifs with various information-processing
42 functions. Feed-forward loop (FFL), which consists of two regulators and a target, represents a
43 major class of network motifs [1]. Each of interactions among the components of a FFL can be either
44 positive (activation) or negative (repression). As a result, there are eight possible structural
45 configurations of FFL. Of these configurations, incoherent type-4 FFL (I4-FFL), in which a
46 regulator has a positive effect on the target but a negative effect on the other regulator that positively
47 regulates the target, is rare in biological networks and, therefore, its biological function has rarely
48 been described.

49 In nature, plants are in constant contact with a wide variety of microbes, which often
50 produce common molecular signatures known as microbe-associated molecular patterns (MAMPs)
51 [2]. Plants sense MAMPs by plasma membrane-localized pattern recognition receptors and feed this
52 information into signaling networks that finely control the output immune reaction designated as
53 pattern-triggered immunity (PTI) [2-5]. Since recognized MAMPs are often common to a class of
54 microbes [2], PTI could be triggered by both pathogenic and non-pathogenic microbes. Therefore, it
55 is vital for plants to avoid unnecessary PTI against non-pathogenic microbes, as there is a trade-off
56 between immunity and growth [6-9]. At the same time, it is important to retain PTI that is effective
57 against pathogens that deploy virulence effectors to interfere with immune signaling components [10,
58 11] and that can function under perturbation due to diverse environmental conditions [12]. The
59 molecular mechanisms that allow these properties to emerge from PTI signaling networks are poorly
60 understood.

61 Plants rely on PTI to resist necrotrophs that actively kill hosts to acquire nutrients as well

62 as to resist biotrophs that require living hosts for multiplication [2, 13]. The phytohormone
63 jasmonate (JA) is a major contributor to immunity against necrotrophs [13]. JA is produced in
64 response to MAMPs such as flg22 [14] and chitin [15], a part of bacterial flagellin and a part of
65 fungal cell walls, respectively. JA biosynthesis requires allene oxide synthase encoded by
66 *DELAYED-DEHISCENCE 2* (*DDE2*) [16]. JA and its derivatives including methyl JA (MeJA) can
67 be converted to JA-isoleucine (JA-Ile) [17, 18]. Perception of JA-Ile by the F-box protein
68 CORONATINE INSENSITIVE 1 (COI1) leads to ubiquitination- and proteasome-dependent
69 degradation of JASMONATE ZIM DOMAIN (JAZ) proteins [19-21]. This liberates downstream
70 transcription factors including MYC2 and its homologues MYC3 and MYC4, which are normally
71 repressed by JAZ proteins in the resting state, thereby activating JA-mediated transcriptional
72 responses and immunity [22, 23].

73 Another phytohormone, salicylic acid (SA), is a central regulator of immunity against
74 biotrophs and hemi-biotrophs such as the bacterial pathogen *Pseudomonas syringae* [13, 24]. Indeed,
75 SA production is activated by the bacterial MAMP flg22 [25]. Previous studies have identified a
76 number of genes involved in SA biosynthesis and signaling. *SALICYLIC ACID-INDUCTION*
77 *DEFICIENT 2* (*SID2*) encodes an isochorismate synthase that is essential for SA biosynthesis
78 through the isochorismate pathway [26]. *PHYTOALEXIN-DEFICIENT 4* (*PAD4*) contributes to
79 MAMP-induced SA accumulation [25, 27]. *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*) is
80 essential for pathogen-induced SA accumulation in *Arabidopsis thaliana* [28-30] and encodes a
81 MATE transporter, which was proposed to mediate SA transport from chloroplasts, the site of
82 SID2-mediated SA biosynthesis, to the cytoplasm [31]. SA affects transcriptional regulation of
83 hundreds of genes, including *PATHOGENESIS-RELATED 1* (*PR1*) [32]. SA accumulation and
84 signaling should be tightly controlled, as excessive activation of SA biosynthesis or signaling is
85 associated with growth retardation [6, 33-35]. However, current understanding of the signaling

86 mechanisms regulating SA production is fragmented.

87 Phytohormone signaling pathways form a complex network, which could confer great
88 regulatory potential to control plant responses to diverse internal and external stimuli [36, 37]. For
89 instance, antagonism between JA and SA is thought to be important to activate proper immunity
90 depending on pathogen life styles [13, 38]. Interestingly, cooperation between JA and SA has been
91 also reported [14, 39]. Thus, plants appear to have context-dependent crosstalk between JA and SA.
92 However, the molecular mechanisms and the biological relevance of the JA–SA crosstalk remain
93 elusive.

94 Previously, a quantitative model was built to capture signal flows in the network consisting
95 of the JA, SA, PAD4 and ethylene (ET) signaling sectors during PTI [14]. The model pointed to JA
96 and PAD4 as the sole determinants of SA signaling activity [14]. Here, we report the molecular
97 mechanism by which JA enables robust and tunable SA accumulation during PTI in *A. thaliana*. Our
98 data demonstrate that JA inhibits expression of *PAD4*, a positive regulator of *EDS5* expression.
99 Paradoxically, JA induces *EDS5* expression directly via the transcription factor MYC2. This I4-FFL
100 explains the negative role of JA on SA accumulation in the intact network and its positive role in the
101 absence of *PAD4*. We also show that both of these transcriptional effects of JA occur not only in *A.*
102 *thaliana* but also in other *Brassicaceae* species. Taken together, our results highlight the I4-FFL that
103 allows plants to alleviate the negative impact of SA on fitness as well as to support robust SA
104 accumulation when PAD4 function is compromised.

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106

107 **Results**

108 **JA is defined as a repressor or activator of SA accumulation depending on *PAD4***

109 To investigate the regulatory relationship between JA and PAD4 in MAMP-triggered SA

110 accumulation, we measured SA levels in leaves of wild-type, *dde2*, *pad4* and *dde2 pad4* plants after
111 infiltration with flg22. In the wild-type, an increase in SA level was observed at 9 hours post
112 infiltration (Figure 1A). The SA level was elevated in *dde2*, which is reminiscent of the often
113 described repressive effect of JA on SA. In contrast, in *pad4* SA was increased by flg22 treatment,
114 but to a level lower than in wild-type, which is consistent with PAD4 being a positive regulator of
115 SA accumulation in response to flg22 [25]. Strikingly, flg22-triggered SA accumulation was
116 abolished in *dde2 pad4,* showing a requirement of JA for SA induction in the absence of *PAD4*. A
117 similar pattern was observed for expression of the canonical SA marker gene *PR1* (Figure 1B), as
118 well as that of *At2g26400* and *At2g30550* (Figure 1C and D), which was shown to be induced upon
119 challenge with *P. syringae* pv. *tomato* DC3000 (*Pto*) in an SA-dependent manner [40]. In line with
120 the previous study [14], these results demonstrated that JA acts as a repressor or activator of SA
121 accumulation in the presence or absence of *PAD4*, respectively, during flg22-triggered PTI.

122 **JA represses *PAD4* expression through the action of MYC transcription factors**

123 Since the enhanced SA accumulation in *dde2* was dependent on *PAD4* (Figure 1A;
124 compare *dde2* and *dde2 pad4*), we tested whether JA represses *PAD4* expression. *PAD4* expression
125 was elevated in *dde2* as well as in *coi1* at 9 hours after flg22 treatment (Figure 2A). The
126 transcription factors MYC2 and its homologues MYC3 and MYC4 are important for transcriptional
127 responses to JA, and we found a MYC2-binding motif (G-box; CACATG) in the *PAD4* promoter
128 using the online tool Athena (Figure 2B) [41-43]. These observations led us to test whether MYC2
129 and its homologues MYC3 and MYC4 are responsible for JA-mediated repression of *PAD4*
130 expression. Indeed, increased expression of *PAD4* was observed in *myc2 myc3 myc4* but not in *myc2*
131 (Figure 2A). Thus, these MYCs seem to act redundantly to repress *PAD4* expression during
132 flg22-triggered PTI.

133 We then tested whether MYC2 directly binds to the G box motif in the *PAD4* promoter in

134 planta by chromatin immunoprecipitation (ChIP) using a transgenic *A. thaliana* line constitutively
135 expressing the MYC2-GFP fusion protein (Figure EV1). The enrichment of the G-box sequence in
136 immunoprecipitates from MYC2-GFP plants relative to those from wild-type plants was determined
137 by qPCR. A DNA segment from the coding sequence (CDS) of *PAD4* was used as a negative control.
138 Although these MYC transcription factors contribute to *PAD4* repression (Figure 2A), we did not
139 observe direct binding of MYC2 to the *PAD4* promoter even after the treatment with flg22 or MeJA
140 (Figure 2C and D). Considering that MYC2, MYC3 and MYC4 are transcriptional activators with
141 shared DNA-binding specificity [44], it is likely that these MYC transcription factors indirectly
142 repress *PAD4* expression through an intermediate factor(s).

143 **JA induces *EDS5* expression directly through MYC2**

144 Since JA positively contributes to SA accumulation in the absence of *PAD4*, we examined
145 expression levels of *SID2* and *EDS5,* both of which are essential for pathogen-induced SA
146 accumulation [25, 26, 28, 29]. At five hours after flg22 treatment, expression of *SID2* was similar in
147 *pad4* and *dde2 pad4* (Figure 3A). In contrast, expression of *EDS5* was significantly lower in *dde2*
148 *pad4* than in *pad4*, and *EDS5* induction was abolished in *dde2 pad4* (Figure 3B), indicating that
149 *PAD4* and JA together are responsible for flg22-triggered *EDS5* expression. Importantly, the
150 compromised *EDS5* induction in *dde2 pad4* was correlated well with the compromised SA induction
151 in *dde2 pad4* (Figure 1A), suggesting that *EDS5* is the causal gene for the positive role of JA in SA
152 accumulation.

153 To explore the mechanism by which JA regulates *EDS5* expression, the promoter sequence
154 of *EDS5* was searched for *cis* elements using the Athena analysis tool. We found a canonical G box
155 (CACGTG), the binding site for MYC transcription factors, in close proximity to the transcription
156 start site of *EDS5* (Figure 3D). This prompted us to test whether MYC2 and its homologues MYC3
157 and MYC4 are responsible for *EDS5* induction by JA. In wild-type plants, MeJA treatment induced

158 *EDS5* expression at the three time points tested, while *EDS5* expression was significantly reduced in
159 *myc2* and *myc2 myc3 myc4* (Figure 3C), demonstrating that these MYCs are required for
160 JA-mediated *EDS5* induction. We then performed ChIP experiments using MYC2-GFP plants
161 treated with or without flg22 or MeJA to test if MYC2 directly binds to the *EDS5* promoter. We
162 found a significant enrichment of the promoter sequence containing the G-box motif in all the
163 conditions tested, but no enrichment was observed for a DNA segment in the CDS of *EDS5* used as
164 a negative control (Figure 3E and F). To test whether the G box in the *EDS5* promoter is required for
165 MYC2-mediated transcriptional activation of *EDS5*, we carried out luciferase (Luc) reporter assays
166 using *Arabidopsis* protoplasts. Expression of MYC2 significantly induced the wild type *EDS5*
167 promoter-driven Luc activity, whereas deletion of the G box abolished this MYC2-mediated
168 transcriptional activation (Figure 3G). Taken together, these results indicate that MYC2 directly
169 binds to the *EDS5* promoter and controls *EDS5* induction by JA.

170 **Reconstitution of *EDS5* expression restores flg22-triggered SA accumulation and immunity in**
171 ***dde2 pad4***

172 To test for a causal link between JA-mediated *EDS5* expression and SA accumulation, we
173 generated transgenic lines expressing *EDS5* under two different promoters in *dde2 pad4*. In two
174 independent lines expressing *EDS5* from the constitutive 35S promoter, *EDS5* expression was higher
175 than in the wild-type and was not altered after flg22 treatment (Figure 4A). The expression level of
176 *EDS5* was more than 8-fold higher in p35S:*EDS5* line #1 than in line #2 (Figure 4A). Another
177 transgenic line expressing *EDS5* from the *SID2* promoter showed the wild-type level of *EDS5*
178 expression after mock treatment and slightly higher expression of *EDS5* compared to the wild-type
179 after flg22 treatment (Figure 4A). This is in accordance with our finding that *SID2* was responsive to
180 flg22 in *dde2 pad4* (Figure 3A). Induction of SA accumulation and *PR1* expression by flg22 was
181 detected in p35S:*EDS5* line #1 but not in line #2 (Figures 4B and C). The *pSID2*:*EDS5* line also

182 showed restored SA accumulation and *PR1* expression after flg22 treatment (Figures 4B and C)
183 although the expression level of *EDS5* was lower than in p35S:*EDS5* line #2. Thus, a minimal level
184 of *EDS5* expression, which is not achieved in *dde2 pad4*, is required for flg22-triggered SA
185 accumulation. These results also suggest that transcriptional induction of *EDS5* in response to flg22
186 can overcome the need to constitutively express *EDS5* at a very high level for flg22-triggered SA
187 accumulation. As *EDS5* is inducible by flg22, this transcriptional induction might be a critical part of
188 flg22-triggered SA accumulation. Overall, our data clearly established a causal connection between
189 compromised *EDS5* expression or induction and the compromised SA accumulation in *dde2 pad4* in
190 response to flg22.

191 To test whether the restored SA accumulation in the transgenic lines is relevant for
192 immunity, we measured *Pto* growth. Leaves were co-infiltrated with *Pto* and flg22 and sampled at 2
193 days after infiltration. Co-infiltration of flg22 inhibited *Pto* growth in the wild type but not in *fls2*, a
194 mutant lacking the receptor for flg22 (Figure 4D). This reduction of bacterial growth, termed
195 flg22-triggered PTI, was calculated by subtracting the $\log_{10}$-transformed bacterial titer in
196 flg22-treated leaves from that in mock-treated leaves. Flg22-triggered PTI was much less in *dde2*
197 *pad4* than in the wild type. Importantly, flg22-triggered PTI was significantly higher in the
198 transgenic lines with restored SA accumulation than in *dde2 pad4* plants (Figure 4D). Given the
199 genetic requirement for JA in flg22-triggered *EDS5* expression and SA accumulation in *pad4*
200 (Figures 1A and 3B), we conclude that JA enables robust flg22-triggered PTI by supporting SA
201 accumulation through MYC2-activated *EDS5* expression.

202 **Distinct effects of JA on bacterial resistance depending on *PAD4***

203 Our genetic perturbation and reconstitution approach illustrates an I4-FFL consisting JA
204 (MYC transcription factors), *PAD4* and *EDS5* (Figure 5A). To further investigate the roles of the
205 I4-FFL in plant immunity, we assessed effects of exogenous MeJA application on flg22-triggered

206 PTI against *Pto* in the wild type, *dde2 pad4* and the transgenic *p35S:EDS5* #1 and *pSID2:EDS5* lines
207 with restored flg22-triggered SA accumulation. MeJA reduced flg22-triggered PTI in the wild type
208 but enhanced it in *dde2 pad4* (Figure 5B), demonstrating that the negative effect of JA is dominant
209 in the presence of *PAD4*, whereas the positive effect of JA is evident in the absence of *PAD4*. MeJA
210 had no effect on flg22-triggered immunity in the transgenic lines, suggesting that the positive role of
211 JA in the absence of *PAD4* is to support SA accumulation via *EDS5* expression. These results are
212 consistent with our I4-FFL model, in which JA negatively or positively regulates SA-mediated
213 bacterial resistance in the presence or absence of *PAD4*, respectively.

214 PAD4-regulated signaling to SA activation is perturbed at high temperature such as 28°C
215 [45]. To investigate the biological importance of the I4-FFL in a more natural context, we measured
216 *Pto* growth in the wild type, *dde2*, *pad4* and *dde2 pad4* at 22°C and 28°C. As shown in Figure 5C,
217 *pad4* was more susceptible to *Pto* than the wild type at 22°C. Such enhanced susceptibility of *pad4*
218 was not observed at 28°C, indicating that PAD4 function in *Pto* resistance is compromised at this
219 temperature. Interestingly, *dde2* and *dde2 pad4* supported more *Pto* growth than the wild type and
220 *pad4*, respectively, at 28°C. No significant differences in *Pto* growth between Col and *dde2* and
221 between *pad4* and *dde2 pad4* were observed at 22°C. The effects of *dde2* mutation at 22°C might be
222 masked by coronatine produced by *Pto*, which activates JA signaling by acting as a molecular mimic
223 of JA-Ile [46, 47]. Overall, these results support a biological significance of the I4-FFL for
224 conferring JA-mediated bacterial resistance under perturbation of PAD4 at high temperature, which
225 can naturally occur.

226 **Conservation and diversification of JA-mediated regulation of *PAD4* and *EDS5* in**
227 ***Brassicaceae***

228 The importance of the I4-FFL identified in this study could be reflected by evolutionary
229 conservation in plants. To address this point, we used the *A. thaliana* EDS5 protein sequence to

230 identify related proteins in some *Brassicaceae* species, tomato and rice whose genome sequences
231 and gene annotations are available. Construction of a phylogenetic tree using the related proteins
232 suggests that the EDS5 clade is conserved in the family *Brassicaceae* but not in other plants (Figure
233 EV2). Since our results suggest that MYC2 controls JA-mediated *EDS5* induction through binding to
234 the CACGTG G box motif (Figure 3E-G), we surveyed 500 bp upstream of the transcription start
235 sites (hereafter referred to as "promoters") and 5′-UTRs of these *EDS5* orthologues for this motif.
236 Interestingly, the G box motif was found in the *EDS5* promoters of *A. thaliana*, *Arabidopsis lyrata*,
237 *Capsella grandiflora* and *Eutrema salsugineum*, whereas it was located in the 5′-UTRs in *Capsella*
238 *rubella* and *Brassica rapa* (Figure EV3). MeJA treatment induced *EDS5* expression in *A. thaliana*, *A.*
239 *lyrata*, *E. salsugineum*, but not in *C. rubella* (Figure 6A). This is in line with the presence or absence
240 of the G box motif in the promoters. *C. rubella* was responsive to MeJA in other ways, as
241 exemplified by induction of a homologue of the *A. thaliana VSP2*, a JA responsive gene (Figure
242 EV4). The inducibility of *EDS5* by JA is not correlated to the phylogenetic distance within
243 *Brassicaceae* [48]. Thus, these results may suggest that the JA-mediated *EDS5* regulation emerged
244 in the ancestor of *Brassicaceae* and *C. rubella* has lost it.

245 PAD4 is conserved among flowering plants [49]. We therefore tested whether
246 JA-mediated repression of *PAD4* expression is conserved among *Brassicaceae*. *A. thaliana, A. lyrata*,
247 *C. rubella* and *E. salsugineum* plants were treated with mock or MeJA, followed by flg22 treatment.
248 In *A. thaliana*, MeJA treatment had no effect on *PAD4* expression but inhibited *PAD4* induction by
249 flg22 (Figure 6B). As in *A. thaliana*, MeJA had an inhibitory effect on *PAD4* induction by flg22 in
250 the other three species (Figure 6B). Thus, the repressive effect of JA on *PAD4* expression during
251 flg22-PTI appears to be conserved in *Brassicaceae*.
252
253

254 **Discussion**

255 It is vital for plants to invoke robust immunity against pathogens that interfere with
256 immune signaling and, at the same time, to minimize fitness costs associated with immunity. This is
257 particularly relevant to PTI, since it is activated by MAMPs which do not distinguish pathogens
258 from other beneficial or benign microbes. In this study, we identified an I4-FFL consisting of JA,
259 *PAD4* and *EDS5* in the PTI signaling network in *A. thaliana*. JA induces *EDS5* expression directly
260 via the transcription factor MYC2 while repressing expression of *PAD4* which positively contributes
261 to *EDS5* expression. I4-FFL is rare in biological networks and, therefore, its biological function has
262 rarely been characterized [50, 51]. In the context of PTI, *PAD4* repression by JA is functionally
263 dominant in the intact network of wild-type plants, which explains reduction of SA accumulation in
264 *pad4* and increase in *dde2*. However, in the absence of *PAD4*, the positive contribution of JA to SA
265 accumulation becomes apparent. Consistently, SA induction in response to flg22 was abolished in
266 *dde2 pad4*. The JA-mediated suppression of *PAD4* expression is likely important to alleviate the
267 negative impact of SA on plant growth [6, 33-35]. In contrast, the JA-mediated *EDS5* induction
268 provides robust SA accumulation in flg22-triggered immunity when PAD4 cannot fulfill its function,
269 for example, due to pathogen effectors or environmental factors.

270 A mechanism by which JA inhibits SA accumulation was uncovered by characterizing the
271 mode of action of the JA-mimicking bacterial phytotoxin coronatine produced by *P. syringae* [52]. It
272 was demonstrated that MYC2 transcriptionally activates the NAC (petunia NAM and *Arabidopsis*
273 ATAF1, ATAF2, and CUC2) transcription factors ANAC019, ANAC055 and ANAC072, which
274 repress the SA biosynthesis gene *SID2* and induce the SA catabolism gene *BSMT1*. However, no
275 significant increase in *SID2* expression was observed in *dde2* during flg22-triggered PTI (Figure 3A).
276 Thus, the negative effect of JA on *SID2* expression is not the cause of antagonistic effects of JA on
277 SA accumulation in the context of flg22-triggered PTI at least in our hands. In contrast, our genetic

278 evidence indicates that the repressive effect of JA on SA accumulation is dependent on *PAD4* in
279 flg22-triggered PTI, as introducing *pad4* mutation into *dde2* abolished flg22-triggered SA
280 accumulation. Consistently, JA represses *PAD4* expression in a manner dependent on MYC2,
281 MYC3 and MYC4. The JA-mediated repression of *PAD4* expression could explain the previous
282 observation that expression of a marker gene of PAD4 signaling activity (At5g46960) was elevated
283 in *dde2* [14]. Overall, our genetic evidence suggests a novel mechanism for JA-mediated
284 suppression of SA accumulation through MYC transcription factors. However, our ChIP experiment
285 did not support direct binding of MYC2 to the *PAD4* promoter. It is also unlikely that *PAD4*
286 repression by JA is directly mediated by the NACs downstream of the MYCs because there is no
287 NAC-binding site present in the *PAD4* promoter [53]. Further studies will be required to unravel the
288 mechanism of the negative regulation of *PAD4* expression by JA in PTI.

289 Although most studies of JA-SA crosstalk have reported antagonistic interactions,
290 cooperative interactions between the two phytohormones have been observed under some conditions
291 [14, 39]. However, the underlying mechanism is unknown. In the present study, we show that JA
292 transcriptionally activates *EDS5* directly through MYC2. This transcriptional regulation is causally
293 linked to JA-mediated SA accumulation and immunity in *pad4*, as reconstitution of *EDS5* expression
294 or induction restored flg22-triggered SA accumulation and immunity in *dde2 pad4*. In addition,
295 exogenous MeJA application enhanced flg22-triggered immunity in *dde2 pad4* but not in the
296 transgenic *p35S:EDS5* #1 and *pSID2:EDS5* lines with restored flg22-triggered SA accumulation.
297 By making use of the fact that PAD4-regulated signaling to SA activation is highly influenced by
298 temperature [45], we showed that JA confers bacterial resistance under perturbation of PAD4 at
299 28°C. Thus, we propose that the robust SA accumulation and immunity enabled by JA has a
300 substantial role, when plants face situations in which PAD4 function is perturbed by environmental
301 factors such as high temperature and likely by pathogen effectors. With respect to the latter situation,

302 it is noteworthy that some bacterial effectors target EDS1, which is required for PAD4 function [54,
303 55].

304 It would be interesting to discuss effects of coronatine in the framework of the I4-FFL
305 identified in this study. Coronatine is a JA-mimicking virulence factor that suppresses SA-mediated
306 immunity to promote bacterial growth [46, 47, 52]. Consistently, we observed that MeJA treatment
307 after flg22 infiltration promotes *Pto* growth in the wild type. However, in *dde2 pad4,* MeJA
308 treatment reduced *Pto* growth. Thus, coronatine may have a negative impact on bacterial virulence
309 when combined with other effectors that interfere with PAD4 activity as well as under
310 environmental conditions in which PAD4 cannot fulfill its function.

311 Although *A. thaliana* is an excellent model system to study molecular and genetic aspects
312 of plant biology, it is becoming increasingly important to expand our knowledge to other plant
313 species [48]. In this study, we took advantage of the family *Brassicaceae*, to which *A. thaliana*
314 belongs, for studying evolutionary conservation of the gene regulation that we identified in *A.*
315 *thaliana*. Our results indicate that the repressive effect of JA on *PAD4* expression during PTI is
316 conserved not only in *A. lyrata* and *C. rubella*, close relatives of *A. thaliana*, but also in *E.*
317 *salsugineum*, a relatively phylogenetically distant species from *A. thaliana*. Thus, the repression of
318 *PAD4* by JA may be a common regulatory mechanism for tunable SA accumulation during PTI in
319 *Brassicaceae*. Since PAD4 is conserved in flowering plants [49], it would be interesting to test
320 whether JA represses *PAD4* expression during PTI in plant species outside *Brassicaceae*.

321 In contrast to PAD4, our phylogenetic analysis highlighted a *Brassicaceae*-specific clade
322 to which *A. thaliana* EDS5 belongs, suggesting that the role of EDS5 in SA accumulation might be
323 restricted to this family. Interestingly, our gene expression data together with promoter analysis
324 pointed to a good correlation between the presence or absence of the CACGTG G box motif in the
325 promoters and the inducibility of *EDS5* by JA in *Brassicaceae*. We note that in *C. rubella*, in which

326 JA does not induce *EDS5*, the CACGTG sequence is present downstream of the transcription start
327 site and transcribed as a part of the 5′ UTR [56]. Thus, *C. rubella* might have lost JA-mediated *EDS5*
328 induction by changing the transcription start site. This might also hold true for *B. rapa, as* the G box
329 motif is located in the 5′-UTR (Brassica rapa FPsc v1.3, DOE-JGI, http://phytozome.jgi.doe.gov/).
330 Overall, our comparative analysis suggests that *EDS5* and its transcriptional regulation by JA are an
331 innovation of the family *Brassicaceae*.

332 In conclusion, our results highlight an I4-FFL that simultaneously provides robust and
333 tunable regulation of SA response during PTI in *A. thaliana*. The transcriptional effects of JA on
334 *EDS5* and *PAD4* appear to be highly conserved in the family *Brassicaceae*. Whether or not this
335 reflects evolutionary conservation of the I4-FFL deserves further study.

336

337

338 **Materials and Methods**

339 **Plant materials and growth conditions**

340 *Arabidopsis* plants were grown in a chamber at 22°C with a 10 h light period and 60%
341 relative humidity for 3 weeks and then in another chamber at 22°C with a 12 h light period and 60%
342 relative humidity. The *A. thaliana* accession Col-0 was the background of all *Arabidopsis* mutants
343 used in this study. *Arabidopsis dde2-2* [16], *pad4-1* [27], *dde2-2 pad4-1* [57], *coi1-1* [19],
344 *jin1-9/myc2* (SALK_017005) [58], *myc2 myc3 myc4* [44], and *fls2* (SAIL_691C4) [59] were
345 described previously. The MYC2-GFP overexpression plants were obtained from Dr. Hironaka
346 Tsukagoshi (Nagoya University, JAPAN). Seedlings of *A. thaliana*, *A. lyrata* (MN47), *C. rubella*
347 (N22697) and *E. salsugineum* (Shandong) were grown on solidified half-strength Murashige and
348 Skoog (MS) medium supplemented with 1% sucrose under a 10 h light period at 22°C.

349 **Chemicals**

350 MeJA (392705) and flg22 were purchased from Sigma (Munich, Germany) and EZBiolab
351 Inc. (Westfield, IN, USA), respectively.

352 **Cloning and plant transformation**

353 The coding sequence (without introns) of *EDS5* (AT4G39030) was amplified by PCR
354 using PrimeSTAR HS DNA polymerase (Takara-Clontech, Saint-Germain-en-Laye, France) and
355 cloned into the pENTR/D-TOPO vector following the manufacturer's protocol (Life Technologies,
356 Darmstadt, Germany) to generate pENTR_EDS5. The promoter sequence of *SID2* (At1g74710) [60]
357 and the Nos terminator sequence from pER8 [61] were amplified by PCR and cloned into the NotI
358 and AscI sites of pENTR_EDS5, respectively, to generate pENTR_pSID2_EDS5_Nos.
359 pENTR_EDS5 and pENTR_pSID2_EDS5_Nos were then recombined into the Gateway-compatible
360 binary vectors pFAST-R02 [62] and pFAST-R01 [62], respectively, through the LR reaction
361 (Invitrogen). Primers used are listed in Table EV1. All plasmids constructed in this study were
362 verified by sequencing. *A. thaliana dde2 pad4* plants were transformed using *Agrobacterium*
363 *tumefaciens* stain GV3101 as described [16].

364 **Statistical analysis**

365 Statistical analysis was performed using the mixed linear model function, lmer,
366 implemented in the package lme4 in the R environment. When appropriate, raw data were log
367 transformed to meet the assumptions of the mixed linear model. For the t-tests, the standard errors
368 were calculated using the variance and covariance values obtained from the model fitting. The
369 Benjamini-Hochberg methods were applied to correct for multiple hypothesis testing when all
370 pairwise comparisons of the mean estimates were made in a figure.

371 **RNA extraction, cDNA synthesis and quantitative PCR**

372 Leaves of 4 to 5-week-old plants were infiltrated with 1 μM flg22 or mock (water) using a
373 needleless syringe and collected at the indicated time points. Seedlings were submerged into liquid

374 half-strength MS medium containing 100 μM MeJA or mock (water) for the indicated time period
375 and, if required, transferred to new liquid half-strength MS medium containing 1 μM flg22 or mock.
376 Total RNAs were isolated using TriFast (peqlab, Erlangen, Germany), followed by cDNA synthesis
377 using superscript II (Life Technologies). Real-time PCR was performed using EvaGreen (Biotium,
378 Hayward, CA, USA) on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Munich,
379 Germany) or the CFX Connect Real-Time PCR Detection System (Bio-Rad). Primers used are listed
380 in Table EV1. The following models were fit to the relative Ct value data compared to *Actin2* using
381 the lmer function in the lme4 package in the R environment: $Ct_{gyr} = GY_{gy}+R_r+e_{gyr}$, where GY,
382 genotype:treatment interaction, and random factors; R, biological replicate; e, residual; $Ct_{ytr} =$
383 $YT_{yt}+R_r+e_{ytr}$, where YT, treatment:time interaction and random factors; R, biological replicate; e,
384 residual. The mean estimates of the fixed effects were used as the modeled relative Ct values,
385 visualized as the relative $\log_2$ expression values, and compared by two-tailed t-tests.

386 **SA measurement**

387 Leaves of 4 to 5-week-old plants were infiltrated with mock (water) or 1 μM flg22.
388 Samples were harvested 9 hours after the treatment and stored at -80 °C. SA measurement was
389 performed as described previously [63]. The following model was fit to $\log_2$-transformed SA levels
390 (pmol/g fresh weight); $SA_{gyr} = GY_{gy}+R_r+e_{gyr}$, where GY, genotype:treatment interaction, and
391 random factors; R, biological replicate; e, residual. The mean estimates of the fixed effects were
392 compared by two-tailed t-tests.

393 **Bacterial growth assay**

394 Bacterial growth assays were performed essentially as described previously [57]. For
395 measuring flg22-triggered immunity, bacterial suspensions were co-infiltrated with 1 μM flg22 into
396 leaves of 4 to 5-week-old plants using a needleless syringe. For assessing effects of MeJA, 1 mM
397 MeJA were sprayed onto 4 to 5-week-old plants shortly after infiltration of bacterial suspensions and

398 1 μM flg22. For assessing effects of temperature, 4 to 5-week-old plants were grown, infiltrated with
399 bacterial suspension, and kept at 22°C or 28°C throughout the experiments. Log_{10}-transformed
400 colony-forming units (cfu) per cm^2 leaf surface area were calculated and the following model was fit
401 to the data; $CFU_{gyr} = GY_{gy}+R_r+e_{gyr}$, where GY, genotype:treatment interaction, and random factors;
402 R, biological replicate; e, residual. Flg22-triggered immunity was calculated by subtracting the
403 modeled bacterial titers in flg22-treated plants from those in the mock-treated plants.

404 **Chromatin immunoprecipitation**

405 Tissue fixation and chromatin immunoprecipitation were carried out as described [64] with
406 some modifications. Briefly, 2-week-old seedlings grown in liquid half-strength MS medium
407 supplemented with 1% sucrose were treated with 1 μM flg22 for 1 or 3 hours. Untreated seedlings
408 were also harvested. Alternatively, seedlings were treated with mock (water) or 100 μM MeJA for 3
409 hours. After fixation in 1% formaldehyde solution, tissues were frozen in liquid nitrogen and stored
410 at -80°C. Frozen tissues (~1 g) were ground in liquid nitrogen using a mortar and pestle and
411 suspended in 3 ml of lysis buffer (50 mM Tris-HCl [pH8.0], 2 mM EDTA, 150 mM NaCl, 1%
412 Triton X-100, 50 μM MG132 (Sigma), and complete protease inhibitor cocktails [04693132001;
413 Roche, Mannheim, Germany] or proteases inhibitor cocktail [P9599; Sigma]). The suspension was
414 sonicated twice on the Bioruptor Next Gen UCD-300 sonication system (Diagenode, Seraing,
415 Belgium) for 10 min at 4°C, followed by centrifugation at 20,000×g for 10 min at 4°C. The
416 supernatant was used as the starting material for chromatin immunoprecipitation using anti-GFP
417 antibody (Ab290; Abcam, Cambridge, UK). Aliquots of the supernatant were kept as input samples.
418 The samples were analyzed by quantitative PCR using primers listed in Table EV1. The percentage
419 of input values of the ChIP DNA was further normalized over the value obtained for the *Actin7*
420 promoter (AT5G09810). Fold enrichment was then calculated by taking ratios between normalized
421 results from wild-type plants and from MYC2-GFP plants. For statistical analysis, the following

422 model was fit to $\log_2$-transformed values of the normalized value data; $Ct_{gyr} = GY_{gy}+R_r+e_{gyr}$, where
423 GY, genotype:treatment interaction and random factors; R, biological replicate; e, residual. The
424 mean estimates of the fixed effects were compared by two-tailed t-tests.

425 **Luciferase reporter assay**

426 The WT *EDS5* promoter was amplified by PCR (PrimeSTAR HS DNA polymerase;
427 Takara-Clontech) using pEDS5_F and pEDS5_R (with HindIII and BamHI restriction sites,
428 respectively) listed in Table EV1, designed as recommended by the In-Fusion HD cloning kit. For
429 the *EDS5* promoter without the G box, two fragments were amplified by PCR using two sets of
430 primers, pEDS5_F and pEDS5w/oGbox_R and pEDS5w/oGbox_F and pEDS5_R, respectively
431 (Table EV1) and then fused by PCR using pEDS5_F and pEDS5_R. These promoter sequences were
432 cloned into HindIII/BamHI digested pBI221-LUC using In-Fusion HD cloning kit
433 (Takara-Clontech) to generate pBI221_pEDS5::LUC and pBI221_pEDS5w/oGbox::LUC.
434 pENTR_MYC2 used in this study was obtained from Dr. Haitao Cui (Max Planck Institute for Plant
435 Breeding Research, Germany) and recombined into pAM-PAT vector (35S promoter) with the
436 Gateway LR clonase (Invitrogen) to obtain the pAM-PAT_MYC2 vector.

437 *EDS5* promoter activity assays were performed by transient expression in *Arabidopsis*
438 Col-0 protoplasts as described previously [65]. Protoplasts were transfected with
439 pBI221_pEDS5::LUC or pBI221_pEDS5w/oGbox::LUC in the presence or absence of pAM-PAT_
440 MYC2. The pPTRL plasmid [66] was included for normalization of transformation efficiency, which
441 expresses *Renilla* luciferase under the 35S promoter. Nineteen hours post transfection, protoplasts
442 were harvested and luciferase assay was performed by Dual-Luciferase reporter assay system
443 (Promega) and Centro LB 960 Microplate Luminometer (Berthold Technologies).

444 **Phylogenetic analysis**

445 The whole protein sequences of *A. thaliana*, *A, lyrata*, *C. rubella*, *C. grandiflora*, *E.*

446 *salsugineum*, *B. rapa*, tomato and rice were retrieved from Phytozome [67] and used for
447 identification of putative orthologous groups using the OrthoMCL program [68]. The proteins
448 belonging to the same group as *A. thaliana* EDS5 were aligned using MUSCLE [69]. A maximum
449 likelihood phylogenetic tree was constructed using the MEGA6 software [70]. To visualize
450 conservation of G boxes, 500 bp upstream of the transcription start sites and 5'-UTRs of the
451 *Brassicaceae EDS5* were retrieved from Phytozome and aligned using MUSCLE.

452 **Accession numbers**

453 The accession numbers for the genes discussed in this article are as follows: *AtActin2*
454 (At3g18780), *AtDDE2* (AT5G42650), *AtCOI1* (AT2G39940), *AtMYC2* (AT1G32640), *AtMYC3*
455 (AT5G46760), *AtMYC4* (At4G17880), *AtEDS5* (AT4G39030), *AtPAD4* (AT3G52430), *AtSID2*
456 (At1g74710), *AtPR1* (At2G14610), *AlActin2* (342019), *AlEDS5* (490671), *AlPAD4* (938122),
457 *EsActin2* (Thhalv10020949m), *EsEDS5* (Thhalv10024859m), *EsPAD4* (Thhalv10011112m),
458 *CrActin2* (Carubv10013961m), *CrEDS5* (Carubv10004548m), *CrPAD4* (Carubv10016970m and
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460

461

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474

475 **Author Contributions**

476 A.M. and K.T. conceived and designed the experiments. A.M., T.N., M.C.S.R., T.M.W., S.A. and
477 K.T. performed the experiments: A.M. and K.T. analyzed and discussed the data. D.B. contributed to
478 generation of the plant materials. A.M. and K.T. wrote the paper.

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481 **Competing interests**

482 The authors have declared that no competing interests exist.

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680

681 **Figure Legends**

682 **Figure 1 - JA is genetically defined as a repressor or activator of SA accumulation depending**

683 **on *PAD4***

684 A Measurement of SA levels in leaves infiltrated with water (mock) or 1 μM flg22 at 9 hpi.

685 Bars represent means and standard errors of the SA levels on a $\log_2$ scale calculated from two

686 independent experiments using a mixed linear model.

687 B-D RT-qPCR analysis of *PR1, At2g26400 and At2g30550* expression in leaves infiltrated with

688 water (mock) or 1 μM flg22 at 9 hpi. Bars represent means and standard errors of the $\log_2$ expression

689 level relative to *Actin2* (*At3g18780*) calculated from three independent experiments using a mixed

690 linear model.

691 Data information: In A-D, the Benjamini–Hochberg method was used to adjust p-values (two-tailed

692 t-tests) for correcting multiple hypothesis testing. Statistically significant differences are indicated by

693 different letters (adjusted p-value < 0.05).

694

695 **Figure 2 - JA represses *PAD4* expression through MYC transcription factors**

696 A RT-qPCR analysis of *PAD4* expression in leaves infiltrated with water (mock) or 1 μM

697 flg22 at 9 hpi. Bars represent means and standard errors of the $\log_2$ expression level relative to

698 *Actin2* calculated from four independent experiments using a mixed linear model.

699 B *PAD4* promoter showing the G box motif located 114 bp upstream of the transcription start

700 site. Bold gray horizontal lines show the regions amplified by different qPCR primers.

701 C, D ChIP-qPCR analysis of MYC2 binding to the *PAD4* promoter. MYC2-GFP seedlings were

702 treated with 1 μM flg22 for the indicated time periods (C) or 100 μM MeJA for 3 hours (D). Bars

703 represent means and standard errors of the fold enrichment relative to the wildtype plants set to 1,

704 calculated from two independent experiments.

705 Data information: In A, the Benjamini–Hochberg method was used to adjust p-values (two-tailed
706 t-tests) for correcting multiple hypothesis testing and statistically significant differences are
707 indicated by different letters (adjusted p-value < 0.05).

708

709 **Figure 3 - MYC2 directly regulates *EDS5* induction by JA**

710 A, B RT-qPCR analysis of *SID2* (A) and *EDS5* (B) expression in leaves infiltrated with water
711 (mock) or 1 μM flg22 at 5 hpi. Bars represent means and standard errors of the $\log_2$ expression
712 levels relative to *Actin2* calculated from four independent experiments using a mixed linear model.

713 C RT-qPCR analysis of *EDS5* expression in seedlings treated with water (mock) or 100 μM
714 MeJA for the indicated time periods. Bars represent means and standard errors of the $\log_2$ expression
715 level relative to *Actin2* calculated from two independent experiments using a mixed linear model.

716 D *EDS5* promoter showing the G box motif located 49 bp upstream of the transcription start
717 site. Bold gray horizontal lines show the regions amplified by different qPCR primers.

718 E, F ChIP-qPCR analysis of MYC2 binding to the *EDS5* promoter. MYC2-GFP seedlings were
719 treated with 1 μM flg22 for the indicated time periods (E) or 100 μM MeJA for 3 hours (F). Bars
720 represent means and standard errors of the fold enrichment relative to the wildtype plants set to 1,
721 calculated from two independent experiments.

722 G Luciferase reporter assays using *EDS5* promoters with or without G box. Luc reporter
723 construct driven by the wild type *EDS5* promoter (*pEDS5*) or the *EDS5* promoter without G box
724 (*pEDS5*_w/oGbox) was transfected with or without 35S-MYC2 plasmid to *Arabidopsis* protoplasts.
725 Bars represent means and standard errors of the Luc activity relative to the internal control (Luc
726 derived from *Renilla* spp. driven by 35S promoter) calculated from three independent experiments
727 each with three biological replicates.

728 Data information: In A-C, the Benjamini–Hochberg method was used to adjust p-values (two-tailed

729 t-tests) for correcting multiple hypothesis testing and statistically significant differences are
730 indicated by different letters (adjusted p-value < 0.05). In E-G, asterisks indicate statistically
731 significant differences from the wildtype (E, F) or from the empty vector control (G) (* P < 0.05, **
732 P < 0.01, *** P < 0.001; two-tailed t-tests).
733
734 **Figure 4 - Reconstitution of *EDS5* expression restores flg22-triggered SA accumulation and**
735 **flg22-PTI in *dde2 pad4***
736 A, B RT-qPCR analysis of *EDS5* (A) and *PR1* (B) expression in leaves of Col, *pad4*, *dde2 pad4,*
737 p35S::*EDS5* lines and a pSID2::*EDS5* line infiltrated with water (mock) or 1 μM flg22. The
738 expression levels of *EDS5* and *PR1* were measured at 5 hpi and 9 hpi, respectively. Bars represent
739 means and standard errors of the $\log_2$ expression levels relative to *Actin2* calculated from two
740 independent experiments using mixed linear models.
741 C Measurement of SA levels in leaves of Col, *pad4*, *dde2 pad4,* p35S::*EDS5* lines and a
742 pSID2::*EDS5* line infiltrated with water (mock) or 1 μM flg22 at 9 hpi. The means and standard
743 errors calculated from two independent experiments using a mixed linear model are shown on a $\log_2$
744 scale.
745 D Bacterial growth assay in leaves of Col, *dde2 pad4,* p35S::*EDS5* lines or a pSID2::*EDS5*
746 line infiltrated with *Pto* (OD600 = 0.0002) together with water (mock) or 1 μM flg22. The bacterial
747 titers at 0 or 2 dpi were measured. Bars represent means and standard errors of two independent
748 experiments with at least 4 or 12 biological replicates for 0 dpi or 2 dpi in each experiment,
749 respectively.
750 Data information: In A-D, the Benjamini–Hochberg method was used to adjust p-values (two-tailed
751 t-tests) for correcting multiple hypothesis testing and statistically significant differences are
752 indicated by different letters (adjusted p-value < 0.05). In D, asterisks indicate statistically

significant differences of the differences (adjusted p-value < 0.05).

Figure 5 – Distinct effects of JA on bacterial resistance depending on *PAD4*

A A model of the incoherent type-4 feed-forward loop consisting of JA, *PAD4* and *EDS5*. The blue line and the red arrow indicate negative and positive effects of JA on the network output, respectively.

B Bacterial growth assay in leaves of Col, *dde2 pad4,* p35S::*EDS5* line #1 and pSID2::*EDS5* line infiltrated with *Pto* (OD600 = 0.0002) and 1 μM flg22 with or without treatment of 1 mM MeJA. The bacterial titers at 2 dpi were measured. Bars represent means and standard errors of three independent experiments each with at least 10 biological replicates.

C Bacterial growth assay in leaves of Col, *dde2*, *pad4* and *dde2 pad4* infiltrated with *Pto* (OD600 = 0.0002) at 22 or 28°C. The bacterial titers at 2 dpi were measured. Bars represent means and standard errors of two (22°C) or three (28°C) independent experiments each with at least 10 biological replicates.

Data information: In B and C, the Benjamini–Hochberg method was used to adjust p-values (two-tailed t-tests) for correcting multiple hypothesis testing and statistically significant differences are indicated by different letters (adjusted p-value < 0.05).

Figure 6 - Conservation and diversification of the transcriptional regulation of *EDS5* and *PAD4* by JA in *Brassicaceae*

A, B RT-qPCR analysis of *EDS5* (A) and *PAD4* (B) expression in seedlings of *A. thaliana*, *A. lyrata*, *C. rubella* and *E. salsugineum.* In A, seedlings were treated with mock (water) or MeJA (100 μM) for the indicated time periods. In B, seedlings were treated with mock (water) or MeJA (100 μM) for 3 hours, followed by treatment with mock (water) or flg22 (1 μM) for 30 minutes. Bars

represent means and standard errors of the $\log_2$ expression levels relative to *Actin2* calculated from two independent experiments using mixed linear models.

Data information: In A, asterisks indicate statistically significant differences from the mock controls at each time point (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; two-tailed t-tests). In B, the Benjamini–Hochberg method was used to adjust p-values (two-tailed t-tests) for correcting multiple hypothesis testing and statistically significant differences were indicated by different letters (adjusted $p\text{-value} < 0.05$).

Expanded View Figure Legends

Figure EV1 - Accumulation of MYC2-GFP protein in the p35S:MYC2-GFP transgenic plants

Total protein was extracted from leaves of 4 to 5-weeks-old plants and separated on an SDS-PAGE gel. The MYC2-GFP protein was detected using an anti-GFP antibody. Ponceau S staining is shown as a loading control.

Figure EV2 - Phylogenetic analysis of putative EDS5 orthologues.

The proteins belonging to the same group as *A. thaliana* EDS5 were identified by OrthoMCL. A maximum likelihood phylogenetic tree was constructed based on the amino acid sequences using the MEGA6 software. The EDS5 clade is highlighted by red lines.

Figure EV3 - Conservation of G boxes in *EDS5* promoters of *Brassicaceae* species

The 500 bp upstream of the transcription start sites of *EDS5* and the 5′UTRs were aligned using MUSCLE. The 5′UTRs were highlighted by gray boxes. The CACGTG G box motif was shown in bold red letters.

Figure EV4 - *C. rubella* is responsive to JA

RT-qPCR analysis of *VSP2* expression in *C. rubella* seedlings after treatment with water (mock) or 100 μM MeJA for the indicated time periods. Bars represent means and standard errors of the $\log_2$ expression levels relative to *Actin2* calculated from two independent experiments using a mixed linear model. Asterisks indicate statistically significant differences compared to the mock controls at each time point (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; two-tailed t-tests).

Table EV1 - Primers used in this study.