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1 **Architecture and dynamics of the jasmonic acid gene regulatory network**

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20 **ABSTRACT**

21

22 Jasmonic acid (JA) is a critical hormonal regulator of plant growth and defense. To advance our  
23 understanding of the architecture and dynamic regulation of the JA gene regulatory network, we  
24 performed high-resolution RNA-Seq time series of methyl JA-treated *Arabidopsis thaliana* at 15  
25 time points over a 16-h period. Computational analysis showed that MeJA induces a burst of  
26 transcriptional activity, generating diverse expression patterns over time that partition into  
27 distinct sectors of the JA response targeting specific biological processes. Presence of  
28 transcription factor (TF) DNA-binding motifs correlated with specific TF activity in temporal  
29 MeJA-induced transcriptional reprogramming. Insight into underlying dynamic transcriptional  
30 regulation mechanisms was captured in a chronological model of the JA gene regulatory  
31 network. Several TFs, including MYB59 and bHLH27, were uncovered as early network  
32 components with a role in pathogen and insect resistance. Analysis of subnetworks surrounding  
33 the TFs ORA47, RAP2.6L, MYB59 and ANAC055, using transcriptome profiling of  
34 overexpressors and mutants, provided novel insights into their regulatory role in defined modules  
35 of the JA network. Collectively, our work illuminates the complexity of the JA gene regulatory  
36 network, pinpoints and validates novel regulators, and provides a valuable resource for  
37 functional studies on JA signaling components in plant defense and development.

38

39

## 40 INTRODUCTION

41

42 In nature, plants are subject to attack by a broad range of harmful pests and pathogens. To  
43 survive, plants have evolved a sophisticated immune signaling network that enables them to  
44 mount an effective defense response upon recognition of invaders. The phytohormone jasmonic  
45 acid (JA) and its derivatives are key regulators in this network and are typically synthesized in  
46 response to insect herbivory and infection by necrotrophic pathogens (Wasternack, 2015).  
47 Enhanced JA production mediates large-scale reprogramming of the plant's transcriptome, which  
48 is influenced by the antagonistic or synergistic action of other hormones produced during  
49 parasitic interactions, such as salicylic acid (SA), ethylene (ET) or abscisic acid (ABA) (Pieterse  
50 et al., 2012; Campos et al., 2014; Wasternack, 2015). The JA signaling network coordinates the  
51 production of a broad range of defense-related proteins and secondary metabolites, the  
52 composition of which is adapted to the environmental context and nature of the JA-inducing  
53 condition (Pieterse et al., 2012; Campos et al., 2014; Wasternack, 2015).

54 In the past decade, major discoveries in the model plant *Arabidopsis thaliana* have  
55 greatly advanced our understanding of the JA signaling pathway. In the absence of an invader,  
56 when JA levels are low, activation of JA responsive gene expression is constrained by repressor  
57 proteins of the JASMONATE ZIM-domain (JAZ) family that bind to specific JA-regulated  
58 transcription factors (TFs). The conserved C-terminal JA-associated (Jas) domain of JAZs  
59 competitively inhibits interaction of the TF MYC3 with the MED25 subunit of the  
60 transcriptional Mediator complex (Zhang et al., 2015). Moreover, JAZs recruit the TOPLESS  
61 corepressor, either directly or through the NOVEL INTERACTOR OF JAZ (NINJA) adapter,  
62 which epigenetically inhibits expression of TF target genes. In response to pathogen or insect

63 attack, bioactive JA-Isoleucine (JA-Ile) is synthesized, which promotes the formation of the  
64 coreceptor complex of JAZ (via its Jas domain) with CORONATINE INSENSITIVE1 (COI1),  
65 the F-box protein of the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF<sup>COI1</sup>. Upon  
66 perception of JA-Ile, JAZ repressor proteins are then targeted by SCF<sup>COI1</sup> for ubiquitination and  
67 subsequent proteasomal degradation (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010).  
68 This leads to the release of JAZ-bound TFs and subsequent induction of JA-responsive gene  
69 expression.

70         Several groups of TFs are known to be important for regulation of the JA pathway. Upon  
71 degradation of JAZs, MYC2 acts in concert with the closely related bHLH TFs MYC3 and  
72 MYC4 in activating a large group of JA-responsive genes by directly targeting their promoters  
73 (Dombrecht et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011). While current  
74 evidence indicates that MYC2, MYC3 and MYC4 act as master regulators of the onset of JA  
75 responsive gene expression, additional factors are required for further fine-regulation of the JA  
76 signaling circuitry. Several other bHLH TFs, such as JASMONATE-ASSOCIATED MYC2-  
77 LIKE1 (JAM1)/bHLH017, JAM2/bHLH013, JAM3/bHLH003 and bHLH014 act redundantly to  
78 repress JA-inducible genes by competitive binding to *cis*-regulatory elements, possibly to control  
79 the timing and magnitude of the induced JA response (Nakata et al., 2013; Sasaki-Sekimoto et  
80 al., 2013; Song et al., 2013). Another important family of regulators that shape the JA response is  
81 the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of TFs. AP2/ERF-type  
82 TFs, such as ERF1 and ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF-  
83 domain protein59), integrate the JA and ET response pathways and act antagonistically on  
84 MYC2,3,4-regulated JA-responsive genes (Lorenzo et al., 2003; Pré et al., 2008; Zhu et al.,  
85 2011; Pieterse et al., 2012). In general, AP2/ERF-regulated JA responses in the ERF branch of

86 the JA pathway are associated with enhanced resistance to necrotrophic pathogens (Berrocal-  
87 Lobo et al., 2002; Lorenzo et al., 2003), whereas the MYC TF-regulated JA responses in the  
88 MYC branch of the JA pathway are associated with the wound response and defense against  
89 insect herbivores (Lorenzo et al., 2004; Kazan and Manners, 2008; Verhage et al., 2011).

90 A detailed understanding of how responsiveness to JA is regulated is important in order  
91 to find leads that can improve crop resistance to pathogens and insects, while maintaining plant  
92 growth. Previously, several microarray-based transcriptome profiling studies revealed important  
93 information on the regulation of JA-responsive gene expression (Goda et al., 2008; Pauwels et  
94 al., 2008). However, because these studies analyzed this response at limited temporal resolution,  
95 much has remained unknown about the architecture and dynamics of the JA gene regulatory  
96 network. Here, we performed an in-depth, high-throughput RNA sequencing (RNA-Seq) study in  
97 which we generated a high-resolution time series of the JA-mediated transcriptional response in  
98 leaf number 6 of *Arabidopsis* plants. Computational analysis of the JA-induced transcriptional  
99 landscape provided insight into the structure of the JA gene regulatory network at an  
100 unprecedented level of detail. We accurately identified distinct JA-induced expression profiles,  
101 and used these to predict and validate the biological function of several novel regulators of the  
102 JA immune regulatory network. We resolved the sequence of transcriptional events that take  
103 place following induction of the JA response, constructed a dynamic model of the JA gene  
104 regulatory network, and identified and validated subnetworks surrounding several JA-induced  
105 TFs, confirming the suitability of our systems approach to obtain detailed knowledge on  
106 regulation of the JA response pathway.

107

## 108 RESULTS

109

## 110 **A time course of MeJA-elicited transcriptional reprogramming**

111 A key step towards a systems-level understanding of the architecture of the JA signaling network  
112 is to obtain comprehensive and accurate insight into the dynamic transcriptional reprogramming  
113 that takes place in plants following JA stimulation. To go beyond earlier studies that analyzed the  
114 JA transcriptional response with a limited number of time points, we generated a high-resolution  
115 time series of JA-mediated transcriptional reprogramming in *Arabidopsis* leaves. Previously,  
116 similar types of dense time series experiments with *Arabidopsis* have been successfully utilized  
117 to help decipher gene regulatory networks underpinning a variety of biological processes, such as  
118 senescence and responsiveness to infection by *Botrytis cinerea* and *Pseudomonas syringae*  
119 (Breeze et al., 2011; Windram et al., 2012; Lewis et al., 2015). Here, we used RNA-Seq  
120 technology to profile whole-genome transcriptional expression in *Arabidopsis* leaves just before  
121 the treatments ( $t = 0$  h), and over 14 consecutive time points within 16 h following application of  
122 methyl JA (MeJA; that is readily converted to JA) or a mock solution to the leaves of intact  
123 plants (Supplemental Dataset 1). At all time points and for each treatment, one leaf (true leaf  
124 number 6) was sampled in quadruplicate from four independent 5-week-old Col-0 plants,  
125 yielding 116 samples in total (Supplemental Dataset 1). Read counts were normalized for  
126 differences in sequencing depth between samples (Supplemental Dataset 2) and a generalized  
127 linear model was employed to identify genes whose transcript levels differed significantly over  
128 time between MeJA and mock treatments (see Van Verk et al. (2013) and Methods for details).  
129 This analysis yielded a set of 3611 differentially expressed genes (DEGs; Supplemental Dataset  
130 3). Many of these DEGs were not previously described as MeJA responsive (Figure 1A) in  
131 experiments where MeJA was applied to cell cultures or seedlings and 3 time points were

132 analyzed (Goda et al., 2008; Pauwels et al., 2008). Among the different genes are 596 genes that  
133 are not represented on the ATH1 microarray used in these earlier studies. Comparison of our  
134 DEGs set with that of an experiment in which *Arabidopsis* leaves were fed on by the JA-  
135 inducing insect herbivore *Pieris rapae* revealed an overlap of 49% (Coolen et al., 2016) (Figure  
136 1A), indicating that the transcriptional changes elicited by exogenously applied MeJA in this  
137 study are biologically relevant.

138 Our high-resolution temporal transcriptome data captured a diverse set of dynamical  
139 responses to MeJA stimulation (Supplemental Figure 1). **The majority of expression changes in**  
140 **individual genes followed a clear single-pulse (impulse) pattern, that is often observed in**  
141 **responses to environmental stress in eukaryotic cells, and coordinates the temporal regulation of**  
142 **specific gene expression programs (Yosef and Regev, 2011).** Examples of genes whose  
143 expression is up- or down-regulated for a short period of time **followed by a transition to a steady**  
144 **state, which is often a return to basal expression,** are *JAR1* and *EDSI* (Figure 1B). Yet, there are  
145 also genes that display a longer lasting change in expression level, e.g. *MYC2* and *BESI* (Figure  
146 1B). Because all transcriptional changes were monitored in leaf number 6, we maximally  
147 synchronized the onset of the JA response in intact plant tissue. Hence, the resulting information-  
148 rich time series of MeJA-responsive gene expression profiles are highly suited to computational  
149 approaches that can generate novel biological insights into the regulation of the underlying JA  
150 transcriptional network.

151

### 152 **Process-specific gene clusters**

153 To begin to decode the JA gene regulatory network, the time series-clustering algorithm  
154 SplineCluster was used to partition the set of 3611 DEGs into clusters of co-expressed genes **that**



155 share similar expression dynamics. This yielded 27 distinct clusters with distinct response  
156 patterns (Figure 2A, Supplemental Figure 1, Supplemental Dataset 4), which broadly fall into  
157 two major groups: those that show increased expression in response to application of MeJA  
158 (cluster 1-14), and those that exhibit reduced expression (cluster 15-27). The cluster analysis  
159 highlights a global burst of MeJA-induced up- or down-regulation of gene transcription,  
160 generally starting within 1 h and peaking within 2 h after treatment. Most clusters show a clear  
161 pulse-like, transient change in transcript levels (e.g. cluster 8 and 18, up- and down-regulation,  
162 respectively). A largely sustained induction throughout the time course is displayed in for  
163 example clusters 1 and 2. More complex expression patterns are also revealed; cluster 14  
164 presents two consecutive pulses of activation.

165 The genes in each cluster were tested for overrepresented functional categories using  
166 Gene Ontology (GO) term enrichment analysis to investigate the biological significance of the  
167 distinct dynamic expression patterns (Supplemental Dataset 5). This analysis showed that  
168 clusters representing up-regulated genes are, as expected, overrepresented for functional terms  
169 associated with JA defense responses. Broad annotations such as ‘Response to wounding’ and  
170 ‘Response to herbivory’ are present in multiple up-regulated clusters, while in contrast the more  
171 specific functional categories are linked to distinct clusters. For example, cluster 6 is specifically  
172 overrepresented for the annotation term ‘Anthocyanin-containing compound biosynthetic  
173 process’, cluster 8 for ‘Tryptophan biosynthetic process’, and cluster 14 for ‘Glucosinolate  
174 biosynthetic process’. Each of these clusters contains many of the genes previously implicated in  
175 these secondary metabolite biosynthesis pathways, but also uncharacterized genes which may  
176 have an important function in these specific processes (Supplemental Dataset 5). The significant  
177 enrichment of distinct gene clusters for a specific biological process indicates that the dynamic

178 expression profiles generated in this study possess information that is sufficiently detailed to  
179 capture discrete sectors of the JA-controlled gene network that control specific processes. These  
180 sectors are likely subject to distinct regulation encoded within the promoters of the genes in the  
181 respective clusters.

182 To facilitate the use of the expression data for the *Arabidopsis* community, a searchable  
183 (by gene ID) figure has been made available that visualizes co-expression relationships in time  
184 for all DEGs in the individual clusters (Supplemental Figure 2).

185

### 186 **Discovery of novel defense regulators**

187 Since TFs are the main drivers of transcriptional networks, we mapped the TF families that are  
188 enriched in the 27 clusters of MeJA-responsive DEGs. Within the up-regulated clusters, genes  
189 encoding members of the bHLH, ERF and MYB TF families were most significantly  
190 overrepresented (Figure 2B), suggesting that these TF families dominate the onset of JA-induced  
191 gene expression.

192 The early up-regulated gene clusters 1 and 2 (61 and 165 genes, respectively) contained  
193 an enrichment for known JA-related genes such as the herbivory markers *VSP1* and *VSP2*, as  
194 well as the regulators *JAZ1*, 2, 5, 7, 8, 9, 10 and 13, *MYC2*, *ANAC019*, *ANAC055*, *RGL3*, and  
195 *JAMI* (Wasternack and Hause, 2013). In addition, TF genes with no previously reported roles in  
196 the JA response pathway are present in these clusters, which implies that they may also have  
197 regulatory functions in the JA response relevant to plant defense. To test this hypothesis, we  
198 selected 7 uncharacterized TF genes from clusters 1 and 2 and supplemented this set with 5  
199 uncharacterized TF genes from other clusters, displaying a similarly rapid response to MeJA  
200 treatment. The respective *Arabidopsis* T-DNA knockout lines were functionally analyzed for

201 their resistance against the necrotrophic fungus *Botrytis cinerea* and the generalist insect  
202 herbivore *Mamestra brassicae*, which are both controlled by JA-inducible defenses (Pieterse et  
203 al., 2012). Mutants in the TF genes *bHLH27*, *ERF16* and *MYB59* displayed a significant increase  
204 in disease susceptibility to *B. cinerea* compared to wild-type *Arabidopsis* Col-0, approaching the  
205 disease severity level of the highly susceptible control mutant *ora59* (Figure 2C; full results in  
206 Supplemental Figure 3 and additional mutant alleles in Supplemental Figure 4). Weight gain of  
207 *M. brassicae* larvae was significantly reduced on mutants of *ANAC056* and *bHLH27*, while on  
208 none of the tested mutants larval weight was enhanced, as was the case on the susceptible control  
209 mutant *myc2,3,4* (Figure 2D; full results and additional mutant alleles in Supplemental Figure 4  
210 and 5). Thus, for 4 of the 12 tested MeJA-responsive, previously uncharacterized TF genes a  
211 predicted role in the JA response could be functionally validated for either *B. cinerea* or *M.*  
212 *brassicae* resistance, demonstrating the value of using information-rich time series data to  
213 accurately identify co-expressed genes that may have novel functions in the JA pathway.

214

#### 215 **Contrasting role in pathogen and insect defense by redundant gene pair *MYB48/MYB59***

216 Many TFs originate from duplication events and have overlapping or even redundant  
217 functionality, so that their single mutants may not display the full effects on host immunity in the  
218 above-described analyses. Therefore, we additionally assayed a double mutant of a pair of  
219 genetically unlinked paralogous genes, *MYB48* and *MYB59* (Bolle et al., 2013) to uncover  
220 phenotypes not seen in either single mutant. This can provide further insight into the  
221 functionality of these TFs. The TF gene *MYB59* was upregulated within 30 minutes after  
222 application of MeJA and although the single mutant *myb59* displayed enhanced susceptibility to  
223 *B. cinerea* (Figure 2C and 2E), it was unaffected in resistance to *M. brassicae* (Figure 2D and

224 2F). *MYB48* was transiently downregulated by MeJA, but the single mutant *myb48* did not show  
225 altered resistance to either *B. cinerea* or *M. brassicae* (Figure 2E and 2F). In contrast, the  
226 *myb48myb59* double mutant was highly resistant to *M. brassicae*, reducing the larval growth 5-  
227 fold in comparison to Col-0 and the single mutants. Moreover, the double mutant displayed  
228 significantly more severe disease symptoms following infection by *B. cinerea* than each of the  
229 single mutants. This suggests that MYB48 and MYB59 function in concerted action as negative  
230 regulators of insect resistance and positive regulators of necrotrophic pathogen resistance.

231 To gain insight into the biological processes contributing to the differentially altered  
232 attacker performance on *myb48myb59*, we performed RNA-Seq analysis on the double mutant. A  
233 total of 399 genes were differentially expressed between non-stimulated *myb48myb59* and Col-0  
234 leaves (168 were up-regulated and 231 were down-regulated in the double mutant; Supplemental  
235 Dataset 6). Functional category analysis showed that in the up-regulated DEG set of the mutant  
236 compared to Col-0, processes like ‘Response to wounding’ and ‘Response to jasmonic acid  
237 stimulus’ were enriched (Supplemental Dataset 7). This is in accordance with these *myb48my59*-  
238 upregulated DEGs being overrepresented in co-expression clusters 1, 2, 7 and 9 of the MeJA  
239 responsive DEGs (Figure 2G). Genes that showed enhanced expression by both MeJA treatment  
240 and the *myb48myb59* mutations are for example JA biosynthetic genes *AOC2* and *OPR3*, and TF  
241 gene *MYC2*. Also the downstream herbivore defense marker gene *VSP2* showed > 50-fold higher  
242 expression level in the mutant. This suggests prioritization of the JA pathway towards the anti-  
243 insect MYC branch in *myb48myb59*, explaining its enhanced resistance to *M. brassicae*.  
244 However, MYC branch-mediated antagonism of the ERF branch of the JA pathway, which  
245 would explain the reduction of defense against the necrotrophic pathogen *B. cinerea*, is not  
246 apparent from our transcriptome data. It may be that MYB48/59-regulated genes that are enriched

247 for ‘Secondary metabolite biosynthetic processes’ (represented by clusters 17-19, 21 and 25) and  
248 are down-regulated in the mutant are important for resistance to *B. cinerea*. This example  
249 demonstrates that higher-order mutants can reveal important gene regulatory functions that  
250 would otherwise be masked by genetic redundancy.

251

### 252 **Enrichment of TF DNA-binding motifs**

253 TFs regulate gene expression by binding to *cis*-regulatory elements of target genes in a sequence  
254 specific manner. Mapping of regulatory DNA motifs that are associated with dynamic MeJA-  
255 responsive gene expression profiles can aid in the understanding and reconstruction of JA gene  
256 regulatory networks. Therefore, we investigated which *Arabidopsis* TF-binding site motifs are  
257 overrepresented within the promoters of co-expressed MeJA-responsive DEGs, using recently  
258 identified DNA-binding specificities for 580 *Arabidopsis* TFs derived from studies with protein-  
259 binding microarrays (PBMs) (Franco-Zorrilla et al., 2014; Weirauch et al., 2014). First, we  
260 screened for overrepresentation of these motifs in the unions of up- and down-regulated gene  
261 clusters, respectively (Figure 3A). Motifs corresponding to DNA-binding sites of bHLH, bZIP,  
262 ERF and MYB TFs are clearly overrepresented in the group of up-regulated genes, while WRKY  
263 and TCP TF specific motifs are markedly overrepresented in the down-regulated genes.  
264 Members of the WRKY TF family and their cognate *cis*-elements are key regulators of the SA  
265 response pathway (Pandey and Somssich, 2009), suggesting that WRKYs are important targets  
266 in the transcriptional repression of the SA pathway by MeJA treatment. Secondly, we analyzed  
267 motif enrichment within each of the 27 clusters of co-expressed genes (Figure 3B). To increase  
268 the chance of discovering nuanced sequence motifs among the genes in these clusters, we  
269 supplemented the known motif analysis (Supplemental Dataset 8) with *de novo* motif discovery

270 (Supplemental Dataset 9 and 10). This revealed promoter elements that are selectively enriched  
271 in specific clusters, offering a more precise link between motifs and cluster-specific gene  
272 expression patterns. Strikingly, while motifs that correspond to bHLH-binding sites are enriched  
273 in the majority of the up-regulated gene clusters, ERF- and MYB-binding motifs are only  
274 overrepresented in a small selection of the up-regulated clusters, which are associated with  
275 specific biological processes (Supplemental Dataset 8). For example, clusters 6 and 14, which  
276 are enriched for GO terms describing distinct secondary metabolite biosynthesis pathways, are  
277 enriched for different (*de novo*) predicted MYB DNA-binding motifs (Figure 3B). These  
278 findings suggest that bHLH TFs and their DNA-binding sites are essential components in  
279 activation of the majority of the MeJA-inducible genes, while ERF and MYB TFs have more  
280 specialized roles in modulating the expression of dedicated sets of target genes.

281

### 282 **Chronology of MeJA-mediated transcriptional reprogramming**

283 Next, we utilized the temporal information in our RNA-Seq time series to resolve the chronology  
284 of gene expression events in the JA gene regulatory network. First, we divided the genes in sets  
285 of up- and down-regulated DEGs and sorted them according to the time at which they first  
286 became differentially expressed (Supplemental Figure 6; see Methods for details). From this  
287 analysis, it became clear that a massive onset of gene activation precedes that of gene down-  
288 regulation, and that different waves of coordinated gene expression changes can be identified in  
289 the time series. The majority of all DEGs become first differentially expressed within 2-4 h after  
290 MeJA treatment, which indicates engagement of relatively short transcriptional cascades,  
291 allowing for a rapid response to an external signal (Alon, 2007). Up- and down-regulated DEGs  
292 were then further separated into two additional sets based on their predicted function as

293 transcriptional regulators (termed regulator genes) or as having a different function (termed  
294 regulated genes; Supplemental Dataset 3). We were specifically interested in identifying time  
295 points where coordinated switches in transcriptional activity take place, reasoning that pairs of  
296 adjacent time points that display a weaker correlation indicate important points of coordinated  
297 switches in transcriptional activity (see Methods and Supplemental Figure 7 for details).  
298 Therefore, within each of the four mutually exclusive gene sets, we examined the pairwise  
299 correlations of expression levels between all pairs of time points. Clustering of the resulting  
300 correlation matrices revealed six distinct phases in transcriptional activation, and four phases in  
301 transcriptional repression (Figure 4A). The first two phases of up-regulation (Phase Up1 and  
302 Up2) start within 0.5 h after MeJA treatment in the set of regulator genes, while at 1.5 h a third  
303 phase of up-regulation of regulator genes ensues (phase Up4). For the regulated genes the first  
304 phase of up-regulation starts at 1 h after MeJA treatment (phase Up3), which is clearly later than  
305 the first onset of the regulator genes. A similar sequence of events can be observed in the down-  
306 regulated regulator and regulated genes, although the start is delayed compared to the activation  
307 of up-regulated genes.

308 **Our time series captures the temporal association between the changes in transcript**  
309 **abundance of transcriptional regulators and downstream targets encoding proteins responsible for**  
310 **the biochemical reactions that represent the defensive outputs of the JA response.** To explore the  
311 biological significance and directionality in the regulation of the identified transcriptional phases  
312 in the JA gene regulatory network, all DEGs were assigned to the phase in which they first  
313 became differentially expressed (see Methods and Supplemental Figure 7 for details). The  
314 resulting gene lists of the 10 transcriptional phases were tested for overrepresentation of  
315 functional categories and promoter motifs (Figure 4B; Supplemental Dataset 11-14). Phase Up1

316 represents the immediate transcriptional response with genes encoding bHLHs, JAZs, MYBs,  
317 ERFs, and other transcriptional regulators associated with JA biosynthesis. These early regulator  
318 genes may play a role in the induction of other regulator-encoding genes present in phases Up2  
319 and 4, and of regulated genes present in phases Up3, 5 and 6, which are linked to defense  
320 responses such as glucosinolate, tryptophan and anthocyanin biosynthesis (Figure 4B;  
321 Supplemental Dataset 12). In support of this, in the promoters of DEGs in phase Up3, DNA  
322 motifs that can be bound by TFs transcribed in previous phases Up1 and 2, like bHLH-, ERF-  
323 and MYB-binding motifs, are enriched. In phase Up3, genes involved in JA biosynthesis are also  
324 enriched, suggesting that this process is one of the first targets of JA-mediated transcriptional  
325 reprogramming. Overall, induction of the JA pathway shows a clear chronology of up-regulated  
326 gene expression events, starting with the activation of genes encoding specific classes of TFs and  
327 of JA biosynthesis enzymes, followed by genes encoding enzymes involved in the production of  
328 important defensive secondary metabolites.

329         The first wave of transcriptional repression by MeJA is also marked by genes encoding  
330 transcriptional regulators, and begins at 1 h after MeJA treatment, after which phases Down2, 3  
331 and 4 follow at 2, 3 and 4 h after MeJA treatment, respectively (Figure 4B; Supplemental Dataset  
332 11). These groups of down-regulated genes highlight the antagonistic effects of JA on other  
333 hormone signaling pathways and defense responses in the first two phases. Phase Down1 for  
334 instance is characterized by the repression of different defense-related genes such as *NPR4* and  
335 *MYB51*, which encode regulators that promote SA responses and indolic glucosinolate  
336 biosynthesis, respectively (Gigolashvili et al., 2007; Fu et al., 2012). Accordingly, *MYC2*, which  
337 is induced by MeJA in phase Up1, was previously shown to suppress the accumulation of indolic  
338 glucosinolates (Dombrecht et al., 2007). Phase Down2 is also enriched for genes associated with



339 SA-controlled immunity, including the key immune-regulators *EDSI* and *PAD4* (Feys et al.,  
340 2001). In line with these observations, there is an overrepresentation for WRKY-binding motifs  
341 in the promoters of genes present in phase Down 1 and 2, suggesting that their repressed  
342 expression is mediated by an effect of MeJA on WRKY action. Later phases of transcriptional  
343 repression (phases Down3 and 4) are marked by an overrepresentation of genes related to growth  
344 and development, including primary metabolism and auxin signaling, and an enrichment of DNA  
345 motifs recognized by TCP TFs, which conceivably reflects an effort by the plant to switch  
346 energy resources from growth to defense (Attaran et al., 2014). [A general observation that can be  
347 made from this chronological analysis of the JA gene regulatory network is that despite the  
348 overall relatively short transcriptional cascades controlling gene activation or repression,  
349 distinctive transcriptional signatures, associated with specific biological processes, are initiated  
350 at different phases in time.](#)

351

### 352 **Inference of regulatory interactions reveals key regulators of local JA subnetworks**

353 Next, we made use of the TF DNA-binding motif information of the genes in the temporally  
354 separated transcriptional phases to construct a gene regulatory network that predicts directional  
355 interactions between the JA responsive TF genes and all genes associated with the different  
356 transcriptional phases (Supplemental Dataset 15). The JA gene regulatory network generated via  
357 this analysis is shown in Figure 5, in which a differentially expressed TF gene (represented by a  
358 circular node in the network) is connected by an edge to a transcriptional phase (represented by a  
359 rectangle in the network) when the corresponding DNA-binding motif is overrepresented in that  
360 phase. The generated network model shows that the TFs are predicted to regulate expression of  
361 genes at either single or multiple transcriptional phases. The early phases likely contain key

362 regulators of subsequent phases. Phase Up1 contains the TFs MYC2 and JAM1, which are  
363 among the most active TFs, as their cognate DNA-binding motifs (both share the same  
364 consensus, CACGTG) are enriched in the promoters of genes assigned to a large fraction of the  
365 up-regulated transcriptional phases. This prediction is in line with recent reports suggesting that  
366 the positive regulator MYC2 and the negative regulator JAM1 cooperate to balance JA responses  
367 by competitive binding to their shared target sequences (Nakata et al., 2013; Sasaki-Sekimoto et  
368 al., 2013). What determines the different timing by these regulators to effectively activate or  
369 repress transcription awaits further investigation. Phases Up1 and Up2 also contain the TF genes  
370 *bHLH27*, *ERF16*, *ANAC056* and *MYB59*, of which corresponding mutants showed altered  
371 resistance levels to *B. cinerea* infection and/or *M. brassicae* infestation (Figure 2C and 2D).  
372 **Cognate DNA-binding motifs of these TF families are enriched in genes that are induced in**  
373 **multiple subsequent transcriptional phases (Figure 4B and 5).**

374 Phase Up1 also contains TF genes that are predicted to have a more limited regulatory  
375 scope, such as the ERF TF gene *ORA47*, of which the binding motif (consensus, CCG(A/T)CC)  
376 is only overrepresented in the promoters of genes assigned to phase Up3. These genes include  
377 the JA biosynthesis genes *LOX2*, *AOS*, *AOC1,2,3*, *ACX* and *OPR3*, thus suggesting that this *cis*-  
378 element and its cognate TF *ORA47* may play a role in regulating JA production, which reflects  
379 the positive feedback loop that is known to maintain and boost JA levels upon initiation of the  
380 JA response (Wasternack, 2015). Focusing on this predicted subnetwork (Figure 6A), we found  
381 that *ORA47* and several of the JA biosynthesis genes were predicted to be targets of MYC2,  
382 suggesting that MYC2 together with *ORA47* regulates JA biosynthesis in *Arabidopsis*. Figure  
383 6B shows that the presence of the *ORA47*-binding motif is conserved between the promoters of  
384 *AOS*, *AOC2*, *OPR3* and *LOX3* orthologs of field mustard (*Brassica rapa*), grape (*Vitis vinifera*),

385 and poplar (*Populus trichocarpa*), pointing to a role for ORA47 and its cognate binding element  
386 in the regulation of JA biosynthesis genes. Evidence for this is provided by the direct binding of  
387 ORA47 to promoter elements of *AOCI*, *AOC3* and *LOX3*, as demonstrated by yeast one-hybrid  
388 experiments (Supplemental Figure 8). Moreover, in stimulated  $\beta$ -estradiol-inducible *ORA47*  
389 plants expression of *LOX2*, *LOX3*, *AOS*, *AOCI*, *AOC2* and *OPR3* was increased and  
390 accumulation of JA and JA-Ile was also enhanced (Figure 6C and 6D), which is in line with and  
391 extends previous findings (Pauwels et al., 2008; Chen et al., 2016). We did not observe a  
392 significant increase in expression of *JAR1*, encoding the enzyme responsible for catalyzing  
393 conjugation of JA with isoleucine, suggesting that basal JAR1 levels are sufficient for the  
394 conversion of excess JA into biologically active JA-Ile. Taken together, these experimental  
395 results confirm our model prediction that ORA47 is an important regulator of JA biosynthesis  
396 and highlight the potential of combining time series expression data with motif analysis to infer  
397 novel key regulators and their targets in gene regulatory networks.

398 For the vast majority of TFs in our chronological model, it is unclear which specific JA-  
399 responsive genes they regulate. To validate and extend our chronological network model further,  
400 we made use of transcriptome data sets of three *Arabidopsis* lines that are perturbed in TFs that  
401 are predicted by our model to regulate downstream subnetworks. We investigated the effect of  
402 the TFs RAP2.6L and ANAC055, which have previously been suggested to regulate JA-  
403 responsive genes among others (Bu et al., 2008; Krishnaswamy et al., 2011), by studying their  
404 target genes in *RAP2.6L*-overexpressing and *anac055* mutant *Arabidopsis* lines. Moreover, we  
405 used the transcriptome data derived from the *myb48myb59* mutant analysis, described in Figure  
406 2G. We performed transcriptional profiling of leaves from plants overexpressing *RAP2.6L*  
407 (*RAP2.6L*-OX) under non-stress conditions, leading to the identification of 93 DEGs

408 (Supplemental Dataset 16). Of these, a significant portion of 31 DEGs ( $P < 3.59e-05$ ;  
409 hypergeometric test) was also differentially expressed in the MeJA time series. Projecting the  
410 common set of DEGs onto the transcriptional network model revealed that >90% of these genes  
411 are present in transcriptional phases that are temporally downstream of the phase containing  
412 *RAP2.6L* (phase-Up2, Figure 6E). Analysis of the overlap between *RAP2.6L*-OX DEGs and the  
413 MeJA-induced co-expression clusters from the present study revealed a specific enrichment for  
414 *RAP2.6L* targets in cluster 14, which as described above is itself overrepresented for genes  
415 associated with aliphatic glucosinolate production. Interestingly, a recent study showed that  
416 *RAP2.6L* can interact with several aliphatic glucosinolate biosynthetic gene promoters and  
417 moreover, that *rap2.6l* mutants are perturbed in glucosinolate production (Li, 2014).

418 Using a similar approach, 56 genes differentially expressed in an *anac055* mutant line  
419 compared to wild-type plants (described previously in Hickman et al. (2013)) were overlaid on  
420 the JA gene regulatory network. The overlap between MeJA-responsive and ANAC055-  
421 regulated genes was statistically significant (24 DEGs,  $P < 4.74e-10$ ; hypergeometric test) and >  
422 85% of these genes became for the first time differentially expressed after *ANAC055* was  
423 induced by MeJA (phase-Up2, Supplemental Figure 9). Down-regulated gene co-expression  
424 cluster 20 is overrepresented for ANAC055 targets that are enhanced in the *anac055* mutant, and  
425 is enriched for GO terms related to SA biosynthesis. Interestingly, ANAC055 has previously  
426 been shown to target SA biosynthetic and metabolic genes to negatively regulate SA  
427 accumulation following induction by the bacterial toxin coronatine (Zheng et al., 2012).  
428 Analogously, we also projected the 399 genes that were differentially expressed in the  
429 *myb48myb59* double mutant line compared to Col-0 wild type (as described above; Supplemental  
430 Dataset 6) on the JA gene regulatory network model. The overlap between MeJA-responsive and

431 MYB48/59-regulated genes was highly significant (164 DEGs,  $P < 2.2e-16$ ; hypergeometric test)  
432 and the vast majority of these genes were first differentially expressed after induction of *MYB48*  
433 and *MYB59* by MeJA treatment (Supplemental Figure 10). This suggests that these DEGs may  
434 be downstream targets of MYB48/MYB59 activity during induced JA signaling. This is  
435 confirmed by the enrichment of the MYB-binding motif in the promoter sequences of the down-  
436 regulated DEG set, while the enrichment in the up-regulated DEGs for the bHLH-binding motif  
437 suggests a role for MYB48/MYB59 in attenuation of the MYC branch of the JA pathway.

438 Collectively, analysis of the transcriptomes of *RAP2.6L-OX*, *anac055* and *myb48myb59*  
439 suggests that in the context of the JA gene regulatory network, the studied TFs play a role in  
440 specific biological processes by specific gene targeting. Thus, these three examples demonstrate  
441 the value of leveraging TF perturbation transcriptome data with our information-rich MeJA-  
442 induced dataset to begin to explore specific transcriptional subnetworks, which better define the  
443 mechanistic function of individual TFs, and aids the holistic understanding of the JA gene  
444 regulatory network.

445

## 446 **DISCUSSION**

447 Computational analyses of high-density time series of RNA-Seq data obtained from *Arabidopsis*  
448 leaves of the same developmental stage (leaf number 6), allowed us to provide an  
449 unprecedentedly detailed insight into the architecture and dynamics of the JA gene regulatory  
450 network. Previously, studies on phytohormone-induced transcriptional responses have typically  
451 included only a limited number of time points or focused on the effect of perturbation of specific  
452 regulatory proteins on transcriptional activity in hormone-controlled gene regulatory networks  
453 (Tsuda et al., 2009; Nakata et al., 2013). **Our time series study shows that MeJA induces a burst**

454 of transcriptional activity that generates a variety of detailed temporal expression patterns that  
455 partition into specific gene clusters representing different biological processes (Figure 1, 2 and 4;  
456 Supplemental Figure 1 and 6). Differential expression analysis yielded a considerably more  
457 comprehensive MeJA-responsive gene set compared to previous transcriptomic studies (Figure  
458 1), including a significant number of genes not represented on microarrays. In turn, this  
459 information yielded novel insights into the chronology and regulation of the biologically relevant  
460 JA response.

461

### 462 **Network-informed discovery of novel players in the JA response**

463 Using a dynamic network approach, we systematically determined how the diverse positive and  
464 negative regulatory components in the JA gene regulatory network function over time. MeJA-  
465 induced gene activation or repression is shown to be controlled by short transcriptional cascades,  
466 yet yielding distinctive transcriptional signatures that correspond to specific sets of genes and  
467 biological processes (Figure 2). In general, it appears that bHLH TFs are master regulators  
468 controlling the majority of the MeJA-inducible genes, while ERF and MYB TFs fine-tune the  
469 expression of dedicated sets of target genes in specific sectors of the gene regulatory network  
470 (Figure 2, 3 and 4). Besides the known regulators of the JA pathway, several other TFs, whose  
471 functions were not previously linked to JA responses, were identified in the network. By using a  
472 guilt-by-association approach, twelve early MeJA-induced TFs with unknown roles in the JA  
473 response were selected for validation of their biological function in pathogen or insect resistance.  
474 Four of these (bHLH27, ERF16, MYB59, and ANAC056) were found to play a role in resistance  
475 against the pathogen *B. cinerea* and/or the insect *M. brassicae* (Figure 2), highlighting the high  
476 success rate of our approach in the discovery of biological functions of novel genes in the JA

477 network. Collectively, our gene perturbation data provide an important starting point for the  
478 characterization of so far unexplored components of the JA gene regulatory network, while  
479 numerous other early- and late-expressed TF or enzyme-encoding genes still await further  
480 exploration for functionality.

481 Mutants in *bHLH27* and the double mutant corresponding to *MYB48/59* were more  
482 susceptible to *B. cinerea*, yet more resistant to *M. brassicae* (Figure 2). Although this  
483 necrotrophic pathogen and chewing insect both stimulate JA biosynthesis, many subsequently  
484 induced changes in JA-responsive gene expression are specifically directed to the different  
485 attackers and hence engage different TFs and downstream targets. This is known to be  
486 coordinated by the mutually antagonistic ERF branch of the JA pathway, which is co-regulated  
487 by ET, and the MYC branch of the JA pathway, which is co-regulated by ABA (Pieterse et al.  
488 2012). Several TFs have been documented to differentially affect MYC versus ERF branch-  
489 controlled gene expression and associated defenses. The best-known example of such a regulator  
490 is *MYC2*, a key positive regulator of MYC branch genes and associated defenses against  
491 chewing insects (e.g. *Helicoverpa armigera*, *Spodoptera littoralis*) (Dombrecht et al., 2007;  
492 Fernández-Calvo et al., 2011). In contrast, *MYC2* negatively regulates defense against  
493 necrotrophic pathogens (e.g. *B. cinerea*, *Plectosphaerella cucumerina*) (Lorenzo et al., 2004;  
494 Nickstadt et al., 2004). JA-inducible NAC TF family paralogs, *ANAC019* and *ANAC055*, show  
495 the same effect: they positively regulate MYC branch-associated genes and defenses to *S.*  
496 *littoralis*, while they antagonize ERF branch-associated resistance to *B. cinerea* (Bu et al., 2008;  
497 Schweizer et al., 2013) Oppositely, the positive regulator of the ERF branch, *ORA59*, controls  
498 defenses to *B. cinerea* while it antagonizes MYC branch defenses and *ORA59* overexpression  
499 lines become more attractive to *P. rapae* larvae (Pré et al., 2008; Verhage et al., 2011). Our data

500 suggest that bHLH27 functions as a negative regulator of the MYC branch, which may enhance  
501 ERF branch activation, thereby influencing resistance to *B. cinerea*. Also other bHLH TFs (so  
502 called JAMs) have been reported to antagonize MYC2-activated gene expression and defense to  
503 insects (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013). By contrast, Song et al. (2013)  
504 reported that ERF branch defense marker genes and resistance against *B. cinerea* were enhanced  
505 by the quadruple mutant of bHLH3/13/14/17. This indicates different underlying mechanisms of  
506 the different repressive bHLHs. MYB48/59 also antagonize the MYC branch as signified by the  
507 *myb48myb59* mutant, showing not only enhanced resistance to *M. brassicae*, but also enhanced  
508 expression of MYC branch-associated genes (Figure 2E-G; Supplemental dataset 6 and 7). The  
509 transcriptome analysis of *myb48myb59* did not suggest that the reduced resistance to *B. cinerea*  
510 is due to MYB48/59-mediated antagonism of ERF branch. It may be that down-regulation of  
511 gene clusters enriched in specific secondary metabolism contributes towards compromised  
512 immunity in this mutant, but this awaits further functional analysis.

513

#### 514 **Uncovering redundant function by double mutant analysis**

515 Reverse genetic screens are an important approach in the study of gene functions in *Arabidopsis*,  
516 but when additional genes have either fully or partially redundant functions, which is often the  
517 case with TF genes, their utility can be limited (Bolle et al., 2011). Redundancy may partially  
518 explain why 8 out of the 12 T-DNA insertion lines of the predicted JA-responsive TF genes that  
519 were tested in this study did not display significant changes in JA-associated immunity. By  
520 specifically targeting the highly similar TF-encoding gene pair *MYB48* and *MYB59*, we  
521 generated a double mutant that displayed a more severe perturbation of JA-associated gene  
522 expression and immunity compared to either single mutant (Figure 2E-G and Supplemental



523 Figure 10). Use of higher-order mutants can be critical to understand TF gene regulatory  
524 functions.

525

### 526 **Network reconstruction enables prediction of regulatory interactions**

527 Our time series data discerned a chronology of 10 transcriptional phases, showing that the onset  
528 of up-regulation preceded that of down-regulation, and that the first phase that was initiated  
529 within 15 minutes was represented by transcriptional regulators (Figure 4). JA biosynthesis is  
530 shown to be a first target for activation, followed by secondary metabolism, including activation  
531 of the tryptophan, glucosinolate and anthocyanin biosynthesis pathways. This latter observation  
532 correlates with the later activation of many *MYB* TF genes, which are important regulators of  
533 secondary metabolism, and the enrichment of MYB DNA-binding motifs in the up-regulated  
534 genes in later phases. Down-regulated genes showed enrichment in WRKY TF-binding motifs,  
535 which is linked with the suppressed expression of SA-associated defense genes.

536 Integrating TF DNA-binding motif enrichment data with our chronological JA network  
537 model predicted putative causal regulations between TFs and downstream JA-regulated  
538 subnetworks (Figure 5 and 6). Although subsets of the regulatory predictions were supported by  
539 literature and by novel experimental validation in this study, the presented network model is not  
540 without limitations. Our approach does not consider potential nonlinear relationships between  
541 gene expression profiles, and has limited ability to account for expression of genes that strongly  
542 depend on the joint activity of more than one TF. Thus, a future extension of the work presented  
543 here could be to utilize these data with more formal modeling approaches that better account for  
544 combinatorial regulation of targets and/or are capable of capturing nonlinear characteristics of  
545 the regulatory system, such as approaches based on mutual information or dynamic Bayesian

546 networks (Margolin et al., 2006; Penfold and Wild, 2011). Even when focusing on  
547 transcriptional networks as we have done here, it is important to note that some TFs may not be  
548 regulated transcriptionally themselves and hence are absent from our analysis. Additional  
549 techniques such as ChIP-seq and Y1H will help incorporate such regulators into the JA gene  
550 regulatory network model (Windram et al., 2014).

551

### 552 **Dataset integration validates TF-specific regulatory functions**

553 Exploring the regulatory predictions between TF regulators and their target genes highlighted a  
554 local regulatory module centered around the early JA-responsive AP2/ERF TF ORA47. Based  
555 on the occurrence of the ORA47 DNA-binding motif in their core promoters, we predicted that  
556 this TF targets a large fraction of genes encoding enzymes involved in JA biosynthesis in  
557 *Arabidopsis* (Figure 6A) and evolutionary distant species (Figure 6B). Indeed, yeast one-hybrid  
558 experiments confirm that ORA47 binds to promoter elements of JA biosynthesis genes  
559 (Supplemental Figure 8). Using transgenic lines that allow for the conditional expression of  
560 *ORA47* upon  $\beta$ -estradiol treatment, we showed that induction of *ORA47* expression significantly  
561 increases levels of JA and bioactive JA-Ile, indicating that ORA47 is an important activator of  
562 JA biosynthesis (Figure 6D). Recently, it was demonstrated that ORA47 could bind to the  
563 promoters of many of the JA biosynthesis genes reported here (Chen et al., 2016), however, the  
564 impact on the expression of its target genes was only reported for a small subset. Using the  $\beta$ -  
565 estradiol conditional overexpression system allowed us to demonstrate that induction of *ORA47*  
566 expression indeed leads to the activation of all 7 important JA biosynthesis genes investigated  
567 (Figure 6C). Our *in silico* predictions combined with experimental validation underscore ORA47

568 as a central regulator of JA biosynthesis, which may form part of an evolutionarily conserved JA  
569 amplification loop (Figure 6B).

570 For many known and unknown JA-responsive TFs, their exact role in the JA gene  
571 regulatory network has remained unresolved. We show how integrating either existing or novel  
572 transcriptome data with our models of MeJA-mediated gene expression can generate hypotheses  
573 regarding the roles of specific transcriptional regulators in the context of the JA response. In  
574 particular, transcriptional profiling of plants overexpressing the MeJA-responsive TF RAP2.6L  
575 and subsequent overlay of the gene expression data onto our co-expression clusters, led to the  
576 hypothesis that within the JA gene regulatory network RAP2.6L plays a role in the regulation of  
577 glucosinolate biosynthesis-associated genes (Figure 6E). A similar approach, using the  
578 established stress-associated TF ANAC055, and MYB48/59 (highlighted in this study),  
579 confirmed and extended the predicted regulatory interactions with distinct downstream targets in  
580 the JA network model (Supplemental Figure 9 and 10). Specific co-expressed gene clusters in  
581 the JA network were shown to be affected in the TF-perturbed lines, highlighting the strength of  
582 our clustering analysis for inferring functional regulation mechanisms. A similar transcriptome  
583 overlay approach could be used in future studies to further define the roles of other JA-inducible  
584 TFs in the diverse JA subnetworks.

585

## 586 **Summary**

587 In sum, this study provides detailed insight into the dynamics and architecture of the JA gene  
588 regulatory network **that is activated in *Arabidopsis* upon treatment with MeJA, and rapidly**  
589 **develops a range of transient or longer lasting expression changes in specific groups of co-**  
590 **expressed genes with distinct biological functions.** Our information-rich data set offers a

591 potentially high success rate for the discovery of genes with so-far unknown functions in JA-  
592 regulated responses related to plant immunity, growth and development. Future use of these time  
593 series data could include integration with additional transcriptome data across diverse  
594 environmental conditions, together with other ‘omics’ datasets, which will aid in building a  
595 comprehensive picture of the JA response.

596

## 597 **METHODS**

598 **Plant materials and growth conditions.** All wild-type, mutant, and transgenic *Arabidopsis*  
599 *thaliana* plants used in this study are in the Columbia ecotype (Col-0) background, except for the  
600 *RAP2.6L*-OX line which has the WS background. The following T-DNA insertion mutants and  
601 transgenic lines were obtained from the Nottingham Arabidopsis Stock Centre: *ofp1*  
602 (At5g01840; SALK\_111492C), *myb59* (At5g59780; GK-627C09), *anac056* (At3g15510;  
603 SALK\_137131C), *rap2.6l* (At5g13330; SALK\_051006C), *rap2.6* (At1g43160;  
604 SAIL\_1225G09), *erf16(-1)* (At5g21960; SALK\_053563C), *erf16-2* (At5g21960;  
605 SALK\_096382C), *at1g10586* (At1g10586; SALK\_027725C), *bhlh19* (At2g22760;  
606 GABI\_461E05), *bhlh27(-1)* (At4g29930; SALK\_049808C), *bhlh27-2* (At4g29930;  
607 SALK\_149244C), *bhlh35* (At5g57150; SALK\_100300C), *bhlh92* (At5g43650;  
608 SALK\_033657C), *bhlh113* (At3g19500; GK\_892H04), *myb48* (At3g46130; SALK\_103847),  
609 *ora59* (Zander et al., 2014) (At1g06160; GK-061A12.16), and *ORA47*  $\beta$ -estradiol-inducible  
610 TRANSPLANTA line (Coego et al., 2014) (N2101685). The *myb48* and *myb59* mutants were  
611 crossed to generate the *myb48myb59* double mutant. The *myc2,3,4* triple mutant  
612 (At1g32640/At5g46760/At4g17880) has been described previously (Fernández-Calvo et al.,  
613 2011). Seeds were stratified for 48 h in water at 4°C prior to sowing on river sand. After 2

614 weeks, the seedlings were transferred to 60-mL pots containing a soil:river sand mixture (12:5)  
615 that had been autoclaved twice for 1 h. Plants were cultivated in standardized conditions under a  
616 10-h day ( $75 \mu\text{mol}/\text{m}^2/\text{s}^1$ ) and 14-h night cycle at 21°C and 70% relative humidity. Plants were  
617 watered every other day and received modified half-strength Hoagland nutrient solution  
618 containing 10 mM Sequestreen (CIBA-GEIGY GmbH, Frankfurt, Germany) once a week. To  
619 minimize within-chamber variation, all the trays, each containing a mixture of plant genotypes or  
620 treatments, were randomized throughout the growth chamber once a week. Mutants or treatments  
621 were indicated by colored labels of which the code was unknown by the experimenter. T-DNA  
622 insertion lines were confirmed homozygous for the T-DNA in the relevant genes with PCR using  
623 the gene-specific primers listed in Supplemental Table 1. The *RAP2.6L* overexpressing line  
624 (*RAP2.6L-OX*) (Krishnaswamy et al., 2011) and the background accession (WS), were cultivated  
625 as described previously (Windram et al., 2012).

626

627 **RNA-Seq experimental setups.** For the MeJA time series, 5-week-old *Arabidopsis* Col-0 plants  
628 were treated by dipping the rosette leaves into a mock or MeJA (Duchefa Biochemie BV,  
629 Haarlem, The Netherlands) solution. The mock solution contained 0.015% (v/v) Silwet L77 (Van  
630 Meeuwen Chemicals BV, Weesp, The Netherlands) and 0.1% ethanol. The MeJA solution  
631 contained 0.015% (v/v) Silwet L77 and 0.1 mM MeJA, which was added from a 1,000-fold stock  
632 in 96% ethanol. For time series expression analysis, leaf number 6 (counted from oldest true leaf  
633 to youngest leaf) was harvested from individual *Arabidopsis* plants and snap frozen in liquid  
634 nitrogen for each treatment and time point as indicated in Extended Data Table 1. Each  
635 individual leaf corresponds to one biological replicate and four biological replicates for each  
636 treatment and time point combination were sequenced (see below). For the comparison of the

637 *myb48myb59* mutant with wild-type Col-0, two mature leaves (number 6 and 7) were harvested  
638 per plant from two 5-week-old plants per genotype, resulting in two biological replicates.

639

640 **Induction of the *ORA47*  $\beta$ -estradiol-inducible line and hormone analysis.** Five-week-old  
641 *ORA47* inducible overexpression lines were treated by dipping the rosette leaves into a mock or  
642  $\beta$ -estradiol (Sigma-Aldrich, Steinheim, Germany) solution. The mock solution contained 0.015%  
643 (v/v) Silwet L77 and 0.1% DMSO. The  $\beta$ -estradiol solution contained 0.015% (v/v) Silwet L77  
644 and 10  $\mu$ M  $\beta$ -estradiol, which was added from a 1,000-fold stock in DMSO.

645 Hormone analysis was performed as described previously (Vos et al., 2013). Briefly, for  
646 JA, JA-Ile, SA, and ABA quantification, 0.5 g of leaf tissue was ground to a fine powder using  
647 liquid nitrogen. Samples were homogenized in 0.5 ml of 70% methanol using a Precellys24  
648 tissue homogenizer (Bertin Technologies) by shaking at 6,000 rpm for 40 s. The resulting  
649 homogenates were centrifuged at 10,000 x g for 20 min at 4°C. Hormone levels were analyzed by  
650 liquid chromatography-mass spectrometry (LC-MS) on a Varian 320 Triple Quad LC-MS/MS.  
651 JA and JA-Ile levels were calculated by correcting for the internal standard of JA and for leaf  
652 weight. ABA and SA levels were calculated by correcting for leaf weight and their respective  
653 internal standards.

654

655 **Insect performance and disease bioassays.** *Botrytis cinerea* disease resistance was determined  
656 essentially as described previously (Van Wees et al., 2013). In brief, *B. cinerea* was grown on  
657 half-strength Potato Dextrose Agar (PDA; Difco BD Diagnostics, Franklin Lakes, NJ, USA)  
658 plates for 2 weeks at 22°C. Harvested spores were incubated in half-strength Potato Dextrose  
659 Broth (PDB; Difco) at a final density of  $5 \times 10^5$  spores/mL for 2 h prior to inoculation. Five-

660 week-old plants were inoculated by placing a 5- $\mu$ L droplet of spore suspension onto the leaf  
661 surface. Five leaves were inoculated per plant. Plants were maintained under 100% relative  
662 humidity with the same temperature and photoperiod conditions. Disease severity was scored 3  
663 days after inoculation in four classes ranging from restricted lesion (<2 mm; class I), non-  
664 spreading lesion (2 mm) (class II), spreading lesion (2-4 mm; class III), up to severely spreading  
665 lesion (>4 mm; class IV). The distribution of disease categories between genotypes were  
666 compared using a Chi-squared test.

667 *Mamestra brassicae* eggs were obtained from the laboratory of Entomology at  
668 Wageningen University where they were reared as described previously (Pangesti et al., 2015).  
669 Per 5-week-old *Arabidopsis* plant one freshly hatched first-instar (L1) larva was directly placed  
670 on a leaf using a fine paintbrush. Larval fresh weight was determined after 8-12 days of feeding.  
671 To confine the larvae, every plant was placed in a cup that was covered with an insect-proof  
672 mesh. Significant differences in larval weight between genotypes were determined using a two-  
673 tailed Student's *t* test.

674  
675 **High-throughput RNA-sequencing.** *Arabidopsis* leaves were homogenized for 2 x 1.5 min  
676 using a mixer mill (Retsch, Haan, Germany) set to 30 Hz. Total RNA was extracted using the  
677 RNeasy Mini Kit (Qiagen) including a DNaseI treatment step in accordance with manufacturer's  
678 instructions. Quality of RNA was checked by determining the RNA Integrity Number (RIN)  
679 using an Agilent 2100 Bioanalyzer and RNA 6000 Nano Chips (Agilent, Santa Clara, United  
680 States). For Illumina TruSeq RNA library preparation (see below) only RNA samples with a RIN  
681 value of  $\geq 9$  were used.

682 For the time series experiment, RNA-Seq library preparation and sequencing was  
683 performed by the UCLA Neuroscience Genomics Core (United States). Sequencing libraries  
684 were prepared using the Illumina TruSeq mRNA Sample Prep Kit, and sequenced on the  
685 Illumina HiSeq2000 platform with read lengths of 50 bases. In total, 12 randomized samples  
686 were loaded per lane of a HiSeq2000 V3 flowcell, and each mix of 12 samples was sequenced in  
687 4 different lanes over different flow cells to account for technical variation. A complete scheme  
688 of all biological replicates, technical replicates, barcoding used per sample, lane and flow cell  
689 usage is provided in Extended Data Table 1. For each of the 15 time points, 4 biological  
690 replicates were sequenced in 4 technical replicates, resulting in ~60 million reads per sample  
691 with a read length of 50 bp single end. Complete sequencing setup details can be found in  
692 Supplemental Dataset 1.

693 Basecalling was performed using the Casava v1.8.2. pipeline with default settings except  
694 for the additional argument ‘--use-bases-mask y50,y6n’, to provide an additional Fastq file  
695 containing the barcodes for each read in each sample. Sample demultiplexing was performed by  
696 uniquely assigning each barcode to sample references, allowing for a maximum of 2 mismatches  
697 (the maximum allowed by the barcode) and only considering barcode nucleotides with a quality  
698 score of 28 or greater.

699 For the analysis of the *myb48myb59* double mutant, RNA-Seq library preparation and  
700 sequencing was performed by the Utrecht Sequencing Facility (the Netherlands). Sequencing  
701 libraries were prepared using the Illumina Truseq mRNA Stranded Sample Prep Kit, and  
702 sequenced on the Illumina NextSeq5000 platform with read lengths of 75 bases.

703 The raw RNA-Seq read data are deposited in the Short Read Archive  
704 (<http://www.ncbi.nlm.nih.gov/sra/>) and are accessible through accession number PRJNA224133.



705

706 **Processing of RNA-Seq data.** Read alignment, summarization and normalization followed the  
707 pipeline as previously described (Van Verk et al., 2013). Reads were aligned to the *Arabidopsis*  
708 genome (TAIR version 10) using TopHat v2.0.4 (Trapnell et al., 2009) with the parameter  
709 settings: ‘transcriptome-mismatches 3’, ‘N 3’, ‘bowtie1’, ‘no-novel-juncs’, ‘genome-read-  
710 mismatches 3’, ‘p 6’, ‘read-mismatches 3’, ‘G’, ‘min-intron-length 40’, ‘max-intron-length  
711 2000’. Aligned reads were summarized over annotated gene models using HTSeq-count v0.5.3p9  
712 (Anders et al., 2015) with settings: ‘-stranded no’, ‘-i gene\_id’. Sample counts were depth-  
713 adjusted using the median-count-ratio method available in the DESeq R package (Anders and  
714 Huber, 2010).

715

716 **Differential gene expression analysis.** Genes that were significantly differentially expressed  
717 after MeJA treatment compared to mock were identified using a generalized linear model (GLM)  
718 with a log link function and a negative binomial distribution. Within this model we considered  
719 both the time after treatment and the treatment itself as factors. To assess the treatment effect on  
720 the total read count for each gene, a saturated model (total counts ~ treatment + time +  
721 treatment:time) was compared to a reduced model considering time alone (total counts ~ time)  
722 using ANOVA with a Chi-squared test. For all genes, the *P* values obtained from the Chi-  
723 squared test were corrected for multiple testing using a Bonferroni correction. All genes that did  
724 not meet the following requirement were omitted from further analysis: a minimum 2-fold  
725 difference in expression on at least one of the 14 time points, supported by a minimum of 10  
726 counts in the lowest expressed sample, and a *P* value  $\leq 0.01$  for that time point. Remaining genes  
727 with Bonferroni-corrected *P* value  $\leq 0.05$  were called as differentially expressed genes (DEGs).

728 All statistics associated with testing for differential gene expression were performed with R  
729 (<http://www.r-project.org>).

730 Of all the DEGs, the time point of first differential expression was predicted. To this end  
731 the significance of the treatment effect at each time point was obtained from the GLM,  
732 represented by its  $z$  score. These values were used as a basis to interpolate the significance of the  
733 treatment effect in between the sampled time points. This was done using the `interpSpline`  
734 function in R using 249 segments. The first time point of differential expression was set where  
735 the  $z$  score was higher than 2.576 (equivalent of  $P$  value 0.01) for up-regulation or lower than -  
736 2.576 for down-regulation.

737 Differentially expressed genes between Col-0 and *myb48myb59* ( $|\log_2\text{-fold change}| > 1$ ;  
738  $\text{FDR} \leq 0.05$ ) were identified using DESeq (Anders and Huber, 2010). For analysis of DEGs  
739 between WS and *RAP2.6L-OX* see “Microarray analysis of *RAP2.6L* transgenic plants”.

740  
741 **Clustering of gene expression profiles.** Clustering of DEGs was performed using `SplineCluster`  
742 (Heard et al., 2006) on the profiles of  $\log_2$ -fold changes at each time point (MeJA-treated versus  
743 mock), with a prior precision value of  $10^{-4}$ , the default normalization procedure and cluster  
744 reallocation step (Heard, 2011). All other optional parameters remained as default.

745  
746 **TF family and promoter motif analyses.** To determine which TF families are enriched among  
747 the genes differentially expressed in response to application of MeJA, we tested for  
748 overrepresentation of 58 TF families described in the TF database PlantTFDB version 3.0 (Jin et  
749 al., 2014). Overrepresented TF families within a set of genes were analyzed using the cumulative

750 hypergeometric distribution, with the total number of protein coding genes (TAIR version 10) as  
751 the background. *P* values were corrected for multiple testing with the Bonferroni method.

752 For promoter motif analysis, the promoter sequences defined as the 500 bp upstream of  
753 the predicted transcription start site (TSS) were retrieved from TAIR (version 10). *De novo*  
754 promoter motifs were identified by applying the motif-finding programs MEME (Bailey and  
755 Elkan, 1994) and XXmotif (Hartmann et al., 2013) to the promoters of all genes present in a  
756 given co-expression cluster. This approach exploits the strengths of different motif-finding  
757 strategies, which has been demonstrated to improve the quality of motif detection (Tompa et al.,  
758 2005). Both algorithms searched for motifs on the forward and reverse strands and used the zero-  
759 or-one occurrences per sequence (ZOOOPS) motif distribution model. MEME was run using a  
760 3rd-order Markov model learned from the promoter sequences of all genes in the *Arabidopsis*  
761 genome, using parameter settings: ‘-minw 8 -maxw 12 -nmotifs 10’. XXmotif was run using a  
762 3rd-order Markov model and the medium similarity threshold for merging motifs, with all other  
763 parameters kept as default. This analysis yielded a large number of motifs, many of which were  
764 highly similar. To reduce redundancy amongst motifs, a post-processing step was performed  
765 using the TAMO software package (Gordon et al., 2005). Motifs were converted to TAMO  
766 format, clustered using the UPGMA algorithm, and merged to produce consensus motifs. The set  
767 of processed motifs were converted to MEME format for all subsequent analyses using the  
768 *tamo2meme* function available in the MEME Suite (Bailey et al., 2009). For the analysis of  
769 known motifs originating from protein-binding microarray (PBM) studies (Franco-Zorrilla et al.,  
770 2014; Weirauch et al., 2014), the published weight matrices were converted into MEME format.

771 The presence or absence of a given motif within a promoter was determined using FIMO  
772 (Grant et al., 2011). A promoter was considered to contain a motif if it had at least one match

773 with a  $P$  value  $\leq 10^{-4}$ . For each *de novo*- and PBM-derived motif, the statistical enrichment of  
774 each motif within the promoters of co-expression gene clusters or transcriptional phases was  
775 tested using the cumulative hypergeometric distribution. This test computes the probability that a  
776 motif is present within a set of promoter sequences at a frequency greater than would be  
777 expected if the promoters were selected at random from the *Arabidopsis* genome.

778 Analysis of the *ORA47* DNA-binding motif conservation across different plant species  
779 was performed using the promoters of genes orthologous to *Arabidopsis AOC2*, *AOS*, *OPR3* and  
780 *LOX3*. Orthologs were identified in *Vitis vinifera*, *Populus trichocarpa* and *Brassica rapa*  
781 genomes (Ensembl database release 25) using the reciprocal best BLAST hit method (Tatusov et  
782 al., 1997). Presence or absence of the *ORA47* motif in the promoters (500 bp upstream of  
783 predicted TSS) of these orthologous genes was determined using FIMO as described above.

784  
785 **Gene Ontology analysis.** Gene ontology (GO) enrichment analysis on gene clusters was  
786 performed using GO term finder (Boyle et al., 2004) and an *Arabidopsis* gene association file  
787 downloaded from ftp.geneontology.org on 2nd May 2013. Overrepresentation for the GO  
788 categories 'Biological Process' and 'Molecular Function' were identified by computing a  $P$  value  
789 using the hypergeometric distribution and false discovery rate for multiple testing ( $P \leq 0.05$ ).

790  
791 **Identification of chronological phases in MeJA-induced gene expression.** To identify phases  
792 of MeJA-induced changes in transcription we first divided all DEGs depending on whether they  
793 were either up- or down-regulated in response to MeJA and then further according to their  
794 function as either a transcriptional regulator (termed regulator genes) or having a different  
795 function (termed regulated genes). To identify DEGs that encode transcriptional regulators we

796 used the comprehensive list of Arabidopsis TFs and transcriptional regulators described by  
797 (Pruneda-Paz et al., 2014) and subjected it to minor additional manual literature curation. This  
798 filtering yielded four mutually exclusive sets of MeJA-responsive genes (i.e. regulator genes up  
799 and down, regulated genes up and down). For each of the four gene sets, the depth-normalized  
800 expression values (see above) for all pairs of time points were compared pairwise using the  
801 Pearson correlation measure. Each resulting correlation matrix was then clustered using the  
802 Euclidean distance measure with average linkage. The resulting dendrograms were used to infer  
803 distinct phases of MeJA-induced transcription, where each phase has a start and end time. Each  
804 gene present in one of the four final gene sets was assigned to a transcriptional phase based on its  
805 time point of first differential expression (Supplemental Figure 6). All genes that were for the  
806 first time differentially expressed before, or equal to, the final time point in a given phase  
807 (clustered group of time points), and after the final time point of a preceding phase, were  
808 assigned to that transcriptional phase (see Supplemental Figure 7 for overview of the method).

809

810 **Network construction.** The identification of potential regulatory network connections between  
811 TFs and transcriptional phases was performed with a set of TFs that met two criteria: (1) They  
812 were differentially expressed in response to application of MeJA (and thus belong to a phase).  
813 (2) They have an annotated DNA-binding motif (as described in “TF family and promoter motif  
814 analyses”). Each set of genes that constitute a transcriptional phase (10 phases in total) was  
815 tested for overrepresentation of each motif using the hypergeometric distribution as described  
816 above. A directional edge was drawn from a TF to a phase when its cognate binding motif was  
817 overrepresented in the promoters of genes belonging to that phase (hypergeometric distribution;  
818  $P \leq 0.005$ ). The resulting network was visualized using Cytoscape (Shannon et al., 2003).

819

820 **Quantitative RT-PCR analysis.** For quantitative RT-PCR (qRT-PCR), RNA was extracted as  
821 previously described (Oñate-Sánchez and Vicente-Carbajosa, 2008) and subsequently treated  
822 with DNaseI (Fermentas, St. Leon-Rot, Germany) to remove genomic DNA. Genomic DNA-free  
823 total RNA was reverse transcribed by using RevertAid H minus Reverse Transcriptase  
824 (Fermentas, St. Leon-Rot, Germany). PCR reactions were performed in optical 384-well plates  
825 with a ViiA 7 realtime PCR system (Applied Biosystems, Carlsbad, CA, USA), with SYBR®  
826 Green (Applied Biosystems, Carlsbad, CA, USA). A standard thermal profile was used: 50°C for  
827 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplicon  
828 dissociation curves were recorded after cycle 40 by heating from 60 to 95°C with a ramp speed  
829 of 0.05°C/sec. All primers used for qRT-PCR are listed in Supplemental Table 1. The gene  
830 *At1g13320* was used as reference for normalization of expression (Czechowski et al., 2004).

831

832 **Microarray analysis of *RAP2.6L* transgenic plants.** Total RNA was extracted from three  
833 leaves per plant (28-days-old), labeled and hybridized to CATMA v4 arrays (Allemeersch et al.,  
834 2005) as described previously (Breeze et al., 2011). Three biological replicates of WS and  
835 *RAP2.6L*-OX samples were pooled separately and labeled three times with each dye to give six  
836 technical replicates. Analysis of expression differences between WS and *RAP2.6L*-OX was  
837 performed with the R Bioconductor package limmaGUI (Wettenhall and Smyth, 2004) using  
838 Print-Tip lowess transformation and quantile-normalization.

839

840 **Yeast-1-Hybrid (Y1H) protein-DNA interaction assays.** Cloning of bait promoter DNA and  
841 yeast transformation was performed as previously described (Hickman et al., 2013). All primers

842 that were used to clone promoter fragments are listed in Extended Data Table 13. *ORA47* coding  
843 sequence was isolated from the TF library as described in Hickman et al., (2013) and the correct  
844 sequence confirmed by sequencing. Prey strains were constructed by cloning the *ORA47* coding  
845 sequence into pDEST22 (Invitrogen) and transforming AH109 yeast (Clontech), while empty  
846 pDEST22 was used to transform AH109 as a negative control. Three  $\mu\text{L}$  of bait strain cultures  
847 were spotted onto YPDA (yeast, peptone, dextrose, adenine) plates and dried before being  
848 overlaid with 3  $\mu\text{L}$  of prey strain culture and left to grow overnight at 30°C. Colonies were  
849 subcultured in 1 mL mating selective media (SD-Leu-Trp, Clontech) and grown for two nights at  
850 30°C with shaking. Cultures were diluted to  $10^8$  cells/mL in SD-Leu-Trp liquid media before  
851 four 10-fold serial dilutions were made. Three  $\mu\text{L}$  of each diploid strain was plated to mating  
852 selective (SD-Leu-Trp, Clontech) and interaction selective (SD-Leu-Trp-His, Clontech) media  
853 and incubated at 30°C for 72 h before being photographed using a G:Box EF2 (Syngene). For  
854 promoter D, 5 mM 3-Aminotriazole (Sigma-Aldrich) was required to suppress autoactivation of  
855 *HIS3* expression by this promoter region. For promoters A, B and D experiments were performed  
856 using two independent promoter transformants and four transcription factor transformants, for a  
857 total of eight replicates. For promoter C, there were three replicates across two independent  
858 promoter transformants and two transcription factor transformants.

859

## 860 **Accession numbers**

861 Arabidopsis gene names and identifiers referred to in this article are:

862 *OFPI* (At5g01840), *MYB59* (At5g59780), *MYB48* (At3g46130), *ANAC056* (At3g15510),  
863 *RAP2.6L* (At5g13330), *RAP2.6* (At1g43160), *ERF16* (At5g21960), *AT1G10586* (At1g10586),  
864 *bHLH19* (At2g22760), *bHLH27* (At4g29930), *bHLH35* (At5g57150), *bHLH92* (At5g43650),

865 *bHLH113* (At3g19500), *COII* (At2g39940), *AOS* (At5g42650), *AOC1* (At3g25760), *AOC3*  
866 (At3g25780), *LOX2* (At3g45140), *LOX3* (AT1G17420), *OPR3* (At2g06050), *JAR1*  
867 (At2g46370), *JAZ1* (At1g19180), *JAZ2* (At1g74950), *JAZ3* (At3g17860), *JAZ4* (At1g48500),  
868 *JAZ5* (At1g17380), *JAZ6* (At1g72450), *JAZ7* (At2g34600), *JAZ8* (At1g30135), *JAZ9*  
869 (At1g70700), *JAZ10* (At5g13220), *JAZ11* (At3g43440), *JAZ12* (At5g20900), *MYC2*  
870 (At1g32640), *bHLH003* (At4g16430), *bHLH013* (At1g01260), *bHLH014* (At4g00870),  
871 *bHLH017/JAM1* (At2g46510), *MYC3* (At5g46760), *MYC4* (At4g17880), *MYB29* (At5g07690),  
872 *ANAC019* (At1g52890), *ANAC055* (At3g15500), *NINJA* (At4g28910), *RGL3* (At5g17490),  
873 *ORA47* (At1g74930), *ORA59* (At1g06160), *VSP1* (At5g24780), *VSP2* (At5g24770), *NPR4*  
874 (At4g19660), *MYB51* (At1g18570), *EDS1* (At3g48090), *PAD4* (At3g52430).

875

## 876 **Supplemental Data**

877 Supplemental Figure 1. SplineCluster analysis of MeJA-responsive gene expression profiles.

878 Supplemental Figure 2. Gene ID-searchable significance of differential expression over time for  
879 all DEGs in the 27 clusters of co-expressed genes in response to MeJA treatment.

880 Supplemental Figure 3. *B. cinerea* disease severity assay with selected mutant lines.

881 Supplemental Figure 4. *B. cinerea* disease severity and growth of *M. brassicae* larvae on  
882 additional mutant alleles.

883 Supplemental Figure 5. Growth of *M. brassicae* larvae on selected mutant lines.

884 Supplemental Figure 6. Timing of differential expression for all differentially expressed genes.

885 Supplemental Figure 7. Identification of transcriptional phases induced in response to MeJA  
886 treatment.



887 Supplemental Figure 8. ORA47 can bind to the promoters of multiple *Arabidopsis* genes  
888 encoding JA biosynthesis enzymes in yeast.

889 Supplemental Figure 9. Projection of ANAC055 target genes on the JA network model.

890 Supplemental Figure 10. Projection of MYB48/MYB59 target genes on the JA network model.

891 Supplemental Dataset 1. Time series experimental set-up and mRNA sequencing details.

892 Supplemental Dataset 2. Median-count ratio normalized expression values of all genes and  
893 biological replicates for  $t = 0$  h, and the 14 time points after MeJA and mock treatments.

894 Supplemental Dataset 3. Mean expression values for all genes across the time series following  
895 MeJA treatment.

896 Supplemental Dataset 4. *Arabidopsis* Gene Identifier (AGI) codes for members of each of the 27  
897 gene co-expression clusters identified by SplineCluster.

898 Supplemental Dataset 5. GO-terms overrepresented in each of the 27 co-expression gene  
899 clusters.

900 Supplemental Dataset 6. Lists of genes differentially expressed in *myb48myb59* compared to  
901 Col-0.

902 Supplemental Dataset 7. GO-terms overrepresented in the up-regulated and down-regulated  
903 *myb48myb59* differentially expressed gene sets.

904 Supplemental Dataset 8. Enrichment of known TF DNA-binding motifs in each of the 27 co-  
905 expression gene clusters.

906 Supplemental Dataset 9. *De novo*-derived motif enrichment in each of the 27 gene co-  
907 expression clusters.

908 Supplemental Dataset 10. *De novo*-derived sequence motifs in Weblogo and position weight  
909 matrix format.

910 Supplemental Dataset 11. *Arabidopsis* Gene Identifier (AGI) codes for members of each of the  
911 10 transcriptional phases that are initiated after MeJA treatment.

912 Supplemental Dataset 12. GO-terms overrepresented in each of the 10 transcriptional phases that  
913 are initiated after MeJA treatment.

914 Supplemental Dataset 13. Known TF DNA-binding motif enrichment in each of the 10  
915 transcriptional phases that are initiated after MeJA treatment.

916 Supplemental Dataset 14. *De novo*-derived motif enrichment in each of the 10 transcriptional  
917 phases that are initiated after MeJA treatment.

918 Supplemental Dataset 15. List of differentially expressed TF genes and enrichment of their  
919 corresponding TF DNA-binding motif in the promoters of genes within a transcriptional  
920 phase.

921 Supplemental Dataset 16. List of differentially expressed genes obtained from microarray  
922 analysis of *RAP2.6L-OX*.

923

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936

937

938 **Author contributions**

939 SCMvW and CMJP conceived the approach and together with RJH and MCvV designed the  
940 study. RJH and MCvV designed all bioinformatics approaches. KD provided analytical and  
941 intellectual contributions. RJH and MCvV performed data analysis. RJH, MCvV, AJHvD, MPM,  
942 IAVV, LC, MS, GJW and IvdN performed mutant genotyping and validation experiments. MdV  
943 and RCS performed hormone measurements. AJ and AT performed Y1H assays. JR performed  
944 microarray experiments. RJH, MCvV, CMJP and SCMvW wrote the manuscript. All authors  
945 discussed the results and commented on the manuscript.

946

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## 1203 **FIGURE LEGENDS**

1204 **Figure 1. Temporal expression profiles following application of MeJA. (A)** Circos plots of  
1205 time series expression profiles from our MeJA experiment in comparison to previously published  
1206 MeJA- or *P. rapae*-induced transcriptome data (Pauwels et al., 2008; Goda et al., 2008; Coolen  
1207 et al., 2016), as indicated at the top left of each plot. Outermost bands indicate differentially  
1208 expressed gene sets from this study (red, up-regulated; dark blue, down-regulated) and from the  
1209 previously published datasets (orange, up-regulated; light blue; down-regulated). The stacked  
1210 histograms indicate differential expression (colors indicate sampling time point from 0.25 h up to  
1211 16 h after treatment). Genes differentially expressed in both datasets are marked by connecting  
1212 bands (colors indicate first time point of differential expression in our study). Each section  
1213 within the circus plot represents a set of 100 DEGs. **(B)** Examples of expression profiles of  
1214 selected JA and SA pathway marker genes in our study. y-axis, transcript abundance; x-axis,  
1215 time (h) post application of MeJA; error bars indicate SE.

1216

1217 **Figure 2. Clustering of co-expressed genes in the JA gene regulatory network and**  
1218 **identification of novel components of JA-dependent resistance. (A)** The set of 3611 genes  
1219 showing differential expression in *Arabidopsis* leaves following exogenous application of MeJA  
1220 was partitioned into 27 distinct co-expressed gene clusters using SplineCluster. The heatmap  
1221 shows the mean gene expression profile for each cluster, with red and blue indicating up-

1222 regulation and down-regulation of expression ( $\log_2$ -fold change (MeJA/mock)), respectively. **(B)**  
1223 Significantly overrepresented TF families within clusters of genes up-regulated (clusters 1-14;  
1224 red) or down-regulated (clusters 15-27; blue) in response to MeJA treatment (hypergeometric  
1225 test;  $P \leq 0.001$ ). **(C)** Quantification of disease symptoms of wild-type Col-0, highly susceptible  
1226 ERF TF mutant *ora59*, and T-DNA insertion lines for selected genes *ERF16*, *MYB59*, and  
1227 *bHLH27* (members of co-expression clusters 2, 4 and 1, respectively) at 3 days after inoculation  
1228 with *B. cinerea*. Disease severity of inoculated leaves was scored in four classes ranging from  
1229 restricted lesion (class I), non-spreading lesion (class II), spreading lesion (class III), up to  
1230 severely spreading lesion (class IV). The percentage of leaves in each class was calculated per  
1231 plant ( $n > 20$ ). Asterisk indicates statistically significant difference from Col-0 (Chi-squared test;  
1232  $P \leq 0.05$ ). **(D)** Performance of *M. brassicae* larvae on Col-0, highly susceptible triple bHLH TF  
1233 mutant *myc2,3,4* and T-DNA insertion lines for selected genes *ANAC056* (co-expression cluster  
1234 13) and *bHLH27*. The larval fresh weight was determined after 8 days of feeding. Asterisk  
1235 indicates statistically significant difference from Col-0 (two-tailed Student's *t* test for pairwise  
1236 comparisons;  $P \leq 0.05$ ;  $n=30$ ; error bars are SE). **(E)** Quantification of disease symptoms of Col-  
1237 0, *myb48*, *myb59*, *myb48myb59* and *ora59* mutant lines at 3 days after inoculation with *B.*  
1238 *cinerea*. Disease severity of inoculated leaves was scored as described in (C) ( $n > 20$ ). Asterisk  
1239 indicates statistically significant difference from Col-0 (Chi-squared test;  $P \leq 0.05$ ). **(F)**  
1240 Performance of *M. brassicae* larvae on Col-0 and *myb48*, *myb59* and *myb48myb59* mutant lines.  
1241 The larval fresh weight was determined after 12 days of feeding. Asterisk indicates statistically  
1242 significant difference from Col-0 (two-tailed Student's *t* test for pairwise comparisons;  $P \leq 0.05$ ;  
1243  $n=30$ ; error bars are SE). **(G)** Heatmap indicating hypergeometric enrichment *P* value of genes

1244 differentially expressed in *myb48myb59* (compared to Col-0) in each MeJA-induced co-  
1245 expression cluster.

1246

1247 **Figure 3. Enriched *cis*-regulatory motifs and functional categories in MeJA-responsive**  
1248 **gene co-expression clusters. (A)** Overrepresentation of known TF DNA-binding motifs within  
1249 the unions of up-regulated and down-regulated genes. Rows indicate motifs and are colored by  
1250 corresponding TF family. Red boxes indicate a motif that is significantly overrepresented  
1251 (cumulative hypergeometric distribution). **(B)** Representative co-expression clusters with  
1252 overrepresented TF DNA-binding motifs. Top: Profiles of log<sub>2</sub>-fold change in gene expression  
1253 (MeJA/mock), with mean profile (red) and cluster size (n). Selected overrepresented functional  
1254 categories (F) and representative genes (G) are denoted. Sequence logo depiction of selected  
1255 known (middle) and *de novo*-derived (bottom) motifs that are significantly overrepresented. Full  
1256 results used to derive this figure are available in Supplemental Dataset 6 and 7.

1257

1258 **Figure 4. Chronology of changes in the MeJA-triggered gene regulatory network. (A)**  
1259 Phasing of MeJA-induced transcriptional changes. DEGs were divided into four sets according  
1260 to their function as regulator or non-regulator (regulated), and their expression pattern being up-  
1261 (red) or down-regulated (blue) over time. For each set of genes, a correlation matrix of gene  
1262 transcription counts between all pairs of time points was computed using Pearson's correlation  
1263 metric. Shown are the dendrograms produced by hierarchical clustering of the transcriptome  
1264 correlation matrices (yellow, high correlation; cyan, low correlation). Time is in hours. **(B)**  
1265 Analysis of the major transcriptional phases in the JA gene regulatory network. Transcriptional  
1266 phases are indicated by boxes, aligned on the timeline. DEGs are assigned to the phases

1267 according to the time point where they become first differentially expressed; indicated are  
1268 overrepresented functional categories and representative genes. Colored squares indicate known  
1269 TF DNA-binding motifs overrepresented in gene promoters (hypergeometric distribution;  $P \leq$   
1270 0.001). Pie charts indicate the proportion of TF gene families.

1271

1272 **Figure 5. Predicted directional interactions in the JA gene regulatory network.** Network  
1273 plot of inferred connections between MeJA-induced TFs and genes in transcriptional phases. The  
1274 promoter sequences of genes associated with a transcriptional phase were tested for  
1275 overrepresentation of DNA motifs shown to be bound to TFs that are differentially transcribed  
1276 following MeJA treatment. Each TF with a known motif is represented by a colored circle, and is  
1277 plotted at the time point that its corresponding gene is first differentially expressed. Each  
1278 transcriptional phase is represented by a rectangle and plotted in time according to its onset. An  
1279 edge between a TF and a phase indicates significant enrichment of the corresponding binding  
1280 motif in that phase. The size of each TF node is proportional to the number of phases in which its  
1281 binding site is overrepresented. To aid interpretation of the network, nodes are grouped and  
1282 colored according to the transcriptional phase where they first become differentially expressed.

1283

1284 **Figure 6. Prediction and functional analysis of JA-controlled TF subnetworks. (A)**  
1285 Expanded sub-network extracted from the global JA gene regulatory network, indicating inferred  
1286 regulation of JA biosynthesis genes by ORA47. Nodes indicating TFs and JA biosynthesis genes  
1287 are colored grey and orange, respectively. Directed edges indicate occurrence of TF-binding sites  
1288 in the promoter of the target gene. **(B)** Evolutionary conservation of ORA47 DNA-binding motif.  
1289 Occurrences of the ORA47 motif (consensus, CCG(A/T)CC) were identified in promoters of an

1290 orthologous gene from each of the indicated JA biosynthesis genes (top row). Black arrows  
1291 indicate a significant match within a gene promoter to the ORA47 motif. 5'UTR, 5-prime  
1292 untranslated region; CDS, coding sequence. **(C)** Induction of genes encoding JA biosynthesis  
1293 enzymes in estradiol-inducible *ORA47* plants. Expression levels of JA biosynthesis genes were  
1294 measured in leaves 8 h after application of either estradiol or DMSO (mock) using quantitative  
1295 RT-PCR (qRT-PCR). Shown are the mean expression levels of five biological replicates with  
1296 mock treatments set at 1. Asterisk indicates significant differences between mock- and estradiol-  
1297 treated plants (Student's *t* test;  $P \leq 0.05$ ; error bars are SE). **(D)** Production of JA, JA-Ile, ABA,  
1298 and SA in estradiol-inducible *ORA47* lines. Compound levels were measured from the same leaf  
1299 tissue harvested for the qRT-PCR analysis described in C. Asterisk indicates significant  
1300 difference between mock- and estradiol-treated plants (Student's *t* test;  $P \leq 0.05$ ; error bars are  
1301 SE). **(E)** Projection of *RAP2.6L* target genes on the chronological JA network model. Genes that  
1302 are differentially expressed in the *RAP2.6L*-OX line were overlaid onto the network described in  
1303 Figure 5. DEGs are indicated by nodes and positioned according to phase membership. Direction  
1304 of misregulation in *RAP2.6L*-OX is indicated by color; yellow, up-regulated; cyan, down-  
1305 regulated. The gene encoding *RAP2.6L* is shown as a red-colored node. Inset: heatmap  
1306 indicating hypergeometric enrichment *P* value of *RAP2.6L* target genes in each MeJA-induced  
1307 co-expression cluster.

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### 1309 **Competing financial interests:**

1310 The authors declare no competing financial interests.

### 1311 **Materials and Correspondence:**

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