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Architecture and dynamics of the jasmonic acid gene regulatory network 1

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20 ABSTRACT

21

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

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40 INTRODUCTION

41

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

In the past decade, major discoveries in the model plant Arabidopsis thaliana have 54 greatly advanced our understanding of the JA signaling pathway. In the absence of an invader, 55 when JA levels are low, activation of JA responsive gene expression is constrained by repressor 56 proteins of the JASMONATE ZIM-domain (JAZ) family that bind to specific JA-regulated 57 transcription factors (TFs). The conserved C-terminal JA-associated (Jas) domain of JAZs 58 59 competitively inhibits interaction of the TF MYC3 with the MED25 subunit of the transcriptional Mediator complex (Zhang et al., 2015). Moreover, JAZs recruit the TOPLESS 60 corepressor, either directly or through the NOVEL INTERACTOR OF JAZ (NINJA) adapter, 61 62 which epigenetically inhibits expression of TF target genes. In response to pathogen or insect attack, bioactive JA-Isoleucine (JA-Ile) is synthesized, which promotes the formation of the coreceptor complex of JAZ (via its Jas domain) with CORONATINE INSENSITIVE1 (COI1), the F-box protein of the E3 ubiquitin-ligase Skip-Cullin-F-box complex SCF^{COI1}. Upon perception of JA-Ile, JAZ repressor proteins are then targeted by SCF^{COI1} for ubiquitination and subsequent proteasomal degradation (Chini et al., 2007; Thines et al., 2007; Sheard et al., 2010). This leads to the release of JAZ-bound TFs and subsequent induction of JA-responsive gene expression.

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

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

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

107

108 **RESULTS**

109

110 A time course of MeJA-elicited transcriptional reprogramming

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

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

Our high-resolution temporal transcriptome data captured a diverse set of dynamical 138 responses to MeJA stimulation (Supplemental Figure 1). The majority of expression changes in 139 140 individual genes followed a clear single-pulse (impulse) pattern, that is often observed in responses to environmental stress in eukaryotic cells, and coordinates the temporal regulation of 141 specific gene expression programs (Yosef and Regev, 2011). Examples of genes whose 142 expression is up- or down-regulated for a short period of time followed by a transition to a steady 143 state, which is often a return to basal expression, are JAR1 and EDS1 (Figure 1B). Yet, there are 144 also genes that display a longer lasting change in expression level, e.g. MYC2 and BES1 (Figure 145 1B). Because all transcriptional changes were monitored in leaf number 6, we maximally 146 synchronized the onset of the JA response in intact plant tissue. Hence, the resulting information-147 rich time series of MeJA-responsive gene expression profiles are highly suited to computational 148 approaches that can generate novel biological insights into the regulation of the underlying JA 149 transcriptional network. 150

151

152 **Process-specific gene clusters**

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

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

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

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

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

185

186 **Discovery of novel defense regulators**

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

The early up-regulated gene clusters 1 and 2 (61 and 165 genes, respectively) contained 192 an enrichment for known JA-related genes such as the herbivory markers VSP1 and VSP2, as 193 well as the regulators JAZ1, 2, 5, 7, 8, 9, 10 and 13, MYC2, ANAC019, ANAC055, RGL3, and 194 JAM1 (Wasternack and Hause, 2013). In addition, TF genes with no previously reported roles in 195 the JA response pathway are present in these clusters, which implies that they may also have 196 197 regulatory functions in the JA response relevant to plant defense. To test this hypothesis, we selected 7 uncharacterized TF genes from clusters 1 and 2 and supplemented this set with 5 198 uncharacterized TF genes from other clusters, displaying a similarly rapid response to MeJA 199 200 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 herbivore Mamestra brassicae, which are both controlled by JA-inducible defenses (Pieterse et 202 al., 2012). Mutants in the TF genes bHLH27, ERF16 and MYB59 displayed a significant increase 203 in disease susceptibility to *B. cinerea* compared to wild-type *Arabidopsis* Col-0, approaching the 204 disease severity level of the highly susceptible control mutant ora59 (Figure 2C; full results in 205 Supplemental Figure 3 and additional mutant alleles in Supplemental Figure 4). Weight gain of 206 M. brassicae larvae was significantly reduced on mutants of ANAC056 and bHLH27, while on 207 none of the tested mutants larval weight was enhanced, as was the case on the susceptible control 208 209 mutant myc2,3,4 (Figure 2D; full results and additional mutant alleles in Supplemental Figure 4 and 5). Thus, for 4 of the 12 tested MeJA-responsive, previously uncharacterized TF genes a 210 predicted role in the JA response could be functionally validated for either B. cinerea or M. 211 brassicae resistance, demonstrating the value of using information-rich time series data to 212 accurately identify co-expressed genes that may have novel functions in the JA pathway. 213

214

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

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

To gain insight into the biological processes contributing to the differentially altered 231 232 attacker performance on myb48myb59, we performed RNA-Seq analysis on the double mutant. A total of 399 genes were differentially expressed between non-stimulated myb48myb59 and Col-0 233 leaves (168 were up-regulated and 231 were down-regulated in the double mutant; Supplemental 234 Dataset 6). Functional category analysis showed that in the up-regulated DEG set of the mutant 235 compared to Col-0, processes like 'Response to wounding' and 'Response to jasmonic acid 236 stimulus' were enriched (Supplemental Dataset 7). This is in accordance with these *mvb48mv59*-237 upregulated DEGs being overrepresented in co-expression clusters 1, 2, 7 and 9 of the MeJA 238 responsive DEGs (Figure 2G). Genes that showed enhanced expression by both MeJA treatment 239 and the myb48myb59 mutations are for example JA biosynthetic genes AOC2 and OPR3, and TF 240 gene *MYC2*. Also the downstream herbivore defense marker gene *VSP2* showed > 50-fold higher 241 expression level in the mutant. This suggests prioritization of the JA pathway towards the anti-242 243 insect MYC branch in myb48myb59, explaining its enhanced resistance to M. brassicae. However, MYC branch-mediated antagonism of the ERF branch of the JA pathway, which 244 would explain the reduction of defense against the necrotrophic pathogen B. cinerea, is not 245 246 apparent from our transcriptome data. It may be that MYB48/59-regulated genes that are enriched

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

251

252 Enrichment of TF DNA-binding motifs

TFs regulate gene expression by binding to *cis*-regulatory elements of target genes in a sequence 253 specific manner. Mapping of regulatory DNA motifs that are associated with dynamic MeJA-254 255 responsive gene expression profiles can aid in the understanding and reconstruction of JA gene regulatory networks. Therefore, we investigated which Arabidopsis TF-binding site motifs are 256 overrepresented within the promoters of co-expressed MeJA-responsive DEGs, using recently 257 identified DNA-binding specificities for 580 Arabidopsis TFs derived from studies with protein-258 binding microarrays (PBMs) (Franco-Zorrilla et al., 2014; Weirauch et al., 2014). First, we 259 screened for overrepresentation of these motifs in the unions of up- and down-regulated gene 260 clusters, respectively (Figure 3A). Motifs corresponding to DNA-binding sites of bHLH, bZIP, 261 ERF and MYB TFs are clearly overrepresented in the group of up-regulated genes, while WRKY 262 and TCP TF specific motifs are markedly overrepresented in the down-regulated genes. 263 Members of the WRKY TF family and their cognate cis-elements are key regulators of the SA 264 response pathway (Pandey and Somssich, 2009), suggesting that WRKYs are important targets 265 266 in the transcriptional repression of the SA pathway by MeJA treatment. Secondly, we analyzed motif enrichment within each of the 27 clusters of co-expressed genes (Figure 3B). To increase 267 the chance of discovering nuanced sequence motifs among the genes in these clusters, we 268 269 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 in specific clusters, offering a more precise link between motifs and cluster-specific gene 271 expression patterns. Strikingly, while motifs that correspond to bHLH-binding sites are enriched 272 in the majority of the up-regulated gene clusters, ERF- and MYB-binding motifs are only 273 overrepresented in a small selection of the up-regulated clusters, which are associated with 274 specific biological processes (Supplemental Dataset 8). For example, clusters 6 and 14, which 275 are enriched for GO terms describing distinct secondary metabolite biosynthesis pathways, are 276 enriched for different (de novo) predicted MYB DNA-binding motifs (Figure 3B). These 277 278 findings suggest that bHLH TFs and their DNA-binding sites are essential components in activation of the majority of the MeJA-inducible genes, while ERF and MYB TFs have more 279 specialized roles in modulating the expression of dedicated sets of target genes. 280

281

282 Chronology of MeJA-mediated transcriptional reprogramming

Next, we utilized the temporal information in our RNA-Seq time series to resolve the chronology 283 of gene expression events in the JA gene regulatory network. First, we divided the genes in sets 284 of up- and down-regulated DEGs and sorted them according to the time at which they first 285 became differentially expressed (Supplemental Figure 6; see Methods for details). From this 286 analysis, it became clear that a massive onset of gene activation precedes that of gene down-287 regulation, and that different waves of coordinated gene expression changes can be identified in 288 289 the time series. The majority of all DEGs become first differentially expressed within 2-4 h after MeJA treatment, which indicates engagement of relatively short transcriptional cascades, 290 allowing for a rapid response to an external signal (Alon, 2007). Up- and down-regulated DEGs 291 292 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 regulated genes; Supplemental Dataset 3). We were specifically interested in identifying time 294 points where coordinated switches in transcriptional activity take place, reasoning that pairs of 295 adjacent time points that display a weaker correlation indicate important points of coordinated 296 switches in transcriptional activity (see Methods and Supplemental Figure 7 for details). 297 Therefore, within each of the four mutually exclusive gene sets, we examined the pairwise 298 correlations of expression levels between all pairs of time points. Clustering of the resulting 299 correlation matrices revealed six distinct phases in transcriptional activation, and four phases in 300 301 transcriptional repression (Figure 4A). The first two phases of up-regulation (Phase Up1 and Up2) start within 0.5 h after MeJA treatment in the set of regulator genes, while at 1.5 h a third 302 phase of up-regulation of regulator genes ensues (phase Up4). For the regulated genes the first 303 phase of up-regulation starts at 1 h after MeJA treatment (phase Up3), which is clearly later than 304 the first onset of the regulator genes. A similar sequence of events can be observed in the down-305 regulated regulator and regulated genes, although the start is delayed compared to the activation 306 of up-regulated genes. 307

Our time series captures the temporal association between the changes in transcript 308 abundance of transcriptional regulators and downstream targets encoding proteins responsible for 309 310 the biochemical reactions that represent the defensive outputs of the JA response. To explore the biological significance and directionality in the regulation of the identified transcriptional phases 311 312 in the JA gene regulatory network, all DEGs were assigned to the phase in which they first became differentially expressed (see Methods and Supplemental Figure 7 for details). The 313 resulting gene lists of the 10 transcriptional phases were tested for overrepresentation of 314 315 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, ERFs, and other transcriptional regulators associated with JA biosynthesis. These early regulator 317 genes may play a role in the induction of other regulator-encoding genes present in phases Up2 318 and 4, and of regulated genes present in phases Up3, 5 and 6, which are linked to defense 319 responses such as glucosinolate, tryptophan and anthocyanin biosynthesis (Figure 4B; 320 Supplemental Dataset 12). In support of this, in the promoters of DEGs in phase Up3, DNA 321 motifs that can be bound by TFs transcribed in previous phases Up1 and 2, like bHLH-, ERF-322 and MYB-binding motifs, are enriched. In phase Up3, genes involved in JA biosynthesis are also 323 324 enriched, suggesting that this process is one of the first targets of JA-mediated transcriptional reprogramming. Overall, induction of the JA pathway shows a clear chronology of up-regulated 325 gene expression events, starting with the activation of genes encoding specific classes of TFs and 326 of JA biosynthesis enzymes, followed by genes encoding enzymes involved in the production of 327 important defensive secondary metabolites. 328

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

339 SA-controlled immunity, including the key immune-regulators EDS1 and PAD4 (Feys et al., 2001). In line with these observations, there is an overrepresentation for WRKY-binding motifs 340 in the promoters of genes present in phase Down 1 and 2, suggesting that their repressed 341 expression is mediated by an effect of MeJA on WRKY action. Later phases of transcriptional 342 repression (phases Down3 and 4) are marked by an overrepresentation of genes related to growth 343 and development, including primary metabolism and auxin signaling, and an enrichment of DNA 344 motifs recognized by TCP TFs, which conceivably reflects an effort by the plant to switch 345 energy resources from growth to defense (Attaran et al., 2014). A general observation that can be 346 made from this chronological analysis of the JA gene regulatory network is that despite the 347 overall relatively short transcriptional cascades controlling gene activation or repression, 348 distinctive transcriptional signatures, associated with specific biological processes, are initiated 349 at different phases in time. 350

351

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

Next, we made use of the TF DNA-binding motif information of the genes in the temporally 353 separated transcriptional phases to construct a gene regulatory network that predicts directional 354 interactions between the JA responsive TF genes and all genes associated with the different 355 transcriptional phases (Supplemental Dataset 15). The JA gene regulatory network generated via 356 this analysis is shown in Figure 5, in which a differentially expressed TF gene (represented by a 357 358 circular node in the network) is connected by an edge to a transcriptional phase (represented by a rectangle in the network) when the corresponding DNA-binding motif is overrepresented in that 359 phase. The generated network model shows that the TFs are predicted to regulate expression of 360 361 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 among the most active TFs, as their cognate DNA-binding motifs (both share the same 363 consensus, CACGTG) are enriched in the promoters of genes assigned to a large fraction of the 364 up-regulated transcriptional phases. This prediction is in line with recent reports suggesting that 365 the positive regulator MYC2 and the negative regulator JAM1 cooperate to balance JA responses 366 367 by competitive binding to their shared target sequences (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013). What determines the different timing by these regulators to effectively activate or 368 repress transcription awaits further investigation. Phases Up1 and Up2 also contain the TF genes 369 370 bHLH27, ERF16, ANAC056 and MYB59, of which corresponding mutants showed altered resistance levels to *B. cinerea* infection and/or *M. brassicae* infestation (Figure 2C and 2D). 371 Cognate DNA-binding motifs of these TF families are enriched in genes that are induced in 372 multiple subsequent transcriptional phases (Figure 4B and 5). 373

Phase Up1 also contains TF genes that are predicted to have a more limited regulatory 374 scope, such as the ERF TF gene ORA47, of which the binding motif (consensus, CCG(A/T)CC) 375 is only overrepresented in the promoters of genes assigned to phase Up3. These genes include 376 the JA biosynthesis genes LOX2, AOS, AOC1,2,3, ACX and OPR3, thus suggesting that this cis-377 element and its cognate TF ORA47 may play a role in regulating JA production, which reflects 378 the positive feedback loop that is known to maintain and boost JA levels upon initiation of the 379 JA response (Wasternack, 2015). Focusing on this predicted subnetwork (Figure 6A), we found 380 381 that ORA47 and several of the JA biosynthesis genes were predicted to be targets of MYC2, suggesting that MYC2 together with ORA47 regulates JA biosynthesis in Arabidopsis. Figure 382 6B shows that the presence of the ORA47-binding motif is conserved between the promoters of 383 384 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 in the regulation of JA biosynthesis genes. Evidence for this is provided by the direct binding of 386 ORA47 to promoter elements of AOC1, AOC3 and LOX3, as demonstrated by yeast one-hybrid 387 experiments (Supplemental Figure 8). Moreover, in stimulated β-estradiol-inducible ORA47 388 plants expression of LOX2, LOX3, AOS, AOC1, AOC2 and OPR3 was increased and 389 accumulation of JA and JA-Ile was also enhanced (Figure 6C and 6D), which is in line with and 390 extends previous findings (Pauwels et al., 2008; Chen et al., 2016). We did not observe a 391 significant increase in expression of JAR1, encoding the enzyme responsible for catalyzing 392 393 conjugation of JA with isoleucine, suggesting that basal JAR1 levels are sufficient for the conversion of excess JA into biologically active JA-Ile. Taken together, these experimental 394 results confirm our model prediction that ORA47 is an important regulator of JA biosynthesis 395 and highlight the potential of combining time series expression data with motif analysis to infer 396 novel key regulators and their targets in gene regulatory networks. 397

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

(Supplemental Dataset 16). Of these, a significant portion of 31 DEGs (P < 3.59e-05; 408 hypergeometric test) was also differentially expressed in the MeJA time series. Projecting the 409 common set of DEGs onto the transcriptional network model revealed that >90% of these genes 410 are present in transcriptional phases that are temporally downstream of the phase containing 411 RAP2.6L (phase-Up2, Figure 6E). Analysis of the overlap between RAP2.6L-OX DEGs and the 412 MeJA-induced co-expression clusters from the present study revealed a specific enrichment for 413 RAP2.6L targets in cluster 14, which as described above is itself overrepresented for genes 414 associated with aliphatic glucosinolate production. Interestingly, a recent study showed that 415 416 RAP2.6L can interact with several aliphatic glucosinolate biosynthetic gene promoters and moreover, that *rap2.61* mutants are perturbed in glucosinolate production (Li, 2014). 417

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

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

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

445

446 **DISCUSSION**

Computational analyses of high-density time series of RNA-Seq data obtained from *Arabidopsis* leaves of the same developmental stage (leaf number 6), allowed us to provide an unprecedentedly detailed insight into the architecture and dynamics of the JA gene regulatory network. Previously, studies on phytohormone-induced transcriptional responses have typically included only a limited number of time points or focused on the effect of perturbation of specific regulatory proteins on transcriptional activity in hormone-controlled gene regulatory networks (Tsuda et al., 2009; Nakata et al., 2013). Our time series study shows that MeJA induces a burst of transcriptional activity that generates a variety of detailed temporal expression patterns that partition into specific gene clusters representing different biological processes (Figure 1, 2 and 4; Supplemental Figure 1 and 6). Differential expression analysis yielded a considerably more comprehensive MeJA-responsive gene set compared to previous transcriptomic studies (Figure 1), including a significant number of genes not represented on microarrays. In turn, this information yielded novel insights into the chronology and regulation of the biologically relevant JA response.

461

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

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

Mutants in bHLH27 and the double mutant corresponding to MYB48/59 were more 481 susceptible to B. cinerea, yet more resistant to M. brassicae (Figure 2). Although this 482 necrotrophic pathogen and chewing insect both stimulate JA biosynthesis, many subsequently 483 induced changes in JA-responsive gene expression are specifically directed to the different 484 attackers and hence engage different TFs and downstream targets. This is known to be 485 coordinated by the mutually antagonistic ERF branch of the JA pathway, which is co-regulated 486 by ET, and the MYC branch of the JA pathway, which is co-regulated by ABA (Pieterse et al. 487 2012). Several TFs have been documented to differentially affect MYC versus ERF branch-488 controlled gene expression and associated defenses. The best-known example of such a regulator 489 is MYC2, a key positive regulator of MYC branch genes and associated defenses against 490 chewing insects (e.g. Helicoverpa armigera, Spodoptera littoralis) (Dombrecht et al., 2007; 491 Fernández-Calvo et al., 2011). In contrast, MYC2 negatively regulates defense against 492 necrotrophic pathogens (e.g. B. cinerea, Plectosphaerella cucumerina) (Lorenzo et al., 2004; 493 Nickstadt et al., 2004). JA-inducible NAC TF family paralogs, ANAC019 and ANAC055, show 494 the same effect: they positively regulate MYC branch-associated genes and defenses to S. 495 496 *littoralis*, while they antagonize ERF branch-associated resistance to *B. cinerea* (Bu et al., 2008; Schweizer et al., 2013) Oppositely, the positive regulator of the ERF branch, ORA59, controls 497 defenses to B. cinerea while it antagonizes MYC branch defenses and ORA59 overexpression 498 499 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 ERF branch activation, thereby influencing resistance to B. cinerea. Also other bHLH TFs (so 501 called JAMs) have been reported to antagonize MYC2-activated gene expression and defense to 502 insects (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013). By contrast, Song et al. (2013) 503 reported that ERF branch defense marker genes and resistance against *B. cinerea* were enhanced 504 505 by the quadruple mutant of bHLH3/13/14/17. This indicates different underlying mechanisms of the different repressive bHLHs. MYB48/59 also antagonize the MYC branch as signified by the 506 myb48myb59 mutant, showing not only enhanced resistance to M. brassicae, but also enhanced 507 508 expression of MYC branch-associated genes (Figure 2E-G; Supplemental dataset 6 and 7). The transcriptome analysis of myb48myb59 did not suggest that the reduced resistance to B. cinerea 509 is due to MYB48/59-mediated antagonism of ERF branch. It may be that down-regulation of 510 gene clusters enriched in specific secondary metabolism contributes towards compromised 511 immunity in this mutant, but this awaits further functional analysis. 512

513

514 Uncovering redundant function by double mutant analysis

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

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

525

526 Network reconstruction enables prediction of regulatory interactions

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

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

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

551

552 Dataset integration validates TF-specific regulatory functions

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

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

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

585

586 Summary

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

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

596

597 **METHODS**

Plant materials and growth conditions. All wild-type, mutant, and transgenic Arabidopsis 598 599 thaliana plants used in this study are in the Columbia ecotype (Col-0) background, except for the RAP2.6L-OX line which has the WS background. The following T-DNA insertion mutants and 600 transgenic lines were obtained from the Nottingham Arabidopsis Stock Centre: ofp1 601 (At5g01840; SALK 111492C), myb59 (At5g59780; GK-627C09), anac056 (At3g15510; 602 SALK 137131C), (At5g13330; SALK 051006C), (At1g43160; 603 rap2.6l *rap2.6* SAIL 1225G09), *erf16(-1)* (At5g21960; SALK 053563C), *erf16-2* 604 (At5g21960; SALK 096382C), at1g10586 (At1g10586; SALK 027725C), bhlh19 (At2g22760; 605 GABI 461E05), (At4g29930; SALK 049808C), *bhlh27(-1) bhlh27-2* (At4g29930; 606 (At5g57150; 607 SALK 149244C), bhlh35 SALK 100300C), bhlh92 (At5g43650; SALK 033657C), bhlh113 (At3g19500; GK 892H04), myb48 (At3g46130; SALK 103847), 608 ora59 (Zander et al., 2014) (At1g06160; GK-061A12.16), and ORA47 β-estradiol-inducible 609 TRANSPLANTA line (Coego et al., 2014) (N2101685). The myb48 and myb59 mutants were 610 crossed to generate the myb48myb59 double mutant. The myc2,3,4 triple mutant 611 (At1g32640/At5g46760/At4g17880) has been described previously (Fernández-Calvo et al., 612 2011). Seeds were stratified for 48 h in water at 4°C prior to sowing on river sand. After 2 613

weeks, the seedlings were transferred to 60-mL pots containing a soil:river sand mixture (12:5) 614 that had been autoclaved twice for 1 h. Plants were cultivated in standardized conditions under a 615 10-h day (75 μ mol/m²/s¹) and 14-h night cycle at 21°C and 70% relative humidity. Plants were 616 watered every other day and received modified half-strength Hoagland nutrient solution 617 containing 10 mM Sequestreen (CIBA-GEIGY GmbH, Frankfurt, Germany) once a week. To 618 minimize within-chamber variation, all the trays, each containing a mixture of plant genotypes or 619 treatments, were randomized throughout the growth chamber once a week. Mutants or treatments 620 were indicated by colored labels of which the code was unknown by the experimenter. T-DNA 621 622 insertion lines were confirmed homozygous for the T-DNA in the relevant genes with PCR using the gene-specific primers listed in Supplemental Table 1. The RAP2.6L overexpressing line 623 (RAP2.6L-OX) (Krishnaswamy et al., 2011) and the background accession (WS), were cultivated 624 as described previously (Windram et al., 2012). 625

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

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

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Induction of the *ORA47* β-estradiol-inducible line and hormone analysis. Five-week-old *ORA47* inducible overexpression lines were treated by dipping the rosette leaves into a mock or β-estradiol (Sigma-Aldrich, Steinheim, Germany) solution. The mock solution contained 0.015% (v/v) Silwet L77 and 0.1% DMSO. The β-estradiol solution contained 0.015% (v/v) Silwet L77 and 10 μ M β-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 JA, JA-Ile, SA, and ABA quantification, 0.5 g of leaf tissue was ground to a fine powder using 646 liquid nitrogen. Samples were homogenized in 0.5 ml of 70% methanol using a Precellys24 647 tissue homogenizer (Bertin Technologies) by shaking at 6,000 rpm for 40 s. The resulting 648 homogenates were centrifuged at 10,000 x g for 20 min at 4°C. Hormone levels were analyzed by 649 liquid chromatography-mass spectrometry (LC-MS) on a Varian 320 Triple Ouad LC-MS/MS. 650 651 JA and JA-Ile levels were calculated by correcting for the internal standard of JA and for leaf weight. ABA and SA levels were calculated by correcting for leaf weight and their respective 652 internal standards. 653

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Insect performance and disease bioassays. *Botrytis cinerea* disease resistance was determined essentially as described previously (Van Wees et al., 2013). In brief, *B. cinerea* was grown on half-strength Potato Dextrose Agar (PDA; Difco BD Diagnostics, Franklin Lakes, NJ, USA) plates for 2 weeks at 22°C. Harvested spores were incubated in half-strength Potato Dextrose Broth (PDB; Difco) at a final density of 5 x 10⁵ spores/mL for 2 h prior to inoculation. Five-

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

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

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

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

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

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

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

705

Processing of RNA-Seq data. Read alignment, summarization and normalization followed the 706 pipeline as previously described (Van Verk et al., 2013). Reads were aligned to the Arabidopsis 707 genome (TAIR version 10) using TopHat v2.0.4 (Trapnell et al., 2009) with the parameter 708 settings: 'transcriptome-mismatches 3', 'N 3', 'bowtiel', 'no-novel-juncs', 'genome-read-709 mismatches 3', 'p 6', 'read-mismatches 3', 'G', 'min-intron-length 40', 'max-intron-length 710 711 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 Huber, 2010). 714

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

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

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

Differentially expressed genes between Col-0 and *myb48myb59* ($|log_2$ -fold change| >1; FDR ≤ 0.05) were identified using DESeq (Anders and Huber, 2010). For analysis of DEGs between WS and *RAP2.6L*-OX see "Microarray analysis of *RAP2.6L* transgenic plants".

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

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

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

- For promoter motif analysis, the promoter sequences defined as the 500 bp upstream of 752 the predicted transcription start site (TSS) were retrieved from TAIR (version 10). De novo 753 promoter motifs were identified by applying the motif-finding programs MEME (Bailey and 754 Elkan, 1994) and XXmotif (Hartmann et al., 2013) to the promoters of all genes present in a 755 given co-expression cluster. This approach exploits the strengths of different motif-finding 756 strategies, which has been demonstrated to improve the quality of motif detection (Tompa et al., 757 758 2005). Both algorithms searched for motifs on the forward and reverse strands and used the zeroor-one occurrences per sequence (ZOOPS) motif distribution model. MEME was run using a 759 3rd-order Markov model learned from the promoter sequences of all genes in the Arabidopsis 760 genome, using parameter settings: '-minw 8 -maxw 12 -nmotifs 10'. XXmotif was run using a 761 3rd-order Markov model and the medium similarity threshold for merging motifs, with all other 762 parameters kept as default. This analysis yielded a large number of motifs, many of which were 763 highly similar. To reduce redundancy amongst motifs, a post-processing step was performed 764 using the TAMO software package (Gordon et al., 2005). Motifs were converted to TAMO 765 format, clustered using the UPGMA algorithm, and merged to produce consensus motifs. The set 766 of processed motifs were converted to MEME format for all subsequent analyses using the 767 tamo2meme function available in the MEME Suite (Bailey et al., 2009). For the analysis of 768 769 known motifs originating from protein-binding microarray (PBM) studies (Franco-Zorrilla et al., 2014; Weirauch et al., 2014), the published weight matrices were converted into MEME format. 770
- The presence or absence of a given motif within a promoter was determined using FIMO (Grant et al., 2011). A promoter was considered to contain a motif if it had at least one match

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

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

784

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

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

809

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

819

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

831

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

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Yeast-1-Hybrid (Y1H) protein-DNA interaction assays. Cloning of bait promoter DNA and
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 sequence was isolated from the TF library as described in Hickman et al., (2013) and the correct 843 sequence confirmed by sequencing. Prev strains were constructed by cloning the ORA47 coding 844 sequence into pDEST22 (Invitrogen) and transforming AH109 yeast (Clontech), while empty 845 pDEST22 was used to transform AH109 as a negative control. Three µL of bait strain cultures 846 were spotted onto YPDA (yeast, peptone, dextrose, adenine) plates and dried before being 847 overlaid with 3 µL of prey strain culture and left to grow overnight at 30°C. Colonies were 848 subcultured in 1 mL mating selective media (SD-Leu-Trp, Clontech) and grown for two nights at 849 30°C with shaking. Cultures were diluted to 10⁸ cells/mL in SD-Leu-Trp liquid media before 850 four 10-fold serial dilutions were made. Three uL of each diploid strain was plated to mating 851 selective (SD-Leu-Trp, Clontech) and interaction selective (SD-Leu-Trp-His, Clontech) media 852 and incubated at 30°C for 72 h before being photographed using a G:Box EF2 (Syngene). For 853 promoter D, 5 mM 3-Aminotriazole (Sigma-Aldrich) was required to suppress autoactivation of 854 HIS3 expression by this promoter region. For promoters A, B and D experiments were performed 855 using two independent promoter transformants and four transcription factor transformants, for a 856 total of eight replicates. For promoter C, there were three replicates across two independent 857 promoter transformants and two transcription factor transformants. 858

859

860 Accession numbers

- Arabidopsis gene names and identifiers referred to in this article are:
- 862 OFP1 (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),

bHLH113 (At3g19500), COI1 (At2g39940), AOS (At5g42650), AOC1 (At3g25760), AOC3 865 (At3g25780), LOX2 (At3g45140), LOX3 (AT1G17420), OPR3 (At2g06050), JAR1 866 (At2g46370), JAZ1 (At1g19180), JAZ2 (At1g74950), JAZ3 (At3g17860), JAZ4 (At1g48500), 867 JAZ5 (At1g17380), JAZ6 (At1g72450), JAZ7 (At2g34600), JAZ8 (At1g30135), JAZ9 868 (At1g70700), JAZ10 (At5g13220), JAZ11 (At3g43440), JAZ12 (At5g20900), MYC2 869 (At1g32640), bHLH003 (At4g16430), bHLH013 (At1g01260), bHLH014 (At4g00870), 870 bHLH017/JAM1 (At2g46510), MYC3 (At5g46760), MYC4 (At4g17880), MYB29 (At5g07690), 871 ANAC019 (At1g52890), ANAC055 (At3g15500), NINJA (At4g28910), RGL3 (At5g17490), 872 ORA47 (At1g74930), ORA59 (At1g06160), VSP1 (At5g24780), VSP2 (At5g24770), NPR4 873 (At4g19660), MYB51 (At1g18570), EDS1 (At3g48090), PAD4 (At3g52430). 874

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
- 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.
- Supplemental Figure 4. *B. cinerea* disease severity and growth of *M. brassicae* larvae on
 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
- 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 <i>RAP2.6L</i> -OX.
923	

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924

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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
- and RCS performed hormone measurements. AJ and AT performed Y1H assays. JR performed
- microarray experiments. RJH, MCvV, CMJP and SCMvW wrote the manuscript. All authors
- discussed the results and commented on the manuscript.

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1203 FIGURE LEGENDS

Figure 1. Temporal expression profiles following application of MeJA. (A) Circos plots of 1204 time series expression profiles from our MeJA experiment in comparison to previously published 1205 MeJA- or P. rapae-induced transcriptome data (Pauwels et al., 2008; Goda et al., 2008; Coolen 1206 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 16 h after treatment). Genes differentially expressed in both datasets are marked by connecting 1211 bands (colors indicate first time point of differential expression in our study). Each section 1212 1213 within the circus plot represents a set of 100 DEGs. (B) Examples of expression profiles of selected JA and SA pathway marker genes in our study. y-axis, transcript abundance; x-axis, 1214 time (h) post application of MeJA; error bars indicate SE. 1215

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Figure 2. Clustering of co-expressed genes in the JA gene regulatory network and identification of novel components of JA-dependent resistance. (A) The set of 3611 genes showing differential expression in *Arabidopsis* leaves following exogenous application of MeJA was partitioned into 27 distinct co-expressed gene clusters using SplineCluster. The heatmap shows the mean gene expression profile for each cluster, with red and blue indicating up1222 regulation and down-regulation of expression (log₂-fold change (MeJA/mock)), respectively. (B) Significantly overrepresented TF families within clusters of genes up-regulated (clusters 1-14; 1223 red) or down-regulated (clusters 15-27; blue) in response to MeJA treatment (hypergeometric 1224 1225 test; $P \le 0.001$). (C) Quantification of disease symptoms of wild-type Col-0, highly susceptible ERF TF mutant ora59, and T-DNA insertion lines for selected genes ERF16, MYB59, and 1226 bHLH27 (members of co-expression clusters 2, 4 and 1, respectively) at 3 days after inoculation 1227 with B. cinerea. Disease severity of inoculated leaves was scored in four classes ranging from 1228 restricted lesion (class I), non-spreading lesion (class II), spreading lesion (class III), up to 1229 severely spreading lesion (class IV). The percentage of leaves in each class was calculated per 1230 plant (n > 20). Asterisk indicates statistically significant difference from Col-0 (Chi-squared test; 1231 $P \le 0.05$). (D) Performance of *M. brassicae* larvae on Col-0, highly susceptible triple bHLH TF 1232 1233 mutant myc2,3,4 and T-DNA insertion lines for selected genes ANAC056 (co-expression cluster 13) and bHLH27. The larval fresh weight was determined after 8 days of feeding. Asterisk 1234 indicates statistically significant difference from Col-0 (two-tailed Student's t test for pairwise 1235 comparisons; $P \le 0.05$; n=30; error bars are SE). (E) Quantification of disease symptoms of Col-1236 0, myb48, myb59, myb48myb59 and ora59 mutant lines at 3 days after inoculation with B. 1237 *cinerea*. Disease severity of inoculated leaves was scored as described in (C) (n > 20). Asterisk 1238 indicates statistically significant difference from Col-0 (Chi-squared test; $P \leq 0.05$). (F) 1239 Performance of *M. brassicae* larvae on Col-0 and *mvb48*, *mvb59* and *mvb48mvb59* mutant lines. 1240 1241 The larval fresh weight was determined after 12 days of feeding. Asterisk indicates statistically significant difference from Col-0 (two-tailed Student's t test for pairwise comparisons; $P \le 0.05$; 1242 n=30; error bars are SE). (G) Heatmap indicating hypergeometric enrichment P value of genes 1243

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

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Figure 3. Enriched cis-regulatory motifs and functional categories in MeJA-responsive 1247 gene co-expression clusters. (A) Overrepresentation of known TF DNA-binding motifs within 1248 the unions of up-regulated and down-regulated genes. Rows indicate motifs and are colored by 1249 corresponding TF family. Red boxes indicate a motif that is significantly overrepresented 1250 (cumulative hypergeometric distribution). (B) Representative co-expression clusters with 1251 1252 overrepresented TF DNA-binding motifs. Top: Profiles of log₂-fold change in gene expression (MeJA/mock), with mean profile (red) and cluster size (n). Selected overrepresented functional 1253 categories (F) and representative genes (G) are denoted. Sequence logo depiction of selected 1254 1255 known (middle) and *de novo*-derived (bottom) motifs that are significantly overrepresented. Full results used to derive this figure are available in Supplemental Dataset 6 and 7. 1256

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Figure 4. Chronology of changes in the MeJA-triggered gene regulatory network. (A) 1258 Phasing of MeJA-induced transcriptional changes. DEGs were divided into four sets according 1259 1260 to their function as regulator or non-regulator (regulated), and their expression pattern being up-(red) or down-regulated (blue) over time. For each set of genes, a correlation matrix of gene 1261 transcription counts between all pairs of time points was computed using Pearson's correlation 1262 1263 metric. Shown are the dendrograms produced by hierarchical clustering of the transcriptome correlation matrices (yellow, high correlation; cyan, low correlation). Time is in hours. (B) 1264 Analysis of the major transcriptional phases in the JA gene regulatory network. Transcriptional 1265 1266 phases are indicated by boxes, aligned on the timeline. DEGs are assigned to the phases according to the time point where they become first differentially expressed; indicated are overrepresented functional categories and representative genes. Colored squares indicate known TF DNA-binding motifs overrepresented in gene promoters (hypergeometric distribution; $P \le$ 0.001). Pie charts indicate the proportion of TF gene families.

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

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

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1309 **Competing financial interests:**

1310 The authors declare no competing financial interests.

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