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Architecture and dynamics of the jasmonic acid gene regulatory network

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ABSTRACT

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

INTRODUCTION

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

In the past decade, major discoveries in the model plant *Arabidopsis thaliana* have greatly advanced our understanding of the JA signaling pathway. In the absence of an invader, when JA levels are low, activation of JA responsive gene expression is constrained by repressor proteins of the JASMONATE ZIM-domain (JAZ) family that bind to specific JA-regulated transcription factors (TFs). The conserved C-terminal JA-associated (Jas) domain of JAZs 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 corepressor, either directly or through the NOVEL INTERACTOR OF JAZ (NINJA) adapter, 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 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 (Dombrecht et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011). While current evidence indicates that MYC2, MYC3 and MYC4 act as master regulators of the onset of JA responsive gene expression, additional factors are required for further fine-regulation of the JA signaling circuitry. Several other bHLH TFs, such as JASMONATE-ASSOCIATED MYC2-LIKE1 (JAM1)/bHLH017, JAM2/bHLH013, JAM3/bHLH003 and bHLH014 act redundantly to repress JA-inducible genes by competitive binding to *cis*-regulatory elements, possibly to control the timing and magnitude of the induced JA response (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013). Another important family of regulators that shape the JA response is the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family of TFs. AP2/ERF-type TFs, such as ERF1 and ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF-domain protein59), integrate the JA and ET response pathways and act antagonistically on MYC2,3,4-regulated JA-responsive genes (Lorenzo et al., 2003; Pré et al., 2008; Zhu et al., 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 to find leads that can improve crop resistance to pathogens and insects, while maintaining plant growth. Previously, several microarray-based transcriptome profiling studies revealed important information on the regulation of JA-responsive gene expression (Goda et al., 2008; Pauwels et 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 network. Here, we performed an in-depth, high-throughput RNA sequencing (RNA-Seq) study in 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 landscape provided insight into the structure of the JA gene regulatory network at an unprecedented level of detail. We accurately identified distinct JA-induced expression profiles, and used these to predict and validate the biological function of several novel regulators of the JA immune regulatory network. We resolved the sequence of transcriptional events that take place following induction of the JA response, constructed a dynamic model of the JA gene regulatory network, and identified and validated subnetworks surrounding several JA-induced TFs, confirming the suitability of our systems approach to obtain detailed knowledge on regulation of the JA response pathway.

RESULTS

A time course of MeJA-elicited transcriptional reprogramming

A key step towards a systems-level understanding of the architecture of the JA signaling network is to obtain comprehensive and accurate insight into the dynamic transcriptional reprogramming that takes place in plants following JA stimulation. To go beyond earlier studies that analyzed the JA transcriptional response with a limited number of time points, we generated a high-resolution time series of JA-mediated transcriptional reprogramming in *Arabidopsis* leaves. Previously, similar types of dense time series experiments with *Arabidopsis* have been successfully utilized 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* (Breeze et al., 2011; Windram et al., 2012; Lewis et al., 2015). Here, we used RNA-Seq technology to profile whole-genome transcriptional expression in *Arabidopsis* leaves just before the treatments (t = 0 h), and over 14 consecutive time points within 16 h following application of methyl JA (MeJA; that is readily converted to JA) or a mock solution to the leaves of intact plants (Supplemental Dataset 1). At all time points and for each treatment, one leaf (true leaf number 6) was sampled in quadruplicate from four independent 5-week-old Col-0 plants, yielding 116 samples in total (Supplemental Dataset 1). Read counts were normalized for differences in sequencing depth between samples (Supplemental Dataset 2) and a generalized linear model was employed to identify genes whose transcript levels differed significantly over 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 3). Many of these DEGs were not previously described as MeJA responsive (Figure 1A) in 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 JA-inducing 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 responses to MeJA stimulation (Supplemental Figure 1). The majority of expression changes in 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 specific gene expression programs (Yosef and Regev, 2011). Examples of genes whose expression is up- or down-regulated for a short period of time followed by a transition to a steady state, which is often a return to basal expression, are *JAR1* and *EDS1* (Figure 1B). Yet, there are also genes that display a longer lasting change in expression level, e.g. *MYC2* and *BES1* (Figure 1B). Because all transcriptional changes were monitored in leaf number 6, we maximally synchronized the onset of the JA response in intact plant tissue. Hence, the resulting information-rich time series of MeJA-responsive gene expression profiles are highly suited to computational approaches that can generate novel biological insights into the regulation of the underlying JA transcriptional network.

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

The genes in each cluster were tested for overrepresented functional categories using Gene Ontology (GO) term enrichment analysis to investigate the biological significance of the distinct dynamic expression patterns (Supplemental Dataset 5). This analysis showed that clusters representing up-regulated genes are, as expected, overrepresented for functional terms associated with JA defense responses. Broad annotations such as 'Response to wounding' and 'Response to herbivory' are present in multiple up-regulated clusters, while in contrast the more specific functional categories are linked to distinct clusters. For example, cluster 6 is specifically overrepresented for the annotation term 'Anthocyanin-containing compound biosynthetic process', cluster 8 for 'Tryptophan biosynthetic process', and cluster 14 for 'Glucosinolate 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 have an important function in these specific processes (Supplemental Dataset 5). The significant 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).

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 an enrichment for known JA-related genes such as the herbivory markers *VSP1* and *VSP2,* as well as the regulators *JAZ1, 2, 5, 7, 8, 9, 10* and *13, MYC2*, *ANAC019*, *ANAC055*, *RGL3*, and *JAM1* (Wasternack and Hause, 2013). In addition, TF genes with no previously reported roles in the JA response pathway are present in these clusters, which implies that they may also have 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 uncharacterized TF genes from other clusters, displaying a similarly rapid response to MeJA treatment. The respective *Arabidopsis* T-DNA knockout lines were functionally analyzed for

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 al., 2012). Mutants in the TF genes *bHLH27*, *ERF16* and *MYB59* displayed a significant increase in disease susceptibility to *B. cinerea* compared to wild-type *Arabidopsis* Col-0, approaching the disease severity level of the highly susceptible control mutant *ora59* (Figure 2C; full results in Supplemental Figure 3 and additional mutant alleles in Supplemental Figure 4). Weight gain of *M. brassicae* larvae was significantly reduced on mutants of *ANAC056* and *bHLH27*, while on none of the tested mutants larval weight was enhanced, as was the case on the susceptible control 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 predicted role in the JA response could be functionally validated for either *B. cinerea* or *M. brassicae* resistance, demonstrating the value of using information-rich time series data to accurately identify co-expressed genes that may have novel functions in the JA pathway.

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

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

To gain insight into the biological processes contributing to the differentially altered 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 leaves (168 were up-regulated and 231 were down-regulated in the double mutant; Supplemental Dataset 6). Functional category analysis showed that in the up-regulated DEG set of the mutant compared to Col-0, processes like 'Response to wounding' and 'Response to jasmonic acid stimulus' were enriched (Supplemental Dataset 7). This is in accordance with these *myb48my59*-upregulated DEGs being overrepresented in co-expression clusters 1, 2, 7 and 9 of the MeJA responsive DEGs (Figure 2G). Genes that showed enhanced expression by both MeJA treatment and the *myb48myb59* mutations are for example JA biosynthetic genes *AOC2* and *OPR3*, and TF gene *MYC2*. Also the downstream herbivore defense marker gene *VSP2* showed > 50-fold higher expression level in the mutant. This suggests prioritization of the JA pathway towards the anti-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 would explain the reduction of defense against the necrotrophic pathogen *B. cinerea*, is not 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.

Enrichment of TF DNA-binding motifs

TFs regulate gene expression by binding to *cis*-regulatory elements of target genes in a sequence specific manner. Mapping of regulatory DNA motifs that are associated with dynamic MeJA-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 overrepresented within the promoters of co-expressed MeJA-responsive DEGs, using recently identified DNA-binding specificities for 580 *Arabidopsis* TFs derived from studies with protein-binding microarrays (PBMs) (Franco-Zorrilla et al., 2014; Weirauch et al., 2014). First, we screened for overrepresentation of these motifs in the unions of up- and down-regulated gene clusters, respectively (Figure 3A). Motifs corresponding to DNA-binding sites of bHLH, bZIP, ERF and MYB TFs are clearly overrepresented in the group of up-regulated genes, while WRKY and TCP TF specific motifs are markedly overrepresented in the down-regulated genes. Members of the WRKY TF family and their cognate *cis*-elements are key regulators of the SA response pathway (Pandey and Somssich, 2009), suggesting that WRKYs are important targets 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 the chance of discovering nuanced sequence motifs among the genes in these clusters, we supplemented the known motif analysis (Supplemental Dataset 8) with *de novo* motif discovery

(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 expression patterns. Strikingly, while motifs that correspond to bHLH-binding sites are enriched in the majority of the up-regulated gene clusters, ERF- and MYB-binding motifs are only overrepresented in a small selection of the up-regulated clusters, which are associated with specific biological processes (Supplemental Dataset 8). For example, clusters 6 and 14, which are enriched for GO terms describing distinct secondary metabolite biosynthesis pathways, are enriched for different (*de novo*) predicted MYB DNA-binding motifs (Figure 3B). These 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 specialized roles in modulating the expression of dedicated sets of target genes.

Chronology of MeJA-mediated transcriptional reprogramming

Next, we utilized the temporal information in our RNA-Seq time series to resolve the chronology of gene expression events in the JA gene regulatory network. First, we divided the genes in sets of up- and down-regulated DEGs and sorted them according to the time at which they first became differentially expressed (Supplemental Figure 6; see Methods for details). From this analysis, it became clear that a massive onset of gene activation precedes that of gene down-regulation, and that different waves of coordinated gene expression changes can be identified in 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, allowing for a rapid response to an external signal (Alon, 2007). Up- and down-regulated DEGs 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

represents the immediate transcriptional response with genes encoding bHLHs, JAZs, MYBs, ERFs, and other transcriptional regulators associated with JA biosynthesis. These early regulator genes may play a role in the induction of other regulator-encoding genes present in phases Up2 and 4, and of regulated genes present in phases Up3, 5 and 6, which are linked to defense responses such as glucosinolate, tryptophan and anthocyanin biosynthesis (Figure 4B; Supplemental Dataset 12). In support of this, in the promoters of DEGs in phase Up3, DNA motifs that can be bound by TFs transcribed in previous phases Up1 and 2, like bHLH-, ERF- and MYB-binding motifs, are enriched. In phase Up3, genes involved in JA biosynthesis are also 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 gene expression events, starting with the activation of genes encoding specific classes of TFs and of JA biosynthesis enzymes, followed by genes encoding enzymes involved in the production of important defensive secondary metabolites.

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

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 in the promoters of genes present in phase Down 1 and 2, suggesting that their repressed expression is mediated by an effect of MeJA on WRKY action. Later phases of transcriptional repression (phases Down3 and 4) are marked by an overrepresentation of genes related to growth and development, including primary metabolism and auxin signaling, and an enrichment of DNA motifs recognized by TCP TFs, which conceivably reflects an effort by the plant to switch energy resources from growth to defense (Attaran et al., 2014). A general observation that can be made from this chronological analysis of the JA gene regulatory network is that despite the overall relatively short transcriptional cascades controlling gene activation or repression, distinctive transcriptional signatures, associated with specific biological processes, are initiated at different phases in time.

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 separated transcriptional phases to construct a gene regulatory network that predicts directional interactions between the JA responsive TF genes and all genes associated with the different transcriptional phases (Supplemental Dataset 15). The JA gene regulatory network generated via this analysis is shown in Figure 5, in which a differentially expressed TF gene (represented by a 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 phase. The generated network model shows that the TFs are predicted to regulate expression of genes at either single or multiple transcriptional phases. The early phases likely contain key

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 consensus, CACGTG) are enriched in the promoters of genes assigned to a large fraction of the up-regulated transcriptional phases. This prediction is in line with recent reports suggesting that the positive regulator MYC2 and the negative regulator JAM1 cooperate to balance JA responses 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 repress transcription awaits further investigation. Phases Up1 and Up2 also contain the TF genes *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). Cognate DNA-binding motifs of these TF families are enriched in genes that are induced in multiple subsequent transcriptional phases (Figure 4B and 5).

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

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 ORA47 to promoter elements of *AOC1, AOC3* and *LOX3,* as demonstrated by yeast one-hybrid experiments (Supplemental Figure 8). Moreover, in stimulated β-estradiol-inducible *ORA47* plants expression of *LOX2, LOX3, AOS, AOC1, AOC2* and *OPR3* was increased and accumulation of JA and JA-Ile was also enhanced (Figure 6C and 6D), which is in line with and extends previous findings (Pauwels et al., 2008; Chen et al., 2016). We did not observe a significant increase in expression of *JAR1*, encoding the enzyme responsible for catalyzing 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 results confirm our model prediction that ORA47 is an important regulator of JA biosynthesis and highlight the potential of combining time series expression data with motif analysis to infer novel key regulators and their targets in gene regulatory networks.

For the vast majority of TFs in our chronological model, it is unclear which specific JA-responsive genes they regulate. To validate and extend our chronological network model further, we made use of transcriptome data sets of three *Arabidopsis* lines that are perturbed in TFs that are predicted by our model to regulate downstream subnetworks. We investigated the effect of the TFs RAP2.6L and ANAC055, which have previously been suggested to regulate JA-responsive genes among others (Bu et al., 2008; Krishnaswamy et al., 2011), by studying their 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 2G. We performed transcriptional profiling of leaves from plants overexpressing *RAP2.6L* (*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; hypergeometric test) was also differentially expressed in the MeJA time series. Projecting the common set of DEGs onto the transcriptional network model revealed that >90% of these genes are present in transcriptional phases that are temporally downstream of the phase containing *RAP2.6L* (phase-Up2, Figure 6E). Analysis of the overlap between *RAP2.6L*-OX DEGs and the MeJA-induced co-expression clusters from the present study revealed a specific enrichment for RAP2.6L targets in cluster 14, which as described above is itself overrepresented for genes associated with aliphatic glucosinolate production. Interestingly, a recent study showed that RAP2.6L can interact with several aliphatic glucosinolate biosynthetic gene promoters and moreover, that *rap2.6l* mutants are perturbed in glucosinolate production (Li, 2014).

Using a similar approach, 56 genes differentially expressed in an *anac055* mutant line compared to wild-type plants (described previously in Hickman et al. (2013)) were overlaid on the JA gene regulatory network. The overlap between MeJA-responsive and ANAC055-regulated genes was statistically significant (24 DEGs, P < 4.74e-10; hypergeometric test) and > 85% of these genes became for the first time differentially expressed after *ANAC055* was induced by MeJA (phase-Up2, Supplemental Figure 9). Down-regulated gene co-expression cluster 20 is overrepresented for ANAC055 targets that are enhanced in the *anac055* mutant, and is enriched for GO terms related to SA biosynthesis. Interestingly, ANAC055 has previously been shown to target SA biosynthetic and metabolic genes to negatively regulate SA 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 *myb48myb59* double mutant line compared to Col-0 wild type (as described above; Supplemental 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 down-regulated 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 MeJA-induced 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.

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.

Network-informed discovery of novel players in the JA response

Using a dynamic network approach, we systematically determined how the diverse positive and negative regulatory components in the JA gene regulatory network function over time. MeJA-induced gene activation or repression is shown to be controlled by short transcriptional cascades, yet yielding distinctive transcriptional signatures that correspond to specific sets of genes and biological processes (Figure 2). In general, it appears that bHLH TFs are master regulators controlling the majority of the MeJA-inducible genes, while ERF and MYB TFs fine-tune the expression of dedicated sets of target genes in specific sectors of the gene regulatory network (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 guilt-by-association approach, twelve early MeJA-induced TFs with unknown roles in the JA 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 against the pathogen *B. cinerea* and/or the insect *M. brassicae* (Figure 2), highlighting the high success rate of our approach in the discovery of biological functions of novel genes in the JA

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

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

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 called JAMs) have been reported to antagonize MYC2-activated gene expression and defense to insects (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013). By contrast, Song et al. (2013) reported that ERF branch defense marker genes and resistance against *B. cinerea* were enhanced 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 *myb48myb59* mutant, showing not only enhanced resistance to *M. brassicae*, but also enhanced 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* is due to MYB48/59-mediated antagonism of ERF branch. It may be that down-regulation of gene clusters enriched in specific secondary metabolism contributes towards compromised immunity in this mutant, but this awaits further functional analysis.

Uncovering redundant function by double mutant analysis

Reverse genetic screens are an important approach in the study of gene functions in *Arabidopsis*, 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 explain why 8 out of the 12 T-DNA insertion lines of the predicted JA-responsive TF genes that 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 generated a double mutant that displayed a more severe perturbation of JA-associated gene 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.

Network reconstruction enables prediction of regulatory interactions

Our time series data discerned a chronology of 10 transcriptional phases, showing that the onset of up-regulation preceded that of down-regulation, and that the first phase that was initiated within 15 minutes was represented by transcriptional regulators (Figure 4). JA biosynthesis is shown to be a first target for activation, followed by secondary metabolism, including activation 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 secondary metabolism, and the enrichment of MYB DNA-binding motifs in the up-regulated genes in later phases. Down-regulated genes showed enrichment in WRKY TF-binding motifs, which is linked with the suppressed expression of SA-associated defense genes.

Integrating TF DNA-binding motif enrichment data with our chronological JA network model predicted putative causal regulations between TFs and downstream JA-regulated subnetworks (Figure 5 and 6). Although subsets of the regulatory predictions were supported by 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 gene expression profiles, and has limited ability to account for expression of genes that strongly 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 combinatorial regulation of targets and/or are capable of capturing nonlinear characteristics of 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).

Dataset integration validates TF-specific regulatory functions

Exploring the regulatory predictions between TF regulators and their target genes highlighted a 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 this TF targets a large fraction of genes encoding enzymes involved in JA biosynthesis in *Arabidopsis* (Figure 6A) and evolutionary distant species (Figure 6B). Indeed, yeast one-hybrid experiments confirm that ORA47 binds to promoter elements of JA biosynthesis genes (Supplemental Figure 8). Using transgenic lines that allow for the conditional expression of *ORA47* upon β-estradiol treatment, we showed that induction of *ORA47* expression significantly increases levels of JA and bioactive JA-Ile, indicating that ORA47 is an important activator of 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 impact on the expression of its target genes was only reported for a small subset. Using the β-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 (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 regulatory network has remained unresolved. We show how integrating either existing or novel transcriptome data with our models of MeJA-mediated gene expression can generate hypotheses regarding the roles of specific transcriptional regulators in the context of the JA response. In particular, transcriptional profiling of plants overexpressing the MeJA-responsive TF RAP2.6L and subsequent overlay of the gene expression data onto our co-expression clusters, led to the hypothesis that within the JA gene regulatory network RAP2.6L plays a role in the regulation of glucosinolate biosynthesis-associated genes (Figure 6E). A similar approach, using the established stress-associated TF ANAC055, and MYB48/59 (highlighted in this study), confirmed and extended the predicted regulatory interactions with distinct downstream targets in the JA network model (Supplemental Figure 9 and 10). Specific co-expressed gene clusters in the JA network were shown to be affected in the TF-perturbed lines, highlighting the strength of our clustering analysis for inferring functional regulation mechanisms. A similar transcriptome overlay approach could be used in future studies to further define the roles of other JA-inducible TFs in the diverse JA subnetworks.

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 co-expressed 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 JA-regulated 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.

METHODS

Plant materials and growth conditions. All wild-type, mutant, and transgenic *Arabidopsis 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 transgenic lines were obtained from the Nottingham Arabidopsis Stock Centre: *ofp1* (At5g01840; SALK_111492C), *myb59* (At5g59780; GK-627C09), *anac056* (At3g15510; SALK_137131C), *rap2.6l* (At5g13330; SALK_051006C), *rap2.6* (At1g43160; SAIL_1225G09), *erf16(-1)* (At5g21960; SALK_053563C), *erf16-2* (At5g21960; SALK_096382C), *at1g10586* (At1g10586; SALK_027725C), *bhlh19* (At2g22760; GABI_461E05), *bhlh27(-1)* (At4g29930; SALK_049808C), *bhlh27-2* (At4g29930; SALK_149244C), *bhlh35* (At5g57150; SALK_100300C), *bhlh92* (At5g43650; SALK_033657C), *bhlh113* (At3g19500; GK_892H04), *myb48* (At3g46130; SALK_103847), *ora59* (Zander et al., 2014) (At1g06160; GK-061A12.16), and *ORA47* β-estradiol-inducible TRANSPLANTA line (Coego et al., 2014) (N2101685). The *myb48* and *myb59* mutants were crossed to generate the *myb48myb59* double mutant. The *myc2,3,4* triple mutant (At1g32640/At5g46760/At4g17880) has been described previously (Fernández-Calvo et al., 2011). Seeds were stratified for 48 h in water at 4°C prior to sowing on river sand. After 2

weeks, the seedlings were transferred to 60-mL pots containing a soil:river sand mixture (12:5) that had been autoclaved twice for 1 h. Plants were cultivated in standardized conditions under a 10-h day (75 µmol/m^2/s^1) and 14-h night cycle at 21°C and 70% relative humidity. Plants were watered every other day and received modified half-strength Hoagland nutrient solution containing 10 mM Sequestreen (CIBA-GEIGY GmbH, Frankfurt, Germany) once a week. To minimize within-chamber variation, all the trays, each containing a mixture of plant genotypes or treatments, were randomized throughout the growth chamber once a week. Mutants or treatments were indicated by colored labels of which the code was unknown by the experimenter. T-DNA 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 (*RAP2.6L*-OX) (Krishnaswamy et al., 2011) and the background accession (WS), were cultivated as described previously (Windram et al., 2012).

RNA-Seq experimental setups. For the MeJA time series, 5-week-old Arabidopsis Col-0 plants were treated by dipping the rosette leaves into a mock or MeJA (Duchefa Biochemie BV, Haarlem, The Netherlands) solution. The mock solution contained 0.015% (v/v) Silwet L77 (Van Meeuwen Chemicals BV, Weesp, The Netherlands) and 0.1% ethanol. The MeJA solution contained 0.015% (v/v) Silwet L77 and 0.1 mM MeJA, which was added from a 1,000-fold stock in 96% ethanol. For time series expression analysis, leaf number 6 (counted from oldest true leaf 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 individual leaf corresponds to one biological replicate and four biological replicates for each 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* β-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 β-estradiol (Sigma-Aldrich, Steinheim, Germany) solution. The mock solution contained 0.015%
643 (v/v) Silwet L77 and 0.1% DMSO. The β-estradiol solution contained 0.015% (v/v) Silwet L77
644 and 10 μM 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
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 x 10^5 spores/mL for 2 h prior to inoculation. Five-

660 week-old plants were inoculated by placing a 5-µL 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 ≥ 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 ≤ 0.01 for that time point. Remaining genes
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".

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.

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

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 the predicted transcription start site (TSS) were retrieved from TAIR (version 10). *De novo* promoter motifs were identified by applying the motif-finding programs MEME (Bailey and Elkan, 1994) and XXmotif (Hartmann et al., 2013) to the promoters of all genes present in a given co-expression cluster. This approach exploits the strengths of different motif-finding strategies, which has been demonstrated to improve the quality of motif detection (Tompa et al., 2005). Both algorithms searched for motifs on the forward and reverse strands and used the zero-or-one occurrences per sequence (ZOOPS) motif distribution model. MEME was run using a 3rd-order Markov model learned from the promoter sequences of all genes in the *Arabidopsis* genome, using parameter settings: '-minw 8 -maxw 12 -nmotifs 10'. XXmotif was run using a 3rd-order Markov model and the medium similarity threshold for merging motifs, with all other parameters kept as default. This analysis yielded a large number of motifs, many of which were highly similar. To reduce redundancy amongst motifs, a post-processing step was performed using the TAMO software package (Gordon et al., 2005). Motifs were converted to TAMO format, clustered using the UPGMA algorithm, and merged to produce consensus motifs. The set of processed motifs were converted to MEME format for all subsequent analyses using the tamo2meme function available in the MEME Suite (Bailey et al., 2009). For the analysis of 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.

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

773 with a *P* value ≤ 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

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 filtering yielded four mutually exclusive sets of MeJA-responsive genes (i.e. regulator genes up 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 Pearson correlation measure. Each resulting correlation matrix was then clustered using the Euclidean distance measure with average linkage. The resulting dendrograms were used to infer distinct phases of MeJA-induced transcription, where each phase has a start and end time. Each 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 first time differentially expressed before, or equal to, the final time point in a given phase (clustered group of time points), and after the final time point of a preceding phase, were assigned to that transcriptional phase (see Supplemental Figure 7 for overview of the method).

Network construction. The identification of potential regulatory network connections between TFs and transcriptional phases was performed with a set of TFs that met two criteria: (1) They were differentially expressed in response to application of MeJA (and thus belong to a phase). (2) They have an annotated DNA-binding motif (as described in "TF family and promoter motif analyses"). Each set of genes that constitute a transcriptional phase (10 phases in total) was 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 overrepresented in the promoters of genes belonging to that phase (hypergeometric distribution; $P \leq 0.005$). 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 μL of bait strain cultures
847 were spotted onto YPDA (yeast, peptone, dextrose, adenine) plates and dried before being
848 overlaid with 3 μL of prey strain culture and left to grow overnight at 30^{o}C. Colonies were
849 subcultured in 1 mL mating selective media (SD-Leu-Trp, Clontech) and grown for two nights at
850 30^{o}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 μL 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^{o}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 *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),

865 *bHLH113* (At3g19500), *COI1* (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), *MYC*3 (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.

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

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

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

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

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

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

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936
937

Author contributions

SCMvW and CMJP conceived the approach and together with RJH and MCvV designed the study. RJH and MCvV designed all bioinformatics approaches. KD provided analytical and intellectual contributions. RJH and MCvV performed data analysis. RJH, MCvV, AJHvD, MPM, 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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FIGURE LEGENDS

Figure 1. Temporal expression profiles following application of MeJA. (A) Circos plots of time series expression profiles from our MeJA experiment in comparison to previously published MeJA- or *P. rapae*-induced transcriptome data (Pauwels et al., 2008; Goda et al., 2008; Coolen et al., 2016), as indicated at the top left of each plot. Outermost bands indicate differentially expressed gene sets from this study (red, up-regulated; dark blue, down-regulated) and from the previously published datasets (orange, up-regulated; light blue; down-regulated). The stacked 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 bands (colors indicate first time point of differential expression in our study). Each section 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, time (h) post application of MeJA; error bars indicate SE.

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 up-

regulation and down-regulation of expression (log_2-fold change (MeJA/mock)), respectively. **(B)** Significantly overrepresented TF families within clusters of genes up-regulated (clusters 1-14; red) or down-regulated (clusters 15-27; blue) in response to MeJA treatment (hypergeometric test; $P \leq 0.001$). **(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 *bHLH27* (members of co-expression clusters 2, 4 and 1, respectively) at 3 days after inoculation with *B. cinerea*. Disease severity of inoculated leaves was scored in four classes ranging from restricted lesion (class I), non-spreading lesion (class II), spreading lesion (class III), up to severely spreading lesion (class IV). The percentage of leaves in each class was calculated per plant (n > 20). Asterisk indicates statistically significant difference from Col-0 (Chi-squared test; $P \leq 0.05$). **(D)** Performance of *M. brassicae* larvae on Col-0, highly susceptible triple bHLH TF 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 indicates statistically significant difference from Col-0 (two-tailed Student's *t* test for pairwise comparisons; $P \leq 0.05$; n=30; error bars are SE). **(E)** Quantification of disease symptoms of Col-0*, myb48, myb59, myb48myb59* and *ora59* mutant lines at 3 days after inoculation with *B. cinerea*. Disease severity of inoculated leaves was scored as described in (C) (n > 20). Asterisk indicates statistically significant difference from Col-0 (Chi-squared test; $P \leq 0.05$). **(F)** Performance of *M. brassicae* larvae on Col-0 and *myb48, myb59* and *myb48myb59* mutant lines. 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; $P \leq 0.05$; n=30; error bars are SE). **(G)** Heatmap indicating hypergeometric enrichment *P* value of genes

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

Figure 3. Enriched *cis*-regulatory motifs and functional categories in MeJA-responsive gene co-expression clusters. (A) Overrepresentation of known TF DNA-binding motifs within the unions of up-regulated and down-regulated genes. Rows indicate motifs and are colored by corresponding TF family. Red boxes indicate a motif that is significantly overrepresented (cumulative hypergeometric distribution). **(B)** Representative co-expression clusters with overrepresented TF DNA-binding motifs. Top: Profiles of $\log_2$-fold change in gene expression (MeJA/mock), with mean profile (red) and cluster size (n). Selected overrepresented functional categories (F) and representative genes (G) are denoted. Sequence logo depiction of selected 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.

Figure 4. Chronology of changes in the MeJA-triggered gene regulatory network. (A) Phasing of MeJA-induced transcriptional changes. DEGs were divided into four sets according 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 transcription counts between all pairs of time points was computed using Pearson's correlation 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)** Analysis of the major transcriptional phases in the JA gene regulatory network. Transcriptional 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; $P \leq 0.001$). Pie charts indicate the proportion of TF gene families.

Figure 5. Predicted directional interactions in the JA gene regulatory network. Network plot of inferred connections between MeJA-induced TFs and genes in transcriptional phases. The promoter sequences of genes associated with a transcriptional phase were tested for 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 plotted at the time point that its corresponding gene is first differentially expressed. Each 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 motif in that phase. The size of each TF node is proportional to the number of phases in which its binding site is overrepresented. To aid interpretation of the network, nodes are grouped and colored according to the transcriptional phase where they first become differentially expressed.

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

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 untranslated region; CDS, coding sequence. **(C)** Induction of genes encoding JA biosynthesis enzymes in estradiol-inducible *ORA47* plants. Expression levels of JA biosynthesis genes were measured in leaves 8 h after application of either estradiol or DMSO (mock) using quantitative RT-PCR (qRT-PCR). Shown are the mean expression levels of five biological replicates with mock treatments set at 1. Asterisk indicates significant differences between mock- and estradiol-treated plants (Student's *t* test; $P \leq 0.05$; error bars are SE). **(D)** Production of JA, JA-Ile, ABA, 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 difference between mock- and estradiol-treated plants (Student's *t* test; $P \leq 0.05$; error bars are 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 Figure 5. DEGs are indicated by nodes and positioned according to phase membership. Direction of misregulation in *RAP2.6L*-OX is indicated by color; yellow, up-regulated; cyan, down-regulated. The gene encoding RAP2.6L is shown as a red-colored node. Inset: heatmap indicating hypergeometric enrichment *P* value of RAP2.6L target genes in each MeJA-induced co-expression cluster.

Competing financial interests:

The authors declare no competing financial interests.

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