A MYC2/MYC3/MYC4-dependent transcription factor network regulates water spray-responsive gene expression and jasmonate levels

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Edited by Mark Estelle, University of California San Diego, La Jolla, CA, and approved October 6, 2019 (received for review July 10, 2019)

Mechanical stimuli, such as wind, rain, and touch affect plant development, growth, pest resistance, and ultimately reproductive success. Using water spray to simulate rain, we demonstrate that jasmonic acid (JA) signaling plays a key role in early gene-expression changes, well before it leads to developmental changes in flowering and plant architecture. The JA-activated transcription factors MYC2/MYC3/MYC4 modulate transiently induced expression of 266 genes, most of which peak within 30 min, and control 52% of genes induced >100-fold. Chromatin immunoprecipitation-sequencing analysis indicates that MYC2 dynamically binds >1,300 promoters and *trans*-activation assays show that MYC2 activates these promoters. By mining our multiomic datasets, we identified a core MYC2/MYC3/MYC4-dependent "regulon" of 82 genes containing many previously unknown MYC2 targets, including transcription factors *bHLH19* and *ERF109*. bHLH19 can in turn directly activate the *ORA47* promoter, indicating that MYC2/MYC3/MYC4 initiate a hierarchical network of downstream transcription factors. Finally, we also reveal that rapid water spray-induced accumulation of JA and JA-isoleucine is directly controlled by MYC2/MYC3/MYC4 through a positive amplification loop that regulates JA-biosynthesis genes.

mechanical stimulation | touch | jasmonic acid | plants | transcription

Plants are constantly subjected to a changing environment. As sessile organisms, they have evolved defense mechanisms to cope with abiotic and biotic stresses that can interfere with their development and growth. Stresses, such as salt, wounding, and insect herbivory are known to affect plant growth, development, and flowering time (1–4). These phenotypes are also observed in plants that are repeatedly exposed to mechanical stimulation, including wind, rain, neighboring plants, agricultural equipment, and human touch, colloquially termed "thigomorphogenesis" (5, 6). Such mechanical stimulation without observable damaging of leaves also increases disease resistance against insect and fungal pests (7–9). As flowering time and disease resistance are of significance for global food production, understanding the molecular basis of the touch response may aid in rational design of future crops.

At the core of this response are signaling molecules, such as reactive oxygen species (ROS) and the phytohormones jasmonic acid (JA), abscisic acid (ABA), gibberellic acid (GA), brassinosteroids, auxin, and ethylene (10). Furthermore, a single touch results in fast accumulation of early signaling compounds, like calcium (11, 12), activation of membrane-localized mechanosensitive channels (13, 14), and genome-wide transcriptional changes (15, 16), while repeated touch eventually results in stunted growth and delayed flowering (17).

While a well-calibrated touch response in plants should not automatically result in a wound response, a clear overlap between the wound response and the response to touch is apparent. Repeated wounding of leaves results in increased JA accumulation in *Arabidopsis* (18, 19) and stunts its growth in a JA-dependent manner (2, 20). Similarly, regular touch increases JA accumulation (7, 21). Some of the JA genes known to be upregulated by wounding, such as *JASMONATE ZIM-DOMAIN*

Significance

Plants are continuously exposed to mechanical manipulation by wind, rain, neighboring plants, animals, and human activities. These mechanical stimuli cause short-term molecular changes and long-term developmental effects, affecting flowering time, pathogen defence, and plant architecture. Using water spray to simulate rain, we show that jasmonic acid-signaling factors mediate rapid gene-expression changes. Nearly 300 genes are regulated by MYC2/MYC3/MYC4 transcription factors, particularly affecting the most highly responsive genes. This is controlled by induced binding and activation of water spray-inducible promoters by MYC2. We have identified a core MYC2 "regulon," including many secondary transcription factors that in turn activate downstream promoters, creating a hierarchical transcriptional network. Finally, we demonstrate that spray-induced jasmonate accumulation is transcriptionally regulated by a MYC2/MYC3/MYC4-controlled positive-feedback loop.

Author contributions: A.V.M., K.B.S., J.R.E., A.G., A.H.M., and O.V.A. designed research; A.V.M., O.D., M.Z., J.Š., M.B., R.V.B., M.G.L., S.L., and O.V.A. performed research; A.V.M., O.D., M.Z., J.Š., K.L., A.G., and O.V.A. analyzed data; and A.V.M., A.H.M., and O.V.A. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the ArrayExpress database, https://www.ebi.ac.uk/arrayexpress/ (accession nos. E-MTAB-8019 [ArrayExpress RNA-seq set 1], E-MTAB-8021 [ArrayExpress RNA-seq set 2]), the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE132316 [ChIP-seq data]), and the ProteomeXchange Consortium PRIDE repository, https://www.ebi.ac.uk/pride/archive/ (accession no. PXD014008).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1911758116/-/DCSupplemental.

First published October 29, 2019.

(*JAZ*) *10, 12-OXOPHYTODIENOATE REDUCTASE* (*OPR*) *3*, *LYPOXYGENASE* (*LOX*), and *ALLENE OXIDE CYCLASE* (*AOC*) have been also shown to be touch-inducible (22–24). In *Arabidopsis*, this effect of touch on gene expression can be alleviated in *allene oxide synthase* (*aos*) and *opr3* mutants (7). At the same time, some genes are specifically responsive to either wounding or touch and the expression of a significant set of touch genes, including *TOUCH* (*TCH*) *2*, *TCH4*, and *CALMODULIN-LIKE* (*CML*) *39*, is independent of JA (7). Finally, whereas both touch and wounding cause a fast accumulation of calcium (6), the concomitant modulated electric potential in wounded leaves appears not to occur in touched leaves (25), hence further indicative of discriminating signaling cascades between touch and wounding responses.

JA integrates environmental stresses and developmental signals to regulate plant growth and defense (26, 27). A key transcription factor (TF) of the JA-signaling pathway is the basic helix–loop–helix (bHLH) TF MYC2 (28), which is involved in many aspects of plant defense and development (2, 29–33). Importantly, in addition to CORONATINE INSENSITIVE 1 (COI1) (34) and the JAZ repressors (35), MYC2 and its paralogs MYC3 and MYC4 also regulate the JA-dependent delay of flowering time (36) and, whereas untouched *myc2* mutants show no flowering phenotype (34), the *myc2 myc3 myc4* triple (*myc234*) mutant flowers early (36). Although many indirect targets of MYC2 have been identified through analyses of *myc2* and *myc234* mutants (29, 37, 38), few of its direct targets have been identified to date (31, 33, 39–41).

Besides JA, the volatile phytohormone ethylene has been widely linked to touch responses in the past, although it seems that for most touch responses ethylene is not directly involved (10). Both expression of the touch-responsive genes *TCH2*, *TCH3*, and *TCH4*, as well as the developmental changes associated with touch, are not noticeably affected in the ethylene signaling mutants *ein2* and *etr1* (42). Similarly, touch-induced expression of the JA-biosynthesis gene *OPR3* appears independent from ETHYLENE RECEPTOR 1 (ETR1) (43). Nevertheless, some studies have reported ethylene accumulation and expression of the ethylene biosynthesis gene *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE* (*ACS*) is induced after touch (10, 44, 45). Given the cross-talk between ethylene and other hormones like JA in regulation of growth and development (32, 33), a role for ethylene in some aspects of the touch response cannot be excluded (10). To what extent the genome-wide transcriptome is affected after touch in any of the ethylene signaling mutants, or any hormone signaling mutants by extension, has however not yet been investigated.

A single touch can impose fast and wide-spread transcriptional changes (15, 17). Transcriptional, posttranscriptional, and post-translational mechanisms underlying the touch response have been identified. These include the identification of *cis*-regulatory regions, the characterization of active mRNA degradation components, and posttranslational modifications that affect touch-induced transcript accumulation levels (46–50). Although transcription factors are central to such transcriptional reprogramming, a regulatory network underlying the touch response remains to be identified.

Using water spray as a trigger (5), we have screened the responsiveness of hallmark mechanical stimulation-responsive genes in selected core signaling mutants to identify major pathways regulating their transcriptional response. To substantiate our findings, we have undertaken in-depth multiomics profiling of the early water spray-induced response in *Arabidopsis* in the context of JA-signaling components and have discovered a regulatory network governed by MYC2, MYC3, and MYC4.

Results

Identification of Key Regulatory Components of the *Arabidopsis* Touch Response. Touch-responses in *Arabidopsis* involve mechanosensitive channels (14, 51) as well as the accumulation of phytohormones, such as JA, GA, and ethylene (7). We have shown previously that the GA biosynthesis mutant *ga2ox7* displays a normal transcriptional response for some of these genes (52). Here, we first assessed the expression of the touch marker genes *WRKY40*, *JAZ8*, *ACS11*, *ETHYLENE RESPONSE FACTOR* (*ERF*) *1*, and *GIBBERELLIN 2-OXIDASE* (*GA2OX*) (15, 51–53) in mutants affected in the JA and ethylene pathways at the reported peak of their expression 25 min after "touch," using water spray as a trigger, as this causes similar but more reproducible transcriptomic responses compared to mechanical touching by hand, brush, or with tweezers (5, 52) (*SI Appendix*, Fig. S1). The ethylene receptor *etr1* mutant showed wild-type responsiveness for these 5 genes (*SI Appendix*, Fig. S2*A*), but spray-induced expression of all genes except *GA2OX6* was repressed in the JA receptor mutant *coi1-16* (*SI Appendix*, Fig. S2*B*). The *coi1-16* mutant allele contains a secondary mutation in *PENETRATION 2* (*PEN2*; involved in penetration resistance to fungal pathogens), but this is unlikely to affect short-term signaling responses triggered by water spray (54). While the single *myc2* mutant showed wild-type response for *WRKY40* and *JAZ8*, the triple *myc234* mutant showed reduced transcript accumulation 25 min after water spray compared to Col-0 (*SI Appendix*, Fig. S2*C*). In the mechanosensitive channel mutants *msl9,10* and *msl4,5,6,9,10*, no difference in water spray-induction could be observed (*SI Appendix*, Fig. S2*C*). Based on this we assessed the hallmark touch-induced effects on rosette size and flowering time in *myc234*. Untouched *myc234* showed an increased rosette size and flowered early compared to Col-0. The delay in flowering time and reduced rosette size after touch observed in Col-0 did not occur in *myc234* plants (*SI Appendix*, Fig. S2 *D* and *E*). Taken together, these data indicate that the JA pathway and the TFs MYC2/MYC3/MYC4 are involved in thigomorphogenesis and the underlying water spray-induced transcriptional response.

To further assess the role of these TFs, we performed an RNA-sequencing (RNA-seq) transcriptome analysis in *myc234* 25 min after water spray treatment. Overall, 33,602 annotated transcript loci were mapped, of which 27,360 transcripts were detectable in at least 1 of the 4 sample groups (Dataset S1). After multiple testing correction, we found 2,107 differentially expressed genes (DEGs) by water spray treatment in Col-0 (>2-fold change up or down, $P < 0.05$) (Dataset S2), from which 1,638 (77.6%) were up-regulated and 10.5% (222 transcripts) encoded TFs (Fig. 1*A* and Dataset S2). Of the transcripts, 364 were induced and 11 reduced by over 10-fold. Of the 2,107 water spray-responsive genes, 266 were differently regulated in *myc234* compared to Col-0 ($P < 0.05$), of which 88.3% (235 transcripts) were significantly decreased compared to Col-0 (Fig. 1*B*); 85.7% (228 transcripts) of the 266 genes were up-regulated 25 min after spray in Col-0 and 16.9% (45 transcripts) encoded TFs (Fig. 1*B*). In addition to the 266 MYC2/MYC3/MYC4-dependent touch genes, 198 transcripts showed significant changes (>2-fold change up or down, *P* adjusted [P_{adj}] < 0.05) under untreated conditions in the *myc234* mutant. The vast majority of these constitutively changed transcripts were not responsive to touch in Col-0 (85%, 169 transcripts). The remaining genes were water spray-responsive either in a MYC2/MYC3/MYC4-dependent (13 transcripts) or MYC2/MYC3/MYC4-independent (16 transcripts) manner (*SI Appendix*, Fig. S3*A*). Of these 198 genes, 75.3% (149 transcripts) and 24.7% (49 transcripts) were down-regulated and up-regulated, respectively (*SI Appendix*, Fig. S3*B*). The former and latter are enriched for genes encoding enzymes for glucosinolate/camalexin biosynthesis and genes encoding proteins involved in flower development and flowering time, respectively (*SI Appendix*, Fig. S3*C*).

Of 44 genes induced over 100-fold by water spray in Col-0, 23 (52.3%) were significantly less expressed in *myc234* seedlings (Fig. 1*C*), including the known MYC2 target genes *ZAT10* and *LOX4* (Dataset S3). Several JA-signaling genes required functional

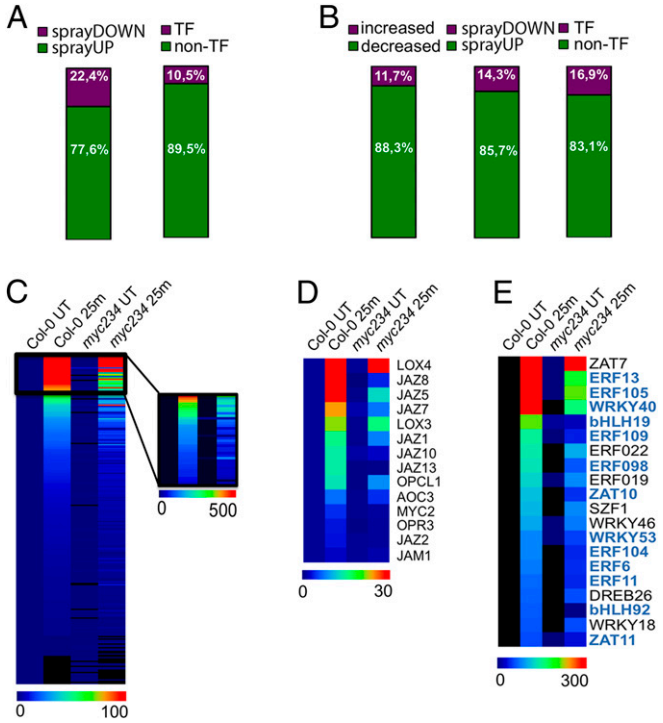

Fig. 1. The MYC2/MYC3/MYC4-dependent water spray-transcriptome. (*A* and *B*) Summary of the RNA-seq experiment in Col-0 (*A*) and *myc234* mutant (*B*) lines in untreated seedlings (UT) and seedlings 25 min (25m) after mechanical stimulation by water spray. (*A*) 77.6% of the genes are up-regulated (sprayUP) after 25 min in Col-0, of which 10.5% encode TFs. (*B*) Of the fraction of genes affected by the *myc234* mutations, 88.3% show reduced inducibility after water spray; 85.7% are up-regulated by spray, and 16.9% encode TFs. (*C*–*E*) Heat maps representing normalized levels of water spray-responsive transcripts in Col-0 and the *myc234* mutant sampled 25 min after waterspray (25m) and untouched (UT). Scale bars represent linear fold-changes normalized to Col-0 untreated as 1. (*C*) Heat map of all 266 *myc234*-dependent spray transcripts. *Inset* represents transcripts more than 100-fold induced by spray treatment in Col-0. (*D*) Heat map of selected MYC2/MYC3/MYC4-dependent transcripts related to JA-signaling and biosynthesis. (*E*) Heat map of the top 20 spray-inducible TF genes in Col-0 and their transcript abundance in *myc234* after touch. TF genes that are significantly altered (at least 2-fold, $P < 0.05$) in *myc234* after spray (*myc234* 25m) compared to Col-0 (Col-0 25m) are indicated in bold and blue.

MYC2/MYC3/MYC4 for their full touch-inducibility (Fig. 1*D*). Notably, expression of the hallmark touch genes *TCH2/3/4* was not affected in *myc234* (*SI Appendix*, Fig. S4*A*). The most highly spray-inducible TF genes appeared to be most affected by the *myc234* mutations (Fig. 1*E* and Dataset S2). Of the 45 MYC2/MYC3/MYC4-dependent touch-inducible TFs, 9 candidate TFs showed at least 40% reduction in their induction in *myc234* compared to Col-0, and literature mining suggested their involvement in plant stress response (Dataset S4). To confirm the RNA-seq results, we performed quantitative RT-PCRs (qPCRs) on Col-0 and *myc234* seedlings before (untreated) and after a 25-min spray treatment (Fig. 2). All selected TF genes were touch-inducible in Col-0 and significantly reduced touch-inducibility in *myc234* was observed for *OCTADECANOID-RESPONSIVE AP2/ERF-DOMAIN TF 47* (*ORA47*), *ERF5*, *ERF6*, *ERF11*, *ERF13*, *ERF109*, and *bHLH19*. The relative effect of the *myc234* mutations was most pronounced for transcript levels of *ERF109* and *bHLH19* (Fig. 2*A*). To confirm the role of MYC2/MYC3/MYC4 in classic touch mechanical stimulation, Col-0 and *myc234* seedlings were mechanically stimulated by brief gentle patting with a soft paint brush (Fig. 2*B*). qPCR analysis clearly shows that representative transcripts *ERF109*, *bHLH19*, and *JAZ8* are significantly less induced in *myc234* than in Col-0 25 min after touching, as observed during water spray mechanical stimulation. Together, these results show that MYC2/MYC3/MYC4 play an important role in regulating touch-responsive expression of particularly the most touch-induced transcripts.

Dynamic Transcript Profiling of the Water Spray-Induced Response. To investigate direct regulation by MYC2 of the TF genes indicated above, we performed a time-resolved water spray experiment in a *myc2* mutant background expressing a *MYC2* promoter-driven FLAG-tagged MYC2 (*myc2* pMYC2:MYC2-FLAG). We sprayed 15-d-old *myc2* MYC2-FLAG seedlings and sampled after 0 (untouched), 10, 25, 40, 60, and 180 min (Dataset S5). This enabled profiling of the speed of responses at the transcript and protein level, as well as subsequent analysis of MYC2 DNA-binding by chromatin immunoprecipitation sequencing (ChIP-seq).

We found 2,612 DEGs in at least 1 time point after water spray (>2-fold change; $P < 0.05$) (Dataset S6). Coexpression analyses were performed to quantify and visualize the dynamics of the response over time (Fig. 3*A*). The list of 2,612 contains many known touch-responsive genes, including *TCH2*, *TCH3*, and *TCH4* (Fig. 3*B*), as well as different genes of the JA-signaling pathway like *OPR3*, *JAZ10*, and *MYC2* (Fig. 3*C*); 1,564 transcripts (60%) were up-regulated and, overall, up-regulation of genes was markedly stronger than down-regulation (Fig. 3*D*). To further capture the dynamics of the response, we grouped water spray-responsive genes based on significant differential expression by at least 2-fold ($P < 0.05$) at each single time point. Using these parameters, 48% of the genes were exclusively expressed at a single time point, of which the majority were expressed at 25 min (Fig. 3*E*). The other genes were differently expressed at multiple time points, including the aforementioned *TCH* and JA-signaling pathway genes, which nevertheless showed similar highly dynamic transcriptional patterns (Fig. 3 *B* and *C*). Of the genes differently regulated at 25 min, 50%, 70%, and 92% regain untreated transcriptional levels by the 40-min, 60-min, and 3-h time points, respectively (Fig. 3 *A* and *E*), illustrating the fast and transient nature of the water spray transcriptional response. To further organize the 1,564 up-regulated genes, we used their peak expression level to assign them into a single "peak expression time point." The majority of genes showed peak expression level 25 min after spray treatment (Fig. 3*F*), including the *TCH* genes, *OPR3* and *JAZ10*, but not *MYC2* that peaked 10 min after spray (Fig. 3 *B* and *C*).

The 2,612 water spray-induced genes comprised 10% TFs, while genes that showed peak expression 10 min after spray were 25% TFs (Fig. 3*G*), representing strong enrichments compared to the 5% TF genes in the *Arabidopsis* genome (Plant Transcription Factor Database, http://planttfdb.cbi.pku.edu.cn/). In total, 55% more TFs were differentially expressed than expected by random sampling ($P < 0.001$), demonstrating that fast reprogramming of a complex transcriptional network may be an important step in the early water spray response of plants.

MYC2/MYC3/MYC4 appear to mostly regulate early responsive genes, with over 75% of the 266 MYC2/MYC3/MYC4-dependent genes peaking in the first 25 min after stimulation (Fig. 3*H*). Two of the most highly affected TF genes in *myc234* were *bHLH19* and *ERF109* (Fig. 2 and Datasets S3 and S4). We assessed transcript accumulation levels in a time-course experiment spanning the first 3 h in Col-0 and *myc234* using qPCR, showing peak expression at 25 min, which was severely impeded in the *myc234* background (Fig. 3*I*).

Between our 2 RNA-seq experiments, 1,273 transcripts were water spray-responsive in both datasets. When combined, the 2 datasets comprise a total of 3,446 spray-responsive transcripts that we can define as a water spray transcriptome, representing ~10% of the 33,602 *Arabidopsis* genes (TAIR10). When combined

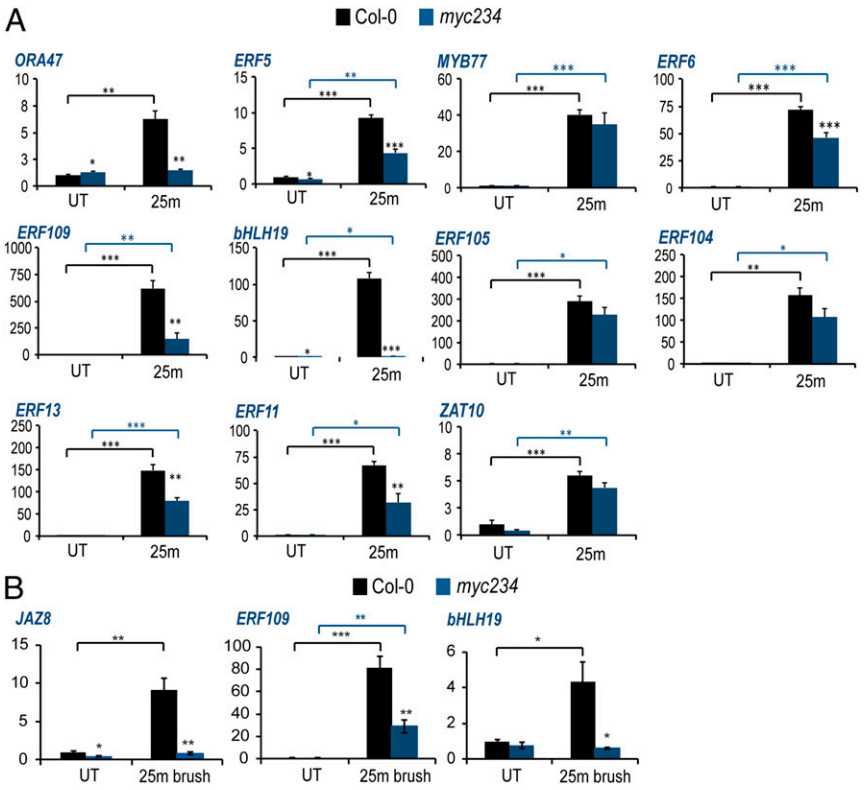

Fig. 2. Analysis of MYC2/MYC3/MYC4-dependent mechano-inducible TF genes. (*A*) qPCR analysis of selected TF genes in seedlings of Col-0 (black bars) and *myc234* (blue bars) untreated (UT) and 25 min after water spray (25m). The *y* axis denotes fold-inductions relative to UT Col-0 (set to 1). Error bars designate SEM ($n = 5$). Statistical significance was determined by Student's *t* test between genotypes and treatments (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$). (*B*) qPCR analysis of selected genes in seedlings of Col-0 (black bars) and *myc234* (blue bars) untreated (UT) and 25 min after touching with a gentle paint brush (25m brush). The *y* axis denotes fold-inductions relative to UT Col-0 (set to 1). Error bars designate SEM ($n = 4$). Statistical significance was determined by Student's *t* test between genotypes and treatments (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

with 2 previously published touch-related transcriptome analyses (15, 16), a total of 3,944 mechanical stimulation-responsive transcripts can be obtained, with 1,671 transcripts (42.3%) represented in at least 2 of the 4 transcriptome datasets (*SI Appendix*, Fig. S5*A*). This so-called "core mechanical stimulation transcriptome" represents ~5% of the *Arabidopsis* genome and contained all 44 genes induced over 100-fold after water spray in Col-0 (*SI Appendix*, Fig. S5*B* and Dataset S7).

MYC2 Orchestrates a Hierarchical Regulatory Network. To gain genome-wide insight into the mechanical stimulation-regulated MYC2 binding sites, ChIP-seq was performed in the pMYC2:MYC2-FLAG seedlings untreated and 25 min after water spray. Overall, 1,316 peaks were mapped to the vicinity of a coding sequence. Some peaks could be assigned to multiple adjacent genes, so in total 1,681 transcripts were represented (Dataset S8). Overlap of these 1,681 transcripts with all transcripts significantly altered by the *myc234* mutations in the RNA-seq ($P < 0.05$; 899 transcripts) and our previously defined core mechanical stimulation transcriptome (1,671 transcripts) resulted in a total of 82 genes (*SI Appendix*, Fig. S5*B*) that we defined as the core MYC2 mechanical stimulation regulon. This regulon contained known direct MYC2-target genes, such as *JAZ2*, *LOX2*, *LOX3*, *OPR3*, *AOS*, *AOC2*, *SULFOTRANSFERASE 16* (*SOT16*), and *ORA47* (31, 41, 55, 56). Importantly, many previously unknown MYC2-targeted TF genes, as well as JA-signaling, JA-biosynthesis, stress-related, and regulatory genes were identified (Dataset S9). About one-quarter (20 genes) of the regulon consisted of TFs (Fig. 4*A*), of which 17 are also JA-inducible, including *bHLH19*, *ERF109*, *ZAT10*, and *ORA47*. Overall, 74.3% (66 transcripts) were inducible by JA, including several JAZ proteins and JA biosynthesis genes (Fig. 4 *A* and *B*). The effect of the *myc234* mutations varied among the regulon genes and many of the previously identified MYC2-target genes were among the most affected transcripts (Fig. 4*B*). Of 26 genes that showed 50% or more reduced expression levels, 8 encoded TFs (Fig. 4*B*). Clustering the regulon TF genes with known MYC2-regulated genes using the water spray time-course transcript data groups *bHLH19* with *JAZ10*, *SOT16* and *LOX3*, and *ERF109* in a clade with *WRKY40* (Fig. 4*C*). Of the 44 genes that are over 100-fold induced by spray after 25 min, 30 (68%) are MYC2-dependent based on the RNA-seq or ChiP-seq results (*SI Appendix*, Fig. S5*B*). In addition, 36 of the regulon genes were present in the 230 mechanical stimulation genes identified in all 4 RNA-seq experiments (*SI Appendix*, Fig. S5*A*).

To further establish a direct role for MYC2 in TF gene activation, we created firefly *LUCIFERASE* (fLUC) reporter constructs with the promoter regions of the 9 selected MYC2/MYC3/MYC4-dependent touch TF genes (Dataset S3) for transient *trans*-activation assays with MYC2 in tobacco protoplasts. A JAZ-inhibition desensitized version of MYC2 ($MYC2^{D105N}$) (41) was used as well. The promoters of *ORA47*, *ZAT10*, *ERF109*, and *bHLH19* could be directly activated by both MYC2 and $MYC2^{D105N}$ (Fig. 5*A*), supporting the *myc234* RNA-seq results (Figs. 4*B* and 5*B*), qPCR results (Fig. 2), and ChIP-seq results (Fig. 5*C*). Analysis of the upstream regions of *bHLH19*, *ERF109*, *ZAT10*, and *ORA47* showed the presence of 1 or more G-boxes (CACGTG) in their promoters (Fig. 5*D* and Dataset S10). None of the other tested promoters could be markedly activated by MYC2 (*SI Appendix*, Fig. S6). We then assessed if the selected promoters could be *trans*-activated by bHLH19 or ERF109. A significant over 2-fold *trans*-activation (5-fold; $P < 0.0005$) could be observed for p*ORA47* by bHLH19 (*SI Appendix*, Fig. S7). These combined experiments delineated the core gene set directly regulated by MYC2 in response to mechanical stimulation and identified *bHLH19* and *ERF109* as direct target genes of MYC2, and *ORA47* as a target gene of bHLH19.

Water Spray-Responsive Expression of *ERF109* and *bHLH19* Depends on JA. Despite an established link between touch and JA, the extent to which the mechanical stimulation transcriptome responds to JA is not known. Combining 2 published large-scale analyses on

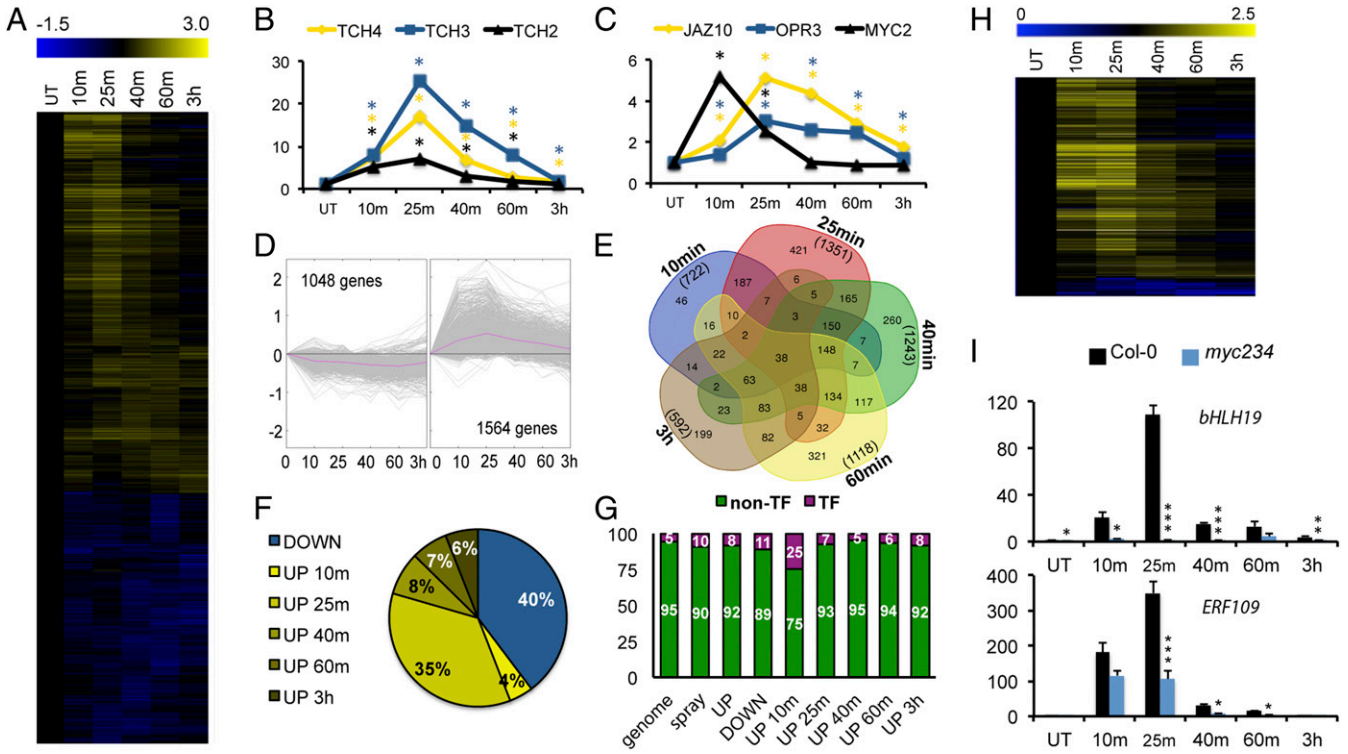

Fig. 3. The dynamic profile of the water spray transcriptome. (*A*) Coexpression analysis of the 2,612 genes that are at least 2-fold and significantly ($P < 0.05$) changed in at least 1 of 5 time points after spray treatment compared to the untreated seedlings. Average linkage hierarchical clustering with Pearson correlation was used. The bar on top shows the log10 scale. Blue and yellow denote down-and up-regulation, respectively. (*B* and *C*) Selection of genes from the RNA-seq dataset involved in the touch-response (*B*) and the JA-signaling pathway (*C*). Asterisks indicate statistically significant differences (see *Methods*) compared to 0 min ($P_{adj} < 0.05$ and 2-fold change). (*D*) *k*-means clustering of the 2,612 water spray-regulated genes showing the numbers and dynamics of down-regulated and up-regulated genes. The *x* axis shows the different time points. The *y* axis represents log10-transformed fold-induction. (*E*) Venn diagram showing the dynamics of genes between the selected time points. For each time point, all genes that are at least 2-fold and significantly ($P < 0.05$) changed were selected. (*F*) Pie-diagram showing the distribution of down-regulated genes (blue) and up-regulated genes (shades of yellow). For the latter, percentages of genes with maximum fold-induction at each time point within the 2,612 water spray-regulated genes is given. (*G*) Fraction of known TFs within defined collections of genes as indicated in *F*. Abbreviations: JAZ, Jasmonate ZIM; OPR, 12-oxophytodienoate reductase; TCH, touch. (*H*) Coexpression analysis using the time-course RNA-seq data of the 266 genes affected by *myc234* (Fig. 1). The color scale shows log10-transformed values with blue and yellow representing down-regulation and up-regulation, respectively. (*I*) qPCR showing the effect of *myc234* at different time points after spray for *bHLH19* (*Upper*) and *ERF109* (*Lower*). The *y* axis represents normalized fold-induction compared to untouched (UT) Col-0 (set to 1). Error bars are SEM ($n = 5$). Statistical significance was determined by the Student's *t* test (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

(Me)JA-treated seedlings resulted in 4,309 unique (Me)JA-responsive transcripts (57, 58). Overlay of the combined 3,944 mechanical stimulation-responsive genes with the 2 combined (Me)JA transcriptomic datasets (4,309 transcripts) yielded 40.2% (1,586 transcripts) transcripts responding to both JA and mechanical stimulation. Similarly, 36.8% of the JA- transcriptome is mechanical stimulation-responsive (Fig. 6*A*).

We tested the water spray-inducible expression of *bHLH19* and *ERF109* in a range of JA-related mutants. In the JA-receptor mutant *coi1-16*, spray-induced expression of both genes was nearly absent (Fig. 6*B*). In contrast, spray-induced transcript accumulation levels of *TCH2/3/4* were COI-independent (*SI Appendix*, Fig. S4*B*), as was shown previously for wound-inducibility (59). In the JA-biosynthesis mutant *opr3*, *bHLH19* and *ERF109* expression was significantly affected (Fig. 6*C*) compared to Col-0. Analogously, in the *jar1* mutant that cannot convert JA into its bioactive form jasmonate-isoleucine (JA-Ile), water spray-induction of *bHLH19* and *ERF109* was almost completely absent (Fig. 6*C*).

Finally, based on a literature search (38, 60) and screening public DNA affinity purification sequencing data (61) for ERF109-target genes, we have selected several mechanical stimulation-inducible genes and assessed their water spray-induced transcript accumulation in an *erf109* mutant line. Neither the basal nor spray-induced gene expression of the selected genes was significantly affected by the *erf109* mutation (*SI Appendix*, Fig. S8). This suggests that either additional factors acting redundantly to ERF109 must be active or that in the context of water spray, these genes are not targeted by ERF109.

Time-Resolved Proteomics of the Water Spray Response. To assess dynamic changes during touch responses in plants at the protein level, an in-depth peptide mass spectrometry (MS) analysis was performed on the same sample sets used for the RNA-seq time-course analysis. For 12,413 proteins, 1 or more peptides were found in at least 1 biological replicate ($n = 4$) of at least 1 time point, with 4,243 proteins considered as reliably quantified. Of these, 347 proteins were significantly altered in abundance compared to the untreated samples ($P < 0.05$, 1.5-fold change) in at least 1 time point based on spectral counting (Fig. 7*A* and Dataset S11). Of the proteins, 139 were more abundant at their peak value, while 208 were less abundant. Different overall abundance patterns could be identified, ranging from rapid increase (cluster A) or decrease (clusters D and E), over initial decrease with recovery at 40 to 60 min (clusters C and F), to later transient decrease (cluster B).

Protein levels of the JA-biosynthesis gene product AOC1 and the JA-signaling component ARABIDOPSIS SKP1 HOMOLOG1 (ASK1), which interacts with COI1 as part of the SCF-complex

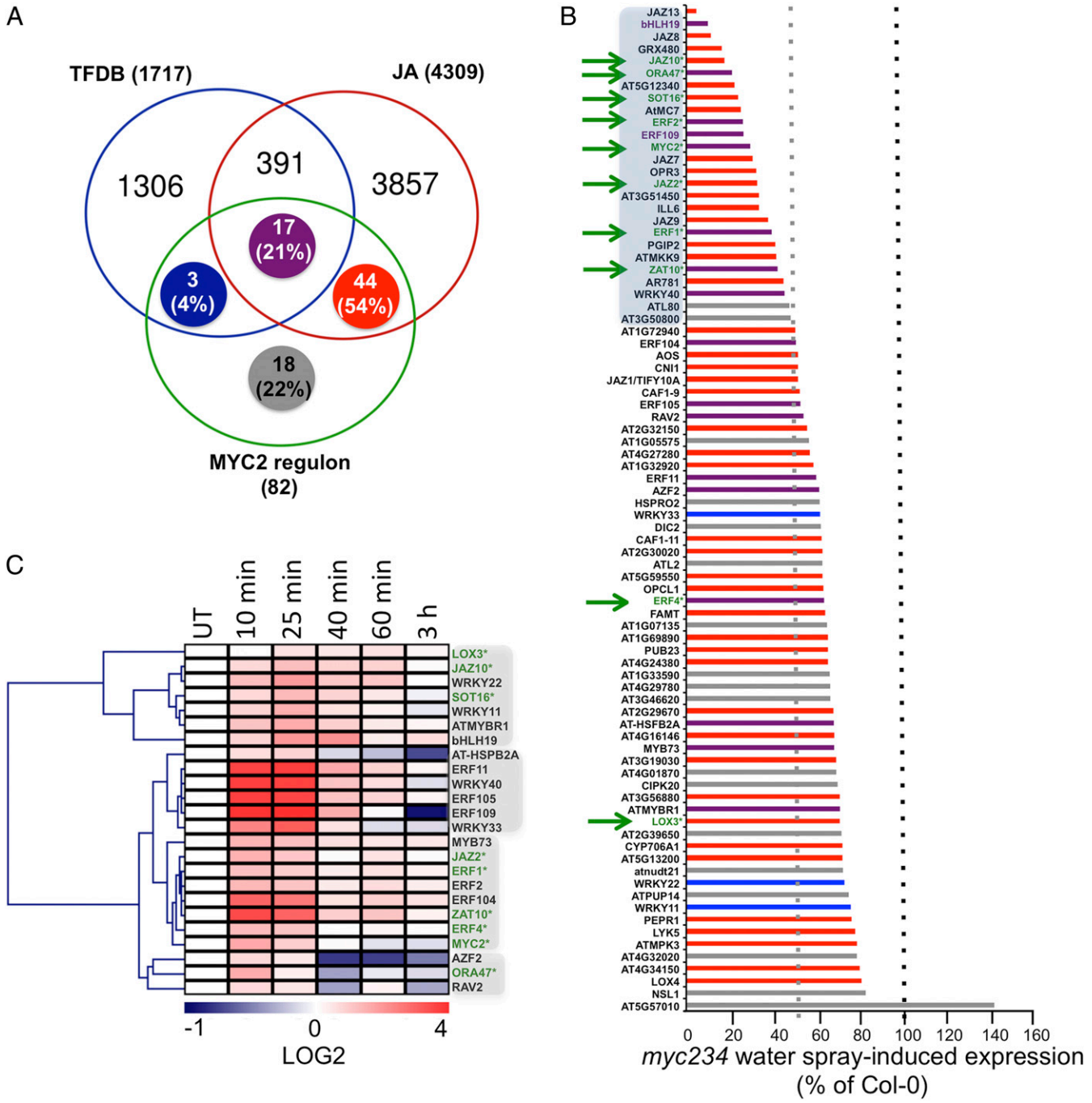

Fig. 4. Characteristics of the MYC2-regulon. (*A*) Venn diagram showing the number of TF genes (Plant Transcription Factor Database, TFDB) and JA-inducible genes (JA) of the MYC2-regulon. (*B*) Distribution of the MYC2 regulon genes based on the water spray RNA-seq dataset in *myc234*. Regulon genes are ranked from most to least affected in *myc234* compared to Col-0 after water spray. Values are percentages of the transcript accumulation levels 25 min after spray in Col-0. The color code of the bars corresponds the color code in the Venn diagram in *A*. Genes previously shown to be bound by MYC2 are indicated with an asterisk and arrow in green. The black dashed line indicates the expression level after water spray for each individual gene in Col-0 (set to 100%) and the gray dashed line marks the 50% value. Boxed genes have transcripts reduced 50% or more in *myc234* compared to Col-0 after touch. (*C*) Hierarchical cluster analysis of the MYC2 regulon TF genes and/or genes previously shown to be bound by MYC2 (indicated with asterisk in green). Clustering was performed using transcript data from Col-0 and *myc234* seedlings untouched (UT) and 25 min after spray (25m), as well as the water spray time course. The scale bar represents log2-transformed values. Red and blue denote up-regulation and down-regulation, respectively.

(62), were responsive to spray. Redox-related proteins are overrepresented in the proteins changing in abundance, compared to the proteome as a whole, including 6 thioredoxins, 4 glutaredoxins, and 6 peroxidases. Glutathione peroxidase GPX2 increased 5-fold after 40 min, while GPX1 and GPX6 decreased in abundance after 10 min and recovered to prespray levels by 40 min. Ten protein kinases, including MITOGEN-ACTIVATED PROTEIN KINASE (MPK) 3, calcium-dependent protein kinases, and receptor-like kinases, and 11 protein phosphatases showed alterations in protein levels, indicating that phosphorylation cascades are likely to be important during early mechanical stimulation responses.

A targeted analysis of peptides derived from proteins whose transcripts were identified as being spray-responsive was conducted

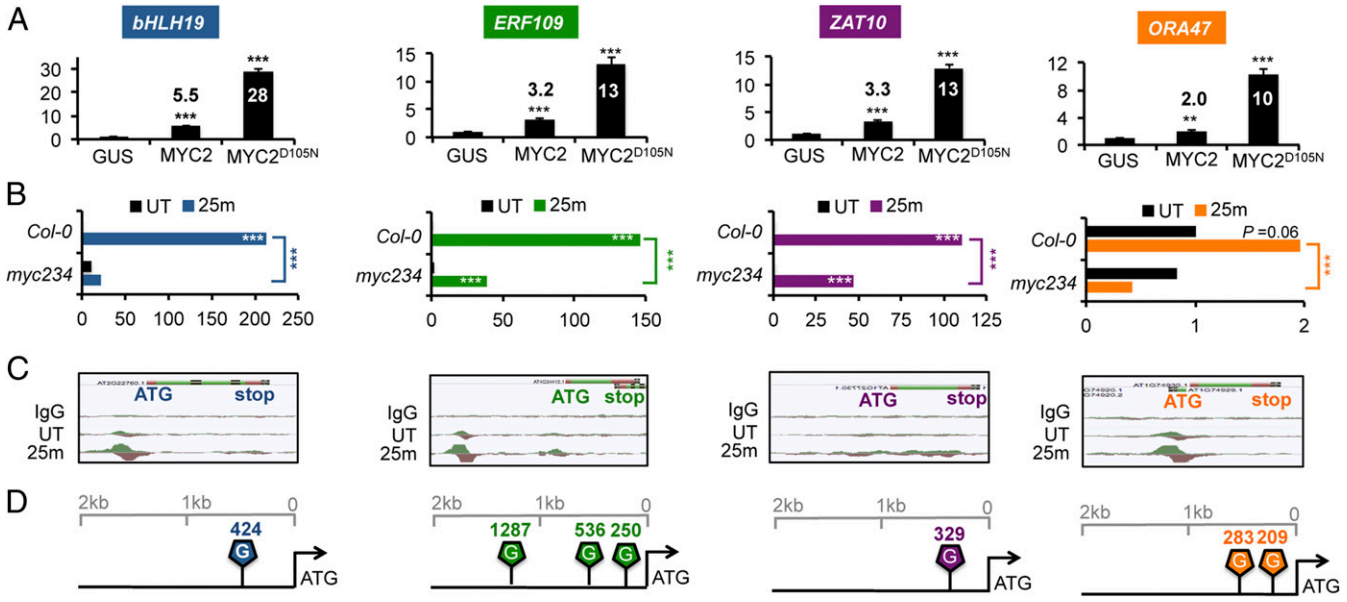

Fig. 5. MYC2 binds and activates touch-inducible TF gene promoters. (*A*) *Trans*-activation assays in *Nicotiana tabacum* protoplast of the selected TF gene promoters by β-glucuronidase (GUS), MYC2, and MYC2^{D105N}. The *y* axes represents normalized firefly luciferase activity (fLUC/*Renilla* luciferase). Values represent fold-induction relative to the GUS control (set to 1) and are presented on top of the bars. Error bars represent SEM ($n = 8$). Statistical significance was determined by Student's *t* test (**$P < 0.005$, ***$P < 0.0005$). (*B*) RNA-seq results of selected TF genes in Col-0 and *myc234* seedlings untouched (UT) and 25 min after water spray. The *x* axis represents fold-induction compared to Col-0 UT (set to 1). Asterisks indicate statistically significant differences (see *Methods* and Dataset S1) between UT (set to 1) and 25 min within each genotype (white) and between genotypes (colored) ($P_{adj} < 0.05$ and 2-fold change). (*C*) ChIP-seq showing MYC2 binding to genome region of the selected TFs in a control transformed line (IgG) and the p*MYC2*:MYC2-FLAG line untouched (UT) and 25 min after water spray (25m). Green indicates reads mapped to the Watson strand and red indicates reads mapped to the Crick strand. (*D*) Organization of the 2-kb upstream regions of the TF genes showing the location of G-boxes (CACGTG) relative to the ATG.

by manual curation of full-scan MS data (MS1 analysis). Fig. 7*B* shows a comparison of protein and RNA levels of representative proteins that were identified as differential during the water spray time course. JA-biosynthetic enzyme OPCL1 and cold-responsive KIN2 showed a positive correlation between transcript induction and protein levels, while LIGHT HARVESTING COMPLEX PHOTOSYSTEM II (LHCB4.2; AT3G08940) was transcriptionally down-regulated and the protein level fell. However, several proteins showed a more negative correlation, with transcripts increasing while protein levels fell: For example, for ERF105, UDP-GLUCOSYL TRANSFERASE (UGT) 74B1, and MAPK3.

MYC2/MYC3/MYC4 Regulate Water Spray-Induced JA Accumulation. About 40% of the mechanical stimulation transcriptome is JA-responsive (Fig. 6*A*) and JA is reported to accumulate after touch (7). Further inspection of selected hormone metabolism pathway genes showed that 10 of 26 JA pathway genes (38%) were water spray-responsive (Dataset S12). Furthermore, 4 of 14 ABA pathway genes (29%) and 9 of 17 indole-3-acetic acid (IAA) pathway genes (53%) were water spray-responsive (>2-fold up or down; $P < 0.05$) (Dataset S12). Half of the water spray-responsive JA pathway genes were dependent on MYC2, whereas none of the spray-responsive IAA and ABA pathway genes were MYC2-dependent (Fig. 8 *A–C*, *SI Appendix*, Fig. S9 *A–C* and S10 *A–C*, and Dataset S12). Thirteen of the JA biosynthesis and degradation genes were identified in the ChIP-seq using MYC2 (*SI Appendix*, Fig. S8).

Therefore, we performed hormone accumulation profiling on a time course spanning the first 3 h after water spray in Col-0 and *myc234* seedlings. We also measured different active forms, precursors, conjugates, and degradation products of JA, auxin (IAA), cytokinins (CK), salicylic acid (SA), and ABA (Dataset S13). Levels of GA were below detection limit using our conditions, and ethylene was not included in the analysis. No difference in CKs between genotypes or as a response to water spray could be observed (Dataset S13).

In untreated conditions (UT), there were no significant difference between Col-0 and *myc234* for ABA (*SI Appendix*, Fig. S9), JA, and its precursor *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA) (Fig. 8*D*). In contrast, IAA levels were significantly reduced by 58% ($P < 0.05$; $n = 5$) (*SI Appendix*, Fig. S10*D*) and JA-Ile levels could not be detected in *myc234* compared to Col-0 (Fig. 8*D*). In response to water spray, IAA levels were unchanged but ABA levels dropped in Col-0 (*SI Appendix*, Figs. S9*D* and S10*D*). For the most highly up-regulated ABA pathway gene, *CYP707A3*, touch without spray could induce expression up to 50-fold, indicating the effect was not merely evoked by water for this gene as well (*SI Appendix*, Fig. S9*E*). Interestingly, JA and its active conjugate JA-Ile transiently peaked 25 min after touch in Col-0, restoring to near untouched levels after 3 h (Fig. 8*D*). The JA precursor *cis*-OPDA remained stable after water spray in Col-0. Significantly different patterns were found in the *myc234* mutant. After water spray, *cis*-OPDA levels in *myc234* were significantly lower than Col-0, pointing to touch-induced MYC2/MYC3/MYC4-dependent activation of OPDA-synthesizing enzymes, such LOX, AOC, and AOS. Whereas JA levels between Col-0 and *myc234* were similar at UT and 10 min, the major peak of JA at 25 min in Col-0 is missing in *myc234* (Fig. 8*D*). JA-Ile levels are drastically lower at all time points measured in *myc234* (Fig. 8*D*). Independently of the *myc234* mutations, levels of the IAA-precursor tryptophan (Trp) were reduced after water spray, although not significantly for the majority of time points. Interestingly, IAA and its conjugate IAA-aspartic acid (IAA-Asp) were depleted in a water spray-independent manner in *myc234* compared to Col-0 (*SI Appendix*, Fig. S10*D*).

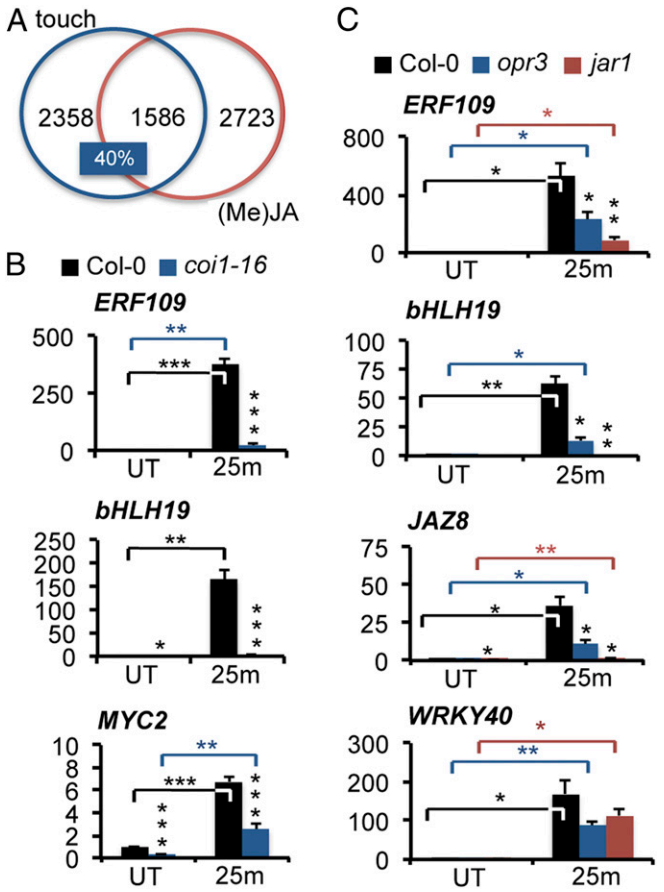

Fig. 6. Mechano-induced expression of *bHLH19* and *ERF109* involves JA. (*A*) Venn diagram showing the connection between the combined mechanical stimulation-related transcriptomes of this and previous studies (15, 16), and 2 MeJA transcriptomes (49, 50). (*B* and *C*) qPCR of the depicted genes in Col-0 and *coi1-16* (*B*), and Col-0, *opr3* and *jar1* (*C*) seedlings untouched (UT) and 25 min after water spray (25m). The *y* values represent normalized fold-induction compared to UT Col-0 (set to 1). Error bars represent SEM: $n = 3$ for UT and $n = 4$ for 25 min (*B*) and $n = 4$ for UT and $n = 5$ for 25 min (*C*). Statistical significance was determined by Student's *t* test (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$). Letters indicate significant differences (ANOVA, $P < 0.005$ according to the least-significant difference post hoc analysis).

Overall, reduced levels of IAA and IAA precursors, conjugates, and degradation products were measured in at least 1 time point of the touch time course, indicating that MYC2/MYC3/MYC4 also modulate overall IAA metabolism. This is supported by subtle but significantly reduced levels for *ANTHRANILATE SYNTHASE 1* (*ASA1*) in *myc234* compared to Col-0, independent of touch (Dataset S12) as previously reported (28). These analyses point to clear differences in hormone profiles when it comes to both water spray response and the contribution of MYC2/MYC3/MYC4.

Discussion

Water Spray Invokes Major Dynamic Transcriptome and Proteome Changes through a Regulatory Network of Transcription Factors. Mechanical stimulation triggers a wide-spread transcriptional response. Our RNA-seq datasets were consistent with 2 published transcriptomic datasets (15, 16), with 230 genes in all 4 and 1,671 genes in 2 of 4 datasets differentially expressed. The increased time resolution of our dataset allowed for a more dynamic dissection. Over 700 genes respond to the water spray treatment within 10 min. Most of these genes continue to increase in expression, peaking at 25 min, returning to near unsprayed levels within 1 h, including *bHLH19*, *ERF109*, and *TCH2/4*. Only very few of the DEGs peak at 10 min with a clear overrepresentation of TFs such as *MYC2* and *ORA47*, suggesting a transcriptional network is being initiated rapidly. Nearly half of the DEGs are differentially expressed at a single time point, illustrating the transient nature of this regulatory network and its implication for the transcriptional response. Accordingly, proteomic analysis revealed that the abundance of over 300 proteins was altered in at least 1 time point after water spray. Several kinases/phosphatases were identified, confirming the importance of phosphorylation cascades in mechanical stimulation signaling (50). In addition, redox status seems to play an important role with many peroxidases, thioredoxins, and glutaredoxins being altered in abundance after water spray. This is in line with previous reports of touch-induced ROS bursts (12).

As a central JA-response regulator, a critical role for MYC2 in insect- and wound-response is well documented (63). Importantly, MYC2 and its paralogs MYC3 and MYC4 are also reported to be involved in the regulation of flowering time (17, 36), a hallmark feature of the touch response. However, their involvement in mechanical stimulation-induced gene expression had not been investigated to date. Here, we have assessed the genome-wide action of MYC2 in response to water spray through RNA-seq on *myc234*, MYC2-tagged ChIP-seq, and promoter *trans*-activation assays. The RNA-seq and ChIP-seq data combined showed that MYC2 (in addition to MYC3/4) (in)directly controls the majority of the most water spray-responsive genes. MYC2/MYC3/MYC4 regulate in particular early-response genes, while *MYC2* gene expression itself peaks 10 min after water spray. This further supports the concept that MYC2 activation is 1 of the first transcriptional events following touch. Generally, our analyses support a preference for MYC2 to directly regulate other TFs, which is largely in agreement with a recent study in tomato (64), and consequently positions MYC2 highly in the hierarchical regulatory network. Indeed, 20 TF genes were found to be directly targeted and regulated by MYC2 after water spray, including *bHLH19* and *ERF109*. ERF109 functions in ROS-related stress response, insect-resistance, and auxin/JA-related lateral root formation (38, 58, 65), among others. Very recently, involvement of ERF109 in JA-dependent wound regeneration was shown (66). bHLH19 has recently been implicated in JA-dependent Fe-metabolism (67). Our results add an additional role in the mechanical stimulation response for these TFs. Given the overlap between the mechanical stimulation response and other (a)biotic stresses, such as wounding, salt, and insect attack, and the prominent role of MYC2 in stress response and development, our MYC2-target gene list could be useful to assess a role for these TFs in other signaling cascades, as well.

MYC2 Regulates JA Biosynthesis and Hormone Levels. Previous reports have shown the importance of JA and the JA-signaling components JAR1, COI1, OPR3, and AOS in thigmomorphogenis (7, 26, 34, 35). However, it was unknown to what extent JA affects the transcriptional responses to mechanical stimulation. Our results show that ~30% of the water spray-induced genes are JA-responsive, whereas a clear non-JA–dependent circuit exists, exemplified by JA- and COI1-independence of the *TCH* genes (5, 57). Accordingly, at the protein level the JA-biosynthesis enzymes OPCL1 and AOC2 were found to be differentially abundant in response to water spray, underlining the importance of JA.

Some of the most striking effects of MYC2/MYC3/MYC4 were observed by hormone profiling. Although JA levels have previously been shown to be induced by touch and wounding in different species (7, 18, 19, 68, 69), the direct role of MYC2 on hormonal levels had not been described before. Our hormone analysis shows that the water spray-induced accumulation of JA and JA-Ile are largely dependent on MYC2/MYC3/MYC4.

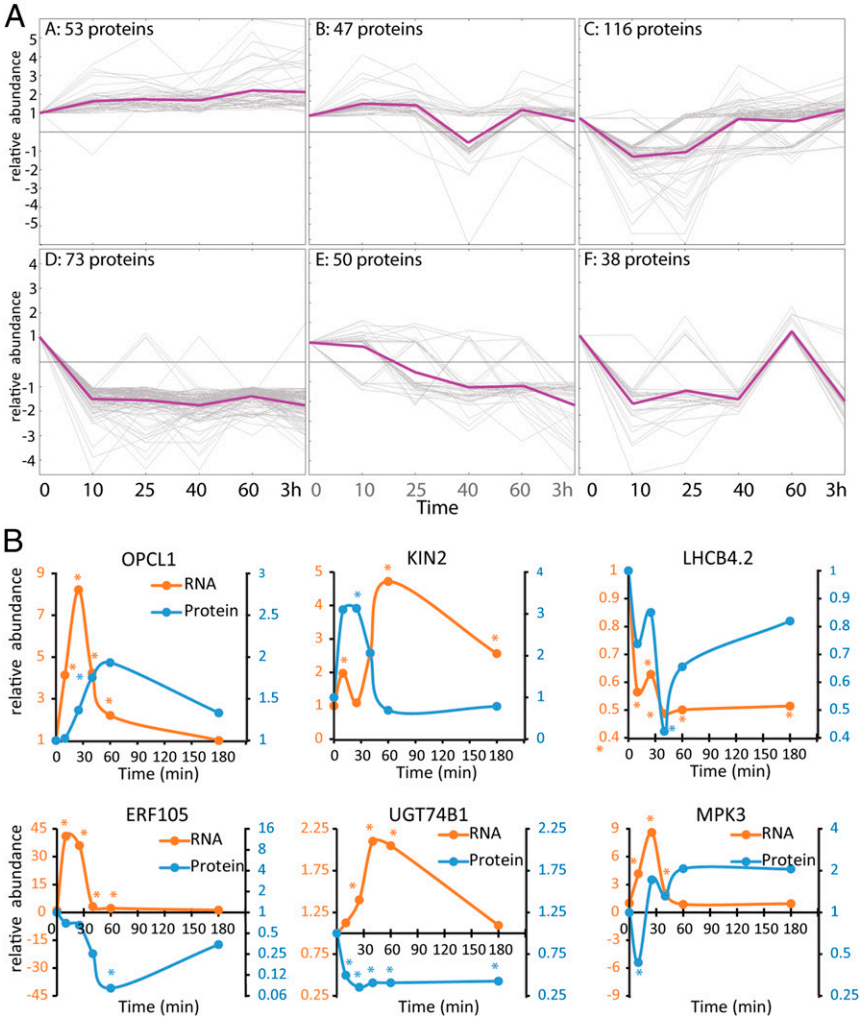

Fig. 7. Water spray triggers wide-spread proteomic changes. (*A*) *k*-means clustering of 355 proteins that significantly change abundance during water spray response ($P < 0.05$, 1.5-fold change). Relative abundance is shown for each time point (minutes/hours after treatment) compared to time point 0 min (UT). (*B*) Peptides derived from proteins whose transcripts were identified as being water spray-responsive were selected for manual curation of full-scan MS data. Comparison of RNA and protein levels for selected proteins over the 3-h time course are shown. Relative abundance compared to time point 0 h were shown. Asterisk indicates statistically significant differences (see *Methods*) compared to UT ($P_{adj} < 0.05$ and 2-fold change for transcripts; $P < 0.05$ and 1.5-fold change for protein levels).

Whereas an initial increase in JA and JA-Ile levels is observed in both Col-0 and *myc234*, the large boost in JA and JA-Ile accumulation in Col-0 is completely absent in *myc234*. This correlates with the widespread MYC2/MYC3/MYC4-dependent up-regulation of JA biosynthesis genes after water spray and is further supported by direct binding of no less than half of the JA-metabolism gene promoters (13 of 26) by MYC2. JA and JA-Ile levels peak at 25 min and drop strongly by 40 to 60 min, which could be the result of enzymatic deactivation of the active hormone, and thus attenuation of the JA signal. This is supported by earlier peak expression for JA-biosynthesis genes like, for example, *LOX3/4* and *OPCL1*, compared to JA-catabolism genes, such as *JASMONIC ACID OXIDASE 2* (*JAO2*) and *JAO4* (70). Interestingly, *JAO2* and *JAO4* are also directly bound by MYC2 in our ChIP-seq analysis, indicating that in addition to JA biosynthesis, JA turnover appears transcriptionally regulated by MYC2 as well.

In conclusion, this study provides a high-resolution landscape of the transcriptional, hormonal, and proteomic effects of water spray in *Arabidopsis*. It clearly shows the direct role of JA and the MYC2/MYC3/MYC4 TFs in the regulation of a large proportion of the transcriptome changes, both by directly setting a secondary network of TFs in motion and directly controlling JA metabolism. Notably, however, this JA- and MYC2/MYC3/MYC4-dependent TF network does not seem to modulate other classic touch marker genes, such as *TCH3* and *TCH4*, meaning that additional touch-induced signaling pathways await discovery.

Methods

Plant Material and Treatment. The *myc2 myc3 myc4* (*myc234*) and *coi1-16* mutant lines have been described previously (30, 71) and were a kind gift from Roberto Solano, Consejo Superior de Investigaciones Científicas (CNB-CSIC), Madrid, Spain. *etr1-1* was kindly donated by Kirk Overmyer, University of Helsinki, Helsinki, Finland, and the *msl* mutants were kindly provided by Elizabeth Haswell, Washington University in St. Louis, St. Louis, MO. The mutant line *erf109* (SALK_150614) originates from the Nottingham *Arabidopsis* Stock Centre and the *myc2 MYC2::MYC2-FLAG* line was described previously (72).

Arabidopsis seeds were dry-sterilized overnight in commercial bleach (1:8 dilution in water) containing 3% HCl. Seeds were placed on 0.5× Murashige and Skoog media (including vitamins), 0.5% 2-ethanesulfonic acid, pH 5.8, 0.7% phytoagar plates, and stratified for 2 to 3 d at 4 °C, after which the plates were transferred to standard growth conditions (21 °C, 16-h/8-h light/dark regime) for 10 to 14 d.

For transcript and metabolite analyses, seedlings were stimulated by spraying downward onto the plate from around 15-cm distance 5 to 10 times (depending on the size of the plate) with milliQ water using a spraying bottle (Black&Gold, 500-mL multipurpose sprayer, cat. no. CLEA0055), except where specified. An example treatment is shown in Movie S1. The average droplet size of the spray is 211 ± 12 μm, as determined by immersion sampling in silicon oil and measurement under a microscope. The water volume of 1 spray is around 625 μL, and we sprayed with enough force to allow the droplets to travel upward against gravity around 52 cm (implying an initial speed of about >3 m/s ignoring air friction). Excess water was then drained

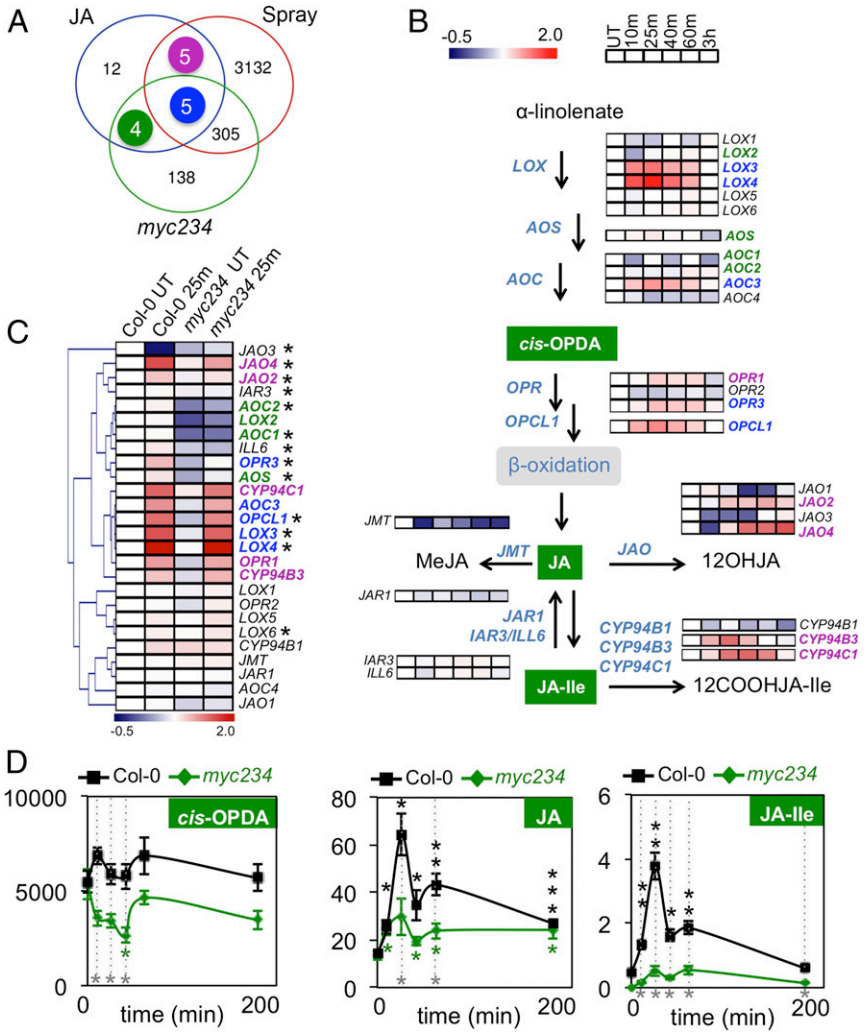

Fig. 8. JA-related metabolite and transcript profiling of the MYC2/MYC3/MYC4-dependent water spray response. (*A*) Venn diagram with selected JA biosynthesis genes (JA), MYC2/MYC3/MYC4-dependent genes (*myc234*) and water spray-responsive genes (spray). The color code corresponds to the genes in *B* and *C*: In green are MYC2/MYC3/MYC4-dependent JA biosynthesis genes, in blue are water spray-responsive MYC2/MYC3/MYC4-dependent JA biosynthesis genes and in pink are water spray-responsive MYC2/MYC3/MYC4-independent JA biosynthesis genes. (*B*) Pathway for JA biosynthesis and expression profiling of the genes encoding the depicted enzymatic steps. Measured metabolites are boxed in green and the enzymatic steps are indicated. Visualization of the time-course expression analysis of the JA-biosynthesis genes are depicted right-hand side of each enzymatic step. The time points are indicated on top and the scale bar represents log10-transformed values. Red and blue denote up-regulation and down-regulation, respectively. (*C*) Hierarchical cluster analysis of the JA biosynthesis genes. Clustering was performed using transcript data of the time series shown in *B*, as well as transcript data from Col-0 and *myc234* seedlings untouched (UT) and 25 min afterwater spray (25m). The scale bar represents log10-transformed values. Red and blue denote up-regulation and down-regulation, respectively. The color code of the transcripts is derived from the Venn diagram in *A*. Asterisks indicate genes identified by MYC2-ChIP (Dataset S8). (*D*) Accumulation of *cis*-OPDA, JA, and JA-Ile after water spray in Col-0 and *myc234* seedlings. The *y* axis denotes pmol·g^{-1} fresh weight. The *x* axis represents sampling time 10, 25, 40, 60, and 180 min after water spray. Black and colored asterisks indicate differences (Student's *t* test; $*P < 0.05$; $**P < 0.005$, $***P < 0.0005$) with UT in Col-0 and *myc234*, respectively. Note that in *myc234* UT, JA-Ile could not be detected. Gray asterisks indicate differences between Col-0 and *myc234* at each time point (Student's *t* test, $P < 0.05$). Gene names can be found in Dataset S13.

off and plates were closed for the indicated time before sampling. Approximately 5 to 10 whole seedlings were sampled for each biological repeat, and the plants were quickly dried with tissue paper before snap-freezing in liquid nitrogen. In case of touching without spraying, seedlings were touched with a blunt forceps for ~10 s (*SI Appendix*, Fig. S1) or using a gentle paint brush (Langnickel Snowhite 4, L4530) (Fig. 2*B* and Movie S2).

For phenotype analysis (*SI Appendix*, Fig. S2 *D* and *E*), seeds were sown in soil, stratified for 2 d at 4 °C, and grown in standard growth conditions (21 °C; 16-h/8-h light/dark regime). From 14 d after transfer to the growth room onward until bolting, leaves were touched 10 times twice per day with blunt tweezers.

Construct Design. All constructs were made using Gateway technology (Invitrogen). Promoter regions of *ORA47*, *ERF13*, *ERF105*, *MYB77*, ZAT10, *ERF5*, *ERF104*, *ERF109*, *ERF13*, and *bHLH19* were isolated using primer P53-72 (Dataset S14) and BP recombined into pDONR221 (Invitrogen). The coding sequences of ERF109 and bHLH19 were isolated using primers P49/50 and P51/52, respectively (Dataset S14) and cloned into pDONR221 (Invitrogen). The entry plasmids were sequence-verified and subsequently LR recombined into pGWL7 for the promoters and p2GW7 for the coding sequences. Cloning of MYC2 and MYC2^{D105N} was described previously (41). Creation of the MYC2::MYC2-FLAG line has been described previously (72).

Expression Analysis. Total RNA extraction was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and 1 μg was used for cDNA synthesis using iScript (Bio-Rad). qPCR was performed with primers P1-P46 (Dataset S14). For normalization housekeeping genes *POLYUBIQUITIN 10* (*UBQ10*; At4g05320) and *UBIQUITIN-CONJUGATING ENZYME 21* (*UBC21*; At5g25760) were used.

Promoter *Trans*-Activation Assays in Tobacco Protoplasts. Transient promoter *trans*-activation assays in tobacco protoplasts were performed as described previously (73).

ChIP-seq. Approximately 100 mg of *myc2 MYC2:MYC2-FLAG* or Col-0 seeds per plate were grown for 2 wk on Murashige and Skoog media in square Petri dishes. Samples were spray-treated with distilled water. At the selected time points, the seedlings were quickly plucked from the plates, with representative seedlings immediately snap-frozen in liquid nitrogen for subsequent transcript and protein analysis. The remaining 5 to 10 g of seedlings was quickly submerged in nylon stockings in 1% formaldehyde (Sigma-Aldrich cat no. F8775) in 10 mM Hepes-NaOH pH 7.4, and vacuum-infiltrated for 10 min. The vacuum was then released and reapplied for 10 min. Next, formaldehyde was replaced with 200 mM glycine and again vacuum-infiltrated for 10 min. Finally, the samples were washed with distilled water, removed from the stockings, and snap-frozen. ChIP-seq experiments were performed as previously described (74), with minor modifications. Approximately 500 mg of 2-wk-old *myc2 MYC2:MYC2-FLAG* and Col-0 seedling tissue was used. Experiments were conducted with antibodies against FLAG (F1804, Millipore Sigma). As a negative control, mouse IgG (015-000-003, Jackson ImmunoResearch) was used. Anti-FLAG antibody and IgG were coupled to 50-μL Protein G Dynabeads (10004D, Thermo Fisher Scientific) 6 h and subsequently incubated overnight with equal amounts of sonicated chromatin. After overnight incubation, beads were washed twice with high salt buffer (50 mM Tris·HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100), low salt (50 mM Tris·HCl pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.5% Triton X-100), and wash buffer (50 mM Tris·HCl pH 7.4, 50 mM NaCl, 2 mM EDTA). After elution, samples were decross-linked and digested with proteinase K digestion before the DNA was precipitated. ChIP-seq libraries were generated following the manufacturer's instructions

(Illumina) and sequenced on the Illumina HiSeq 2500 Sequencing system. Sequencing reads were aligned to the TAIR10 genome assembly using Bowtie2 (75). Overrepresented peaks were called using SICER (76) ($P < 0.01$).

RNA-seq Analysis. Total RNA was extracted from snap-frozen tissues using the Sigma Spectrum Plant RNA kit, and genomic DNA was removed using Ambion DNA-free kit. Libraries for RNA-seq analysis were prepared from 500 ng DNase-treated total RNA using the Illumina Ribo-zero Plant kit (RS-122-2401), following standard procedures, as described previously (77). The number of replicates for the *myc234* time-course experiment were $n = 3$, except for *myc234* 25 min ($n = 4$). For the MYC2-FLAG time course $n = 4$. Libraries were clustered on an Illumina cBot using Truseq SR Cluster Kit v3 cBOT HS (GD-401-3001). Sequencing was then performed on an Illumina HiSeq 1500 using SBS kit v3 for 50 to 61 cycles (FC-401-3002). Reads were aligned to TAIR10 with STAR (78), and 24 to 39 million (*myc234* time course) or 13 to 23 million (MYC2-FLAG time course) uniquely aligned reads were obtained for each sample. Aligned reads were assigned to genes with featureCounts (79). DEGs were called with DEseq2 with no independent filtering (80). Transcripts were considered to be significantly differentially expressed between genotypes when $P_{adj} < 0.05$ (after multiple testing correction) and fold-change > 2×.

Hormone Quantification. Samples ($n = 5$) were extracted, purified, and analyzed according to method described previously (81). Briefly, ∼20 mg of frozen material per sample was homogenized and extracted in 1 mL of ice-cold 50% aqueous acetonitrile (vol/vol) with the mixture of ^{13}C- or deuterium-labeled internal standards using a bead mill (27 Hz, 10 min, 4 °C; MixerMill, Retsch) and sonicator (3 min, 4 °C; Ultrasonic bath P 310 H, Elma) After centrifugation (14,000 rpm, 15 min, 4 °C), the supernatant was purified as following. A solid-phase extraction column Oasis HLB (30 mg 1 cc; Waters) was conditioned with 1 mL of 100% methanol and 1 mL of deionized water (Milli-Q, Merck Millipore). After the conditioning steps, each sample was loaded on an SPE column and flow-through fraction was collected together with the elution fraction 1 mL 30% aqueous acetonitrile (vol/vol). Samples were evaporated to dryness using speed vac (SpeedVac SPD111V, Thermo Scientific). Prior to LC-MS analysis, samples were dissolved in 40 μL of 30% acetonitrile (vol/vol) and transferred to insert-equipped vials. MS analysis of targeted compounds was performed by an UHPLC-electrospray ionization-MS/MS system comprising of a 1290 Infinity Binary LC System coupled to a 6490 Triple Quad LC-MS System with Jet Stream and Dual Ion Funnel technologies (Agilent Technologies). A list of internal standards used in this study is provided in Dataset S15. The quantification was carried out in Agilent MassHunter Workstation Software Quantitative (Agilent Technologies).

Mass Spectrometry. For protein extraction, 200 mg of ground seedlings (4 biological replicates per sample group) were resuspended in 400 μL of 125 m mM Tris·HCl pH 7.0, 7% SDS, 0.5% PVP-40, 25 mM DTT, 1 mM complete protease inhibitor mixture (Roche) and vortexed repeatedly over the course of 5 min. Debris were pelleted by centrifugation and 250 μL of supernatant transferred to fresh tubes. Chloroform:methanol extraction was performed as previously described (82) and the protein layer washed twice in methanol. The pellet was then treated with −20 °C 90% acetone for 2 h with the acetone being changed after 1 h. Pellets were resuspended in 1% SDS, 50 mM ammonium bicarbonate, 10 mM DTT, and treated with 25 mM iodoacetic acid for 30 min in the dark before digestion with trypsin (Life Sciences) 1:20. Samples were cleaned up by combined J4-SDS2 (Nest group) and C18 (Waters) HPLC columns before drying down in a vacuum centrifuge. Peptide samples were analyzed on a ThermoFisher Orbitrap Fusion over the course of 240 min using a 75-μm × 20-mm trap column (ThermoFisher) and a 75-μm × 500-mm analytical column (ThermoFisher). Data files were converted to *.mzML (Msconvert 3.0.9992) before spectral matching through CometMS (2017.01 rev. 4) with reversed decoy database (TAIR10). Peptide scores were cut off at a false-discovery rate of 2% and rescored through PeptideProphet (TPP v5.0.0 Typhoon) and protein lists assembled with ProteinProphet (TPP v5.0.0 Typhoon). Relative abundance measurements were assembled with Abacus (83) and statistical analysis conducted through the DESeq2 packages (80) in the R statistical computing environment (3.5.1). Proteins with at least an average of 5 spectral counts per replicate in at least 1 time point, and a median of the average spectral counts per time point higher than 3 were deemed as reliably quantified and retained for statistical analysis (4,243 proteins) (Dataset S16). Proteins with a fold-change >1.5× and $P_{adj} < 0.05$ (DESeq2) were retained as significantly differential. Additional MS1 data were extracted for a subset of proteins through the MS1 filtering workflow in Skyline (4.1.0.11796).

Cluster Analyses and Venn Diagrams. Average linkage hierarchical clustering with Pearson correlation and *k*-means clustering were performed using the multiple experiment viewer (MeV) software. Venn diagrams were made using a web application (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Statistical Information. Information on statistical processing for the large datasets (RNA-seq, ChIP-seq, and proteomics) are specified in the respective *Methods* sections. Complete lists of *P* values are available in *SI Appendix*. For additional experiments, 2-tailed Student's *t* tests were used, with number of replicates and error bars as indicated in the figure legends.

Data Availability. RNA-seq data have been deposited in the ArrayExpress database under accession nos. E-MTAB-8019 and E-MTAB-8021. The ChIP-seq data have been deposited at Gene Expression Omnibus under accesion no. GSE132316. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (84) partner repository with the dataset identifier PXD014008.

ACKNOWLEDGMENTS. This research was supported by Australian Research Council DP160103573 (to O.V.A., A.H.M., and K.B.S.); and the Salk Pioneer Postdoctoral Endowment Fund, Deutsche Forschungsgemeinschaft research Fellowship Za-730/1-1, and National Science Foundation MCB-1024999 (to M.Z.). J.R.E. is an Investigator of the Howard Hughes Medical Institute. K.L. and J.Š. acknowledge the Knut and Alice Wallenberg Foundation, the Swedish Governmental Agency for Innovation Systems, and the Swedish Research Council, and the Swedish Metabolomics Centre (https://www.swedishmetabolomicscentre.se/) for access to instrumentation. O.V.A. was supported by the Swedish Research Council (Vetenskapsrådet 2017-03854), Crafoord Foundation (20170862), Carl Trygger Foundation (CTS 17: 487), Carl Tesdorpf Stiftelse, and NovoNordiskFonden (NNF18OC0034822). A.V.M. was supported by a postdoctoral fellowship by the Sven and Lily Lawski Foundation. M.G.L. was supported by a European Union Marie Curie FP7 International Outgoing Fellowship (252475).

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