Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells

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Jasmonates (JAs) are plant-specific signaling molecules that steer a diverse set of physiological and developmental processes. Pathogen attack and wounding inflicted by herbivores induce the biosynthesis of these hormones, triggering defense responses both locally and systemically. We report on alterations in the transcriptome of a fast-dividing cell culture of the model plant Arabidopsis thaliana after exogenous application of methyl JA (MeJA). Early MeJA response genes encoded the JA biosynthesis pathway proteins and key regulators of MeJA responses, including most JA ZIM domain proteins and MYC2, together with transcriptional regulators with potential, but yet unknown, functions in MeJA signaling. In a second transcriptional wave, MeJA reprogrammed cellular metabolism and cell cycle progression. Up-regulation of the monolignol biosynthesis gene set resulted in an increased production of monolignols and oligolignols, the building blocks of lignin. Simultaneously, MeJA repressed activation of M-phase genes, arresting the cell cycle in G2. MeJA-responsive transcription factors were screened for their involvement in early signaling events, in particular the regulation of JA biosynthesis. Parallel screens based on yeast one-hybrid and transient transactivation assays identified both positive (MYC2 and the AP2/ERF factor ORA47) and negative (the C2H2 Zn finger proteins STZ/ZAT10 and AZF2) regulators, revealing a complex control of the JA autoregulatory loop and possibly other MeJA-mediated downstream processes.

Results

Dissection of the MeJA Transcriptional Cascade. For transcript profiling of early MeJA responses, an Arabidopsis cell suspension culture was subcultured and grown for 16 h before elicitation with 50 μM MeJA, or an equal volume of DMSO for mock treatment. The cultures were sampled at multiple time points ranging up to 12 h after treatments. Based on a pilot CDNA-amplified fragment length polymorphism assay in which a bird’s eye view on MeJA-mediated transcriptional reprogramming was generated (data not shown), we designed a transcriptome-wide expression profiling experiment with the ATH1 GeneChip. In addition to the zero time point, three other time points were selected, 0.5 h, 2 h, and 6 h, for both mock- and MeJA-treated cultures. RNA from two independent experiments was used for microarray-based transcript profiling, and RNA of a third biological repeat experiment was used for real-time quantitative PCR analysis to validate the data. We considered 15,426 of 22,746 probe sets (68%) as reliably detected and used real-time quantitative PCR analysis to validate the data. We considered 15,426 of 22,746 probe sets (68%) as reliably detected and used real-time quantitative PCR analysis to validate the data. The authors declare no conflict of interest.

Data deposition: The microarray data have been submitted to the ArrayExpress database, www.ebi.ac.uk/arrayexpress (accession no. E-ATMX-13).

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MeJA Inhibits Growth and Halts Cell Cycle Progression in G2. To verify whether the repression of cell cycle gene activation was also reflected at the cellular level, the DNA content of suspension cells grown in the absence or presence of MeJA was analyzed with flow cytometry (SI Fig. 9). At the moment of subculturing, most cells (70%) resided in cell cycle phase G1, confirming previous observations (23). Subculturing was followed by a nearly synchronized reactivation of the cell cycle. At the time of MeJA elicitation, 16 h after subculture, most cells (62%) were in G2 phase. In the mock-treated culture, the G2 population gradually decreased over time leading to 20% G2 cells 96 h after the mock treatment. Upon treatment with MeJA at the concentration used for transcript profiling (50 μM), only a minor shift (2%) toward G2 cells was observed. However, when MeJA concentrations were increased, the applied MeJA dose and the number of cells residing in G2 phase clearly positively correlated. At 200 μM MeJA, 40% of cells were obstructed in G2 phase, doubling the amount of G2 cells compared with those in the mock-treated culture. A similar MeJA dose–response, but with an inverse correlation, was obtained when Arabidopsis callus growth was scored on solid medium with increasing MeJA concentrations (SI Fig. 10), suggesting that MeJA-modulated growth inhibition is at least in part mediated by the imposed G2 arrest of cell cycle progression.

The Early MeJA Response: Identification of Regulators of JA Biosynthesis. As mentioned, MAPMAN analysis pointed to a significant response in the JA metabolism BIN (SI Table 3). Five genes involved in the JA biosynthesis pathway, lipoxygenase 3 (LOX3), allene oxide cyclase 3 (AOC3), OPDA reductase 2 (OPR2), OPR3, and OPC-8:0 CoA ligase 1 (OPCL1), were rapidly up-regulated by MeJA, whereas two other known JA biosynthesis genes, allene oxide synthase (AOS) and acyl-CoA oxidase 1 (ACXI), were significantly induced at later time points (SI Fig. 11). This phenomenon of self-activation of JA biosynthesis has already been extensively reported in the literature (1), but the regulatory mechanism behind this positive feedback loop has not been elucidated yet. Besides the five early responsive JA biosynthesis genes mentioned above, the expression of 70 other genes, of which more than one-third corresponded to genes putatively involved in transcriptional regulation, was simultaneously enhanced 0.5 h after MeJA treatment (SI Fig. 4 and SI Table 1). Transcription factors from eight different TF families (http://attdb.cbi.pku.edu.cn) were found within this set, including JAZ/TIFY, AP2/ERF, WRKY, bHLH, MYB, NAC, and C2H2 Zn finger family members.

To unravel the autoregulation of JA biosynthesis gene expression, TFs controlling the expression of the JA biosynthesis gene LOX3 were identified in two parallel screens. A set of 21 early MeJA-responsive TFs, covering all relevant expression patterns and TF families (SI Fig. 12), was selected and tested (i) for interaction with the LOX3 promoter (PLOX3) by a yeast one-hybrid (Y1H) screen and (ii) for PLOX3 transactivation in a protoplast-based transient expression assay (TEA). For the Y1H analysis, a reporter strain was constructed containing a 1,997-bp PLOX3 fragment fused to the H333 reporter gene. The reporter strain was subsequently supertransformed with expression clones containing the ORFs of the 21 TFs fused to the yeast GAL4 activation domain (GAL4AD). Promoter binding was scored on media with increasing concentrations of 3-amino-1,2,4-triazole (3-AT). Growth of a control reporter strain, as well as of the most other supertransformants, was completely arrested at 2.5 mM 3-AT, except for yeast cells producing the GAL4AD-STZ/ZAT10 and GAL4AD-AZF2 fusion proteins (Fig. 3A). These results indicate that the closely related C2H2 Zn finger proteins STZ/ZAT10 and AZF2, which contain an ERF-associated amphipathic repression domain and act as transcriptional repressors, can bind PLOX3 (SI Fig. 3A) (24).
In parallel, regulation of LOX3 expression was investigated in tobacco protoplasts by an automated TEA (11). To this end, the PLOX3 fragment was fused with the firefly luciferase (fluc) reporter gene, and the resulting construct (PLOX3:fluc) co-transfected with overexpression cassettes of the 21 TFs. No significant effect on PLOX3:fluc expression was observed when cotransfecting with the STZ/ZAT10 or AZF2 overexpression constructs (Fig. 3B). In contrast, PLOX3:fluc expression increased >3-fold when the transcriptional activators ORA47, an AP2/ERF protein, and MYC2, a bHLH protein (Fig. S5B and SI Fig. 13B), were overproduced. Both proteins are known to be positive actors in the MeJA signaling cascade (1), but a direct involvement in the regulation of JA biosynthesis had not been established yet.

**Discussion**

**MeJA Has a Dual and Direct Effect on Growth and Defense of Arabidopsis Cells.** JAs are signaling molecules implicated in the regulation of various biological processes. The transcriptional cascade after MeJA perception in fast-dividing suspension-cultured cells of the model species Arabidopsis was studied. This approach revealed a surprisingly swift- and concurrent-acting dual effect of MeJA on the transcriptome of Arabidopsis cells. On the one hand, MeJA impaired the G2-to-M transition by repressing M-phase gene activation, and, on the other hand, MeJA coordinately induced genes from phenylpropanoid-associated pathways. That both gene expression trends, which might broadly be redefined as repression of multiplication or growth and activation of the defense response, respectively, were set concomitantly within a few hours after MeJA elicitation clearly indicates that both processes occur independently of each other and can consequently be considered as direct effects of MeJA treatment (Fig. 3C).

**The Monolignol Biosynthesis Pathway Is Subject to MeJA Elicitation at Both Transcript and Metabolite Levels.** MeJA elicitation of Arabidopsis cell cultures significantly induced expression of genes involved in general phenylpropanoid and monolignol biosynthesis and associated processes—more specifically, the shikimate pathway that supplies the phenylpropanoid pathway with phenylalanine and the nitrogen-recycling mechanism that salvages the ammonium released by the action of phenylalanine ammonia lyase, the enzyme catalyzing the first committed step of phenylpropanoid metabolism. In agreement with the transcriptome data, targeted metabolite profiling showed that gene induction was accompanied by an increased flux in the monolignol pathway as manifested by elevated levels of extracellular coniferyl alcohol, as well as oligomers thereof. Indeed, once produced, monolignols, i.e., coniferyl and sinapyl alcohols in angiosperms, are mainly exported outside the cell where they can be oxidized by peroxidases and/or laccases and polymerized to lignin by radical–radical combinatorial coupling reactions (20). Therefore, this inducible cell suspension culture might be a suitable model system for monolignol biosynthesis studies.

**M-Phase Gene Repression Correlates with a G2-Phase Blockade.** JA treatment has recently been demonstrated to arrest synchronized tobacco cells in both G2 and G3 phases (27), and the G2 arrest has been found to be accompanied by reduced accumulation of B-type cyclin-dependent kinases and cyclin B1;1 (28), hinting at a G2 checkpoint possibly controlled by JAs. Our data indicated that the G2 arrest could occur together with an unexpectedly rapid blockade at the transcript level, as reflected by the repressive effect of MeJA on M-phase gene expression. Correspondingly, a dose-dependent effect of MeJA on cell cycle progression and growth of Arabidopsis cells was observed. The concentration of MeJA used for elicitation correlated positively with the amount of cells residing in G2 phase 96 h after elicitation and negatively with callus growth. These observations support previous research in which inhibition of cell cycle progression and consequential growth reduction had been linked to altered transcriptional regulation of cell cycle genes (29).

Furthermore, these observations might enlighten the long-reported negative impact of JAs on plant growth (1) that is, for instance, also linked to the dwarfed phenotype of cev1/elil mutants (25, 26). MeJA application repressed activation of a large group of M-phase-associated genes rather than a specific subset. M-phase-specific gene activation has been attributed to M-phase-specific
activator (MSA) promoter elements that are overrepresented in promoters of M-phase-associated genes and are both essential and sufficient for M-phase-specific promoter activity (19, 30). Whether the MSA element plays a role in MeJA-induced repression and whether it involves de novo synthesis or posttranslational modification of a transcriptional regulator remains to be determined. Alternatively, JA signaling may activate secondary messenger molecules that, in turn, affect cell cycle progression. For instance, hydrogen peroxide is believed to act as a secondary messenger in JA-mediated induction of late-responsive defense genes (31). Oxidative stress can impair cell cycle progression and repress cell cycle gene activity in tobacco cells (32). Genes involved in the ascorbate recycling and glutathione biosynthesis pathways are induced by MeJA treatment in cultured cells and seedlings of Arabidopsis (ref. 18 and this study), which could be indicative of an oxidative burst after JA perception.

Early MeJA-Responsive TFs Encode Both Activators and Repressors, Acting at the First Level of the MeJA Signaling Cascade. MeJA perception in Arabidopsis cells was rapidly followed by gene activation. Within 0.5 h, expression of a distinct set of early response genes was significantly enhanced. Besides known regulators of MeJA responses, such as MYC2 (6) and several members of the JAZ repressor family (3–5), this set included several other early MeJA-responsive TFs. These TFs mediate the altered expression of other early MeJA-responsive genes, encoding enzymes involved in JA biosynthesis, corresponding to the autoregulatory JA biosynthesis loop, or later-response genes involved in monolignol biosynthesis and cell cycle progression (Fig. 3C).

Two parallel screens for transcriptional regulators of JA biosynthesis identified both potential activators and repressors. The activators were encoded by MYC2, the bHLH-type TF that directly interacts with the JAZ proteins and seems to act at the first level of the JA cascade (3, 6), and ORA47, the AP2/ERF protein recently postulated to be a positive regulator of JA biosynthesis (33). The repressors were encoded by STZ/ZAT10 and AZF2. These closely related C2H2 Zn finger proteins contain an ERF-associated amphiphilic repression domain and might act as both positive and negative regulators of plant defense responses (24, 34). These findings are summarized in a model (Fig. 3C).

An interesting parallel can be drawn with the transcriptional regulation of the periwinkle gene encoding strictosidine synthase (STR), the enzyme that catalyzes the first step in terpenoid indole alkaloid biosynthesis. Various TFs were shown to bind the STR promoter, including CrMYC1, the putative ortholog of MYC2; CrZCT1 to CrZCT3, homologs of STZ/ZAT10 and AZF2; and CrORCA1 to CrORCA3, encoding AP2/ERF proteins homologous to ORA47 (35). It is tempting to speculate that proteins such as MYC2, ORA47, STZ/ZAT10, AZF2, and perhaps other early-responsive Arabidopsis TFs regulate not only early MeJA responses such as JA biosynthesis, but also later responses, such as activation of phenylpropanoid metabolism. The finding that the MeJA responsiveness of several genes involved in phenylpropanoid/flavonoid biosynthesis was reduced in the jin1myc2 mutant (36) corroborates this hypothesis. It will be exciting to view the outcome of future large-scale TF interaction screens with promoters of the phenylpropanoid and cell cycle genes as baits. Such screens will undoubtedly enrich our current understanding of the complex transcriptional cascade that is steered by JAs and in which both repressors and activators of the same cellular and metabolic pathways are induced to control the plant's defense response.

Materials and Methods

Maintenance, Treatments, and Sampling of the Cell Suspension Culture. Sus- pensions of cultured cells of A. thaliana (L.) Heynh. (ecotype Columbia-0) were maintained as described (37). For elicitation, a 7-day-old culture was diluted 10-fold in fresh medium and grown for 16 h. Subsequently, MeJA (Duchefa) at a final concentration of 50 μM or an equal volume of solvent (DMSO) was added to the cultures. For transcript profiling, cultures were sampled 0, 2, 4, 6, 6, 12 h after treatments. Samples for flow cytometry were taken before treatments and daily for 1–4 days after elicitation. For metabolite profiling, cultures treated with MeJA or DMSO were sampled every day for 1 week after treatments.

Transcript Profiling. Filtered cells were ground in liquid nitrogen, and total RNA was isolated with TRizol Reagent (Invitrogen). The Arabidopsis ATH1 GeneChip (Affymetrix) was hybridized at the Flanders Institute for Biotechnology microarray facility (www.microarrays.be) according to the manufacturer’s instructions. Microarray data processing and statistics as well as real-time quantitative PCR are described in SI Methods. The microarray data have been submitted to the ArrayExpress database (www.ebi.ac.uk/arrayexpress; accession no. E-ATMx-13).

Oligomeric Profiling. Cells and medium were separated by vacuum filtration. Approximately 300 mg of the filtered cells were ground in liquid nitrogen, extracted with 3 ml of methanol, and, after lyophilization of 1 ml of the supernatant, extracted and analyzed by reversed-phase HPLC. Additionally, 350 μl of the culture medium was analyzed (21, 22). Peaks with UV/VIS spectra similar to those of lignans and oligolignols were collected and identified through LC/MS (21, 22). Peak heights of oligolignols were measured with the maxplot option (200–450 nm) in Xcalibur version 1.2. The β-ary ether tetramer was identified as described (SI Methods).

Flow Cytometry. Culture samples were vacuum filtered and frozen in liquid nitrogen. Nuclear DNA content distribution was analyzed (29) and quantified with the multicycle flow cytometry software (Flowcyt Flow System).

TEA and Y1H Analysis. A 1,997-bp PLOX3 fragment was obtained from the Arabidopsis promoterome (www.psb.ugent.be/SAP) and Gateway recombinated with pDONR40018 (Invitrogen) to yield pENTR-PLOX3. For generation of TEA reporter constructs, pENTRY-PLOX3 or pENTRY-PUAS (6xUAS-P35S sensing) were recombinated by Gateway MultiSite LR cloning with pENTRY-FLUC and pm42Gw3,7 (38) to yield PLOX3:FLUC or PUAS:FLUC, respectively. The PLOX3:HIS3 Y1H reporter plasmid was developed by MultiSite LR cloning (39). TF ORFs were amplified by PCR with Platinum Taq High Fidelity (Invitrogen) and cDNA synthesized from MeJA-treated Arabidopsis cells as template. All primers used are listed in SI Table 4. ORFs were introduced by Gateway BP and LR cloning for Y1H into pGAD (40) and for TEA into p2Gw7 (38) and p2Gw12. The latter destination vector was constructed by inserting a Gateway cassette into the P35S-GAL4DB vector (41). TEA and Y1H were performed as described (11, 39). For the growth test on 3-AT, 10- and 100-fold dilutions of a 2-day-old liquid culture were dropped on media containing increasing 3-AT concentrations and allowed to grow for several days at 30°C.

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100.8595–8600.


cluster 1 (51)

cluster 2 (11)

cluster 3 (205)

cluster 4 (31)

cluster 5 (40)

cluster 6 (44)

cluster 7 (106)

cluster 8 (31)

mock  MeJA
**Shikimate pathway**

erythrose-4-phosphate + PEP → shikimate → chorismate

- **DHS3**
- **SK**
- **EPSPS**

**Aromatic AA metabolism, tryptophan**

chorismate → tryptophan

- **ASB**
- **PAT1**
- **IGPS1**
- **TSB**
- **TSA1**

**NH4+ recycling**

\[\text{NH}_4^+\] → glutamate → ketoglutarate → glutamate → glutamine

- **GLT1**
- **GLN1;2**
- **GLN1;3**

**Aromatic AA metabolism, phenylalanine**

chorismate → phenylalanine

- **CM1**
- **ADT5**
- **ADT6**

**NH4+ recycling**

\[\text{NH}_4^+\] → glutamate → glutamine

- **GLN1;2**
- **GLN1;3**

**Phenylpropanoid pathway**

phenylalanine → cinnamate → p-coumaroyl-CoA → caffeoyl-CoA → feruloyl-CoA

- **PAL2**
- **HCT**
- **C3H1**
- **HCT**
- **CCoAOMT1**
- **CCR-like1**
- **CAD2**

flavonoids → coniferyl alcohol → G oligolignols