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 genes corresponding to other probe sets as unexpressed. The MeJA-treated and control cultures were compared pairwise at the three time points of interest. In total, 598 different genes were judged as differentially regulated by MeJA at one or more time points (with a P value cutoff corrected for the false discovery rate.

Jasmonic acid (JA) and its conjugates, such as methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JA-Ile), collectively referred to as jasmonates (JAs), are small signaling molecules, specific for, but ubiquitous in the plant kingdom. Environmental stresses, such as wounding or pathogen attack, can trigger JA production in planta. Upon perception of JA signals, the plant cell activates several defense mechanisms, reflected in a massive reprogramming of gene expression. In addition, JAs are involved in the regulation of many developmental processes, including male fertility, fruit ripening, and root growth (1). The F-box protein CORONATINE INSENSITIVE 1 (COI1) fulfills a key role in JA signaling, which is demonstrated by the JA insensitivity of the coi1-1 loss-of-function mutant (2). In the presence of JA-Ile, COI1 binds to members of the JA ZIM domain (JAZ) protein family (3–5) and marks them for degradation by the 26S proteasome. The JAZ protein JASMONATE INSENSITIVE3 (JAZ3/TIFY6b) physically interacts with the basic helix–loop–helix (bHLH) transcription factor (TF) MYC2 (3), probably repressing its essential function for normal JA signaling (6).

The potency of JAs to elicit secondary metabolism in cell cultures has converted them into powerful tools to unravel this complex cellular process. Combined transcript and metabolite profiling approaches to tackle the MeJA-mediated regulation of secondary metabolism resulted in the establishment of gene-to-metabolite networks involved in the biosynthesis of the pharmaceutically valuable terpenoid indole alkaloids in periwinkle (Catharanthus roseus) cells (7), in the characterization of enzymatic steps in the isoflavone and triterpene biosynthetic pathways in barrel medic (Medicago truncatula) cells (8, 9), and in the isolation of novel regulators of nicotine and phenylpropanoid conjugate biosynthesis in tobacco (Nicotiana tabacum) cells (10–13) or of alphatic glucosinate biosynthesis in Arabidopsis thaliana cells (14).

We monitored the MeJA-triggered transcriptional cascade in a fast-dividing cell culture of the model plant Arabidopsis. In a first wave, MeJA induced expression of genes involved in transcriptional regulation or JA biosynthesis. In a second wave, transcriptional reprogramming resulted in a dual response: repression of cell cycle progression on the one hand and induction of phenylpropanoid metabolism on the other hand. Furthermore, a role was demonstrated for early MeJA-responsive transcriptional regulators of the bHLH, APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/ERF), and C2H2 Zn finger families in the control of the JA autoregulatory loop.

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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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set at 0.05) [supporting information (SI) Table 1]. From a Venn diagram (SI Fig. 4) with the numbers of genes up-regulated or down-regulated at each time point, a few conclusions could already be drawn. First, MeJA clearly activated a transcriptional cascade: the number of MeJA-regulated genes increased from 75 after 0.5 h to 495 after 6 h. Hereafter, genes induced after 0.5 h, 2 h, or 6 h will be referred to as early, intermediate, and late MeJA response genes, respectively. Second, MeJA primarily triggered gene activation: all 75 early response genes had an enhanced expression, and, of the genes responding 2 h after treatment, only 17 were down-regulated. In contrast, genes involved in cell wall modification, the corresponding genes were up-regulated, matching clusters 3 and 4 (Fig. 1B). In other categories, the BIN of JA metabolism was significantly responsive (SI Table 3). Additionally, BINs corresponding to processes directly associated with the phenylpropanoid pathway, such as aromatic amino acid biosynthesis (the shikimic acid pathway) and ammonium metabolism, were also significantly responsive to MeJA (Fig. 1C and SI Fig. 7). MAPMAN analysis also indicated an induction of genes involved in ascorbate and glutathione metabolism. Similarly, Ser and Cys biosynthesis pathways, preceding the glutathione biosynthesis, did also respond to MeJA, as in seedlings (18). In all of the above-mentioned metabolic processes, the corresponding genes were up-regulated, matching clusters 3 and 4 (SI Fig. 5). In contrast, genes involved in cell wall modification, such as pectate lyases and cellulose synthases, appeared to be mainly down-regulated (Fig. 1C). In other categories, the BIN of JA metabolism was significantly responsive (SI Table 3).

Surprisingly, the second most significant MeJA-affected BIN was that of "cell organization." The expression profiles of genes identified in this BIN grouped mostly in cluster 5 with reduced gene activation over time in the presence of MeJA (SI Fig. 5). BiNGO analysis exposed an overrepresentation of the categories...
involved in monolignol biosynthesis were strongly enriched (SI representing phenylpropanoid metabolism, genes known to be abundance differed increasingly in the MeJA-elicited cultures. The monolignol coniferyl alcohol was clearly detectable, and its characteristic of simple phenolic compounds and phenylpropanoids. Separated from the cells by vacuum filtration. The cellular was evaluated by metabolite profiling of both mock- and MeJA-profiling experiment of synchronized associated genes, identified in a transcriptome-wide transcript cluster 5 were compared with a data set of 198 M-phase-related to the M-phase of the cell cycle, the 40 genes of synchronicity are mitotic-associated genes. Based on the annotation of cell cycle-regulated or associated genes (19), a custom cell cycle MAPMAN BIN was designed to visualize the overall influence of MeJA on cell cycle gene expression. The repressive effect of MeJA on cell cycle progression was clearly apparent from the M-phase on, in which it was widespread, and also extended to the G1 phase (Fig. 2).

Together, these findings reveal that MeJA causes a concurrent and dual effect in Arabidopsis cells. MeJA activated defense responses by triggering, for instance, phenylpropanoid biosynthesis gene expression, whereas it repressed expression of M-phase-associated genes.

MeJA Increases Growth Through the Monolignol Biosynthesis Pathway. Within the MeJA-responsive genes in the MAPMAN BIN representing phenylpropanoid metabolism, genes known to be involved in monolignol biosynthesis were strongly enriched (SI Tables 1 and 3) (20). Therefore, phenylpropanoid accumulation was evaluated by metabolite profiling of both mock- and MeJA-treated cells and the corresponding culture media, which were separated from the cells by vacuum filtration. The cellular phenolic profiles revealed UV/VIS absorption spectra characteristic of simple phenolic compounds and phenylpropanoids. The monolignol coniferyl alcohol was clearly detectable, and its abundance differed increasingly in the MeJA-elicited cultures compared with that in the mock-treated cells (Fig. 1B and SI Fig. 8). In addition, MeJA treatment yielded significantly higher levels for the major HPLC peaks corresponding to hydroxycinnamate derivatives (data not shown). No UV/VIS spectra reminiscent of flavonoids were observed. In the medium of both mock- and MeJA-treated cells, HPLC revealed the presence of increasing amounts of oligolignols over time (Fig. 1B and SI Fig. 8), in which the main units were β-aryl ethers, as in lignin (21, 22). In 3-week-old cultures, the culture medium could be stained with phloroglucinol and no more oligolignols could be detected, suggesting that these compounds were used in higher-order lignin (data not shown). Overall, the total amount of oligolignols, expressed as the sum of their individual peak heights, was consistently higher in the MeJA-treated than in the control cells throughout the time course, with a peak after 2 days, supporting a higher monolignol biosynthetic flux due to MeJA treatment.

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%) were 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 (ACX1), 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, 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 simulta- neously enhanced 0.5 h after MeJA treatment (SI Fig. 4 and SI Table 1). Transcription factors from eight different TF families (http://attodb.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 HIS3 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 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 amphiphilic repression domain and act as transcriptional repressors, can bind PLOX3 (SI Fig. 13A) (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 (LUC) reporter gene, and the resulting construct (PLOX3:3LUC) cotransfected with overexpression cassettes of the 21 TFs. No significant effect on PLOX3:3LUC expression was observed when cotransfecting with the STZ/ZAT10 or AZF2 overexpression constructs (Fig. 3B). In contrast, PLOX3:3LUC expression increased >3-fold when the transcriptional activators ORA47, an AP2/ERF protein, and MYC2, a bHLH protein (Fig. 3B 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 coordinate-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 influx 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.

The Arabidopsis mutant constitutive expression of VSPI (cev1) is defective in the cellulose synthase gene CES43 and shows constitutive JA responses together with an increased production of JA and ethylene (25). The *ectopic lignin 1* (*el1*) mutant is allelic to *cev1* and has been identified in a screen for mutants with ectopic lignification patterns. Additionally, isoxaben, an inhibitor of cellulose biosynthesis, phenocopies *el1* lignification in wild-type seedlings. In JA-resistant *coi1* plants, ectopic lignification upon isoxaben treatment is reduced, indicating a partial requirement of JA signaling (26). Our transcriptome analysis clearly supports an active role of JAs in the regulation of *Arabidopsis* monolignol biosynthesis.

**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/el1* 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 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.** Suspensions 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, 0.25, 0.5, 1, 2, 4, 6, 8, and 12 h after treatments. Samples for flow cytometry were taken before elicitation or every 2 days and analyzed daily for 4–7 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 grown 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-ATM1-13).

**Oligolignol 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 super- natant, extracted and analyzed as described (30). In addition, 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 massplot 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 YH Analysis.** A 1,997-bp PLOX3 fragment was obtained from the Arabidopsis promoterome (www.psb.ugent.be/SAP) and Gateway recombined with pDONR4PR18 (Invitrogen) to yield pENTRY-PLOX3. For generation of TEA reporter constructs, pENTRY-PLOX3 or pENTRY-PUAS (6xUAS-P35Smin) were recombined by Gateway MultiSite LR cloning with pENTRY-fLUC and pm42GWF,3 (38) to yield PLOX3:fLUC or PUAS:fLUC, respectively. The PLOX3:HIS3 Y1H reporter plasmid was developed by MultiSite LR cloning (39). 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 p2GAG (41). The latter destination vector was constructed by inserting a Gateway cassette into the P35S-GAL4DB vector (41). TEA and YH were performed as described (11, 39). For the growth test on 3-A, 10- and 100-fold dilutions of a 2-day-old liquid culture were dropped on media containing increasing 3-A concentrations and allowed to grow for several days at 30°C.

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A 0.5  2
19  11  65
44  1  113
134
6

B 0.5  2
0  0  8
0  0  9
194
6
**Shikimate pathway**

erythrose-4-phosphate + PEP → shikimate → chorismate

- *DHS3*
- *SK* (shikimate kinase)
- *EPSPS*

**Aromatic AA metabolism, tryptophan**

- chorismate → tryptophan
  - *ASB* (aromatic side pathway binding)
  - *PAT1* (tryptophan permease)
  - *IGPS1* (imidazoleglycerolphosphate synthase 1)
  - *TSB* (tryptophan synthetase B)
  - *TSA1* (tryptophan synthetase A)

**NH₄⁺ recycling**

- glutamate → glutamine → ketoglutarate
  - *GLT1* (glutamate transport)

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

**Aromatic AA metabolism, phenylalanine**

- chorismate → arogenate → phenylalanine
  - *CM1* (chorismate mutase)
  - *ADT5* (amide transporter)
  - *ADT6* (amide transporter)

**Phenylpropanoid pathway**

- phenylalanine → cinnamate → p-coumaroyl-CoA → caffeoyl-CoA → feruloyl-CoA
  - *PAL2* (phenylalanine ammonia-lyase 2)
  - *4CL2* (4-coumarate:CoA ligase 2)
  - *HCT C3H1 HCT CCoAOMT1 CCR-like1 CAD2* (isoflavonoid and flavonoid metabolism)
  - flavonoids
  - coniferyl alcohol
  - G oligolignols
mock MeJA
6 2 0.5 0 0.5 2 6

Plamid membrane

At1g17420 LOX3
At5g42650 AOS
At3g25780 AOC3

chloroplast

α-LeA

13-HPOT

At1g17420 LOX3
At5g42650 AOS
At3g25780 AOC3

peroxisome

Acyl-CoA

At4g16760 ACX1

At2g06050 OPR3
At1g76690 OPR2
At1g20510 OPCL1

At1g20510 OPCL1

Acyl(n-2)-CoA

(+)-7-iso-jasmonate

other conjugates

MeJA

JA-Ile

Enoyl-CoA

3-ketoacyl-CoA

3 rounds of β-oxidation

Acyl-CoA

Enoyl-CoA

cytosol