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Long-term Exposure to Methyl Jasmonate Increases Myrosinases TGG1 and TGG2 in Arabidopsis *coi1* and *myc2,3,4* Mutants

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Abstract

Myrosinase is an enzyme that activates a group of metabolites, namely glucosinolates, which are involved in the defense against herbivores and pathogens. In *Arabidopsis thaliana*, THIOGLUCOSIDE GLUCOHYDROLASE 1 (TGG1) and TGG2 are two myrosinases that accumulate in specialized myrosin cells of rosette leaves. Here, we show that prolonged exposure to the wounding hormone methyl jasmonate (MeJA) enhances *TGG1* and *TGG2* expression independent of the canonical jasmonic acid (JA) signaling pathway. We found that airborne MeJA treatment for up to 5-days enhanced both *TGG1* and *TGG2* gene expression and their protein levels in Arabidopsis leaves. *TGG1* and *TGG2* gene expression did not stop in two JA signaling pathway mutants, namely *coi1-16* and *myc2,3,4*, after 5-days of MeJA treatment, although other typical JA responses were completely stopped in these two mutants. FAMA is a transcription factor that participates in the specification of myrosin cell development, but it does not appear to be involved in *TGG1* and *TGG2* gene expression regulation in the 5-day MeJA treatment, as its expression did not increase. Taken together, our results suggest the existence of an alternative JA signaling pathway that is activated by long-term exposure to MeJA.

Keywords: myrosinase, methyl jasmonate, COI1, myrosin cell

1 | Introduction

The myrosinase-glucosinolate system is a chemical defense mechanism against herbivores and pathogens in plants of the order Brassicales. This defense mechanism was first discovered in

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mustard seeds (Bussy, 1840; Bones & Rossiter, 1996), and consists of an enzyme called myrosinase and a substrate called glucosinolate. Myrosinases and glucosinolates are stored in different tissues or subcellular compartments in plants (Shirakawa et al., 2016). However, these enzymes and substrates come into contact when tissue damage occurs, and the enzymes begin to hydrolyze glucosinolates to produce toxic compounds, such as isothiocyanate, to stop further damage by herbivores and pathogens (Bhat & Vyas, 2019). In *Arabidopsis thaliana*, myrosinase genes are encoded by six *THIOGLUCOSIDE GLUCOHYDROLASE* (*TGG*) genes, namely *TGG1–TGG6* (Rask et al., 2000; Shirakawa & Hara-Nishimura, 2018). Two of the six genes, *TGG1* (*At5g26000*) and *TGG2* (*At5g25980*), are expressed in the leaves, whereas *TGG4* (*At1g47600*) and *TGG5* (*At1g51470*) are expressed in the roots (Shirakawa et al., 2016; Andersson et al., 2009). *TGG3* (*At5g48375*) and *TGG6* (*At1g51490*) were considered pseudogenes because of their open reading frame disruption (Wang et al., 2009).

In Arabidopsis, TGG1 and TGG2 proteins are stored in two types of cells with similar 'idioblast' characteristics: myrosin cells and stomata guard cells (Shirakawa et al., 2022). Myrosin cells are located next to phloem cells, and this particular name was given because myrosinases accumulate extensively in their vacuoles (Guignard, 1980; Heinricher, 1884;Bones & Rossiter, 1996). Although myrosin cells are localized adjacent to the phloem, they develop independently of vascular precursor cells and originate directly from ground meristem cells (Shirakawa et al., 2014; Shirakawa et al., 2016). Basic helix-loop-helix (bHLH) transcription factors (TFs) FAMA, SCREAM1 and SCREAM2, which regulate stomatal guard cell differentiation, regulate the differentiation of myrosin cells (Li and Sack, 2014; Shirakawa et al., 2014).

Jasmonic acid (JA) and its metabolic derivatives, such as jasmonic acid isoleucine (JA-Ile) and methyl jasmonate (MeJA), are cyclopentanone derivatives of linolenic acid (Devoto et al., 2002; Liu & Timko, 2021). These chemicals are widely distributed phytohormones in higher plants and play important roles in the stress response and regulation of several plant developmental processes (Ruan et al., 2019). In the JA signaling pathway, JA-Ile is sensed by CORONATINE INSENSITIVE 1 (COI1), a leucine-rich repeat (LRR)-F-box protein (Xie et al., 1998; Devoto et al., 2002). COI1 is a component of the ubiquitin ligase, and the hormone ligand promotes binding between COI1 and JASMONATE ZIM-DOMAIN (JAZ) proteins for the ubiquitination of JAZ proteins and their subsequent degradation by the 26S proteasome. In the non-JA-signaling state, JAZ proteins physically interact with MYC2/3/4 TFs and repress their functions (Chini et al., 2009;

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Ruan et al., 2019). Therefore, JA-induced JAZ protein degradation releases downstream TFs that activate JA-inducible genes, including *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *BETA GLUCOSIDASE 18* (*BGLU18*) (Liu & Timko, 2021; Schweizer et al., 2013; Stefanik et al., 2020), which participate in plant defense (Mahmud et al., 2022).

It has been reported that myrosinase activity is significantly lower in the *coi1* mutant than in the wild type when sinigrin is used as a substrate (Capella et al., 2001), and MeJA treatment increases *TGG1* expression in a COI1-dependent manner in Arabidopsis (Feng et al., 2021). In the present study, we investigated the mechanism underlying JA responses of *TGG1* and *TGG2*. We unexpectedly found that although *TGG1* and *TGG2* are JA-responsive genes, the response is not fully dependent on canonical JA signaling pathway components, such as COI1 and MYC2/3/4, when plants are continuously exposed to MeJA for a long time.

2 | Materials and Methods

2.1 | Plant materials, plant growth condition

The *Arabidopsis thaliana* Col-0 accession was used as the wild-type. Transgenic plants containing *ProTGG2:VENUS-2sc* and *pFAMA:GFP* constructs were kind gifts from M. Shirakawa and I. Hara-Nishimura (Shirakawa et al., 2014; Shirakawa and Hara-Nishimura, 2018). The *myc2,3,4* mutant was kindly provided by H. Frerigmann, and the *coi1-16* mutant was purchased from the Arabidopsis Biological Resource Center (ABRC). Following sterilization with 70% (v/v) ethanol, seeds were cultivated at 4 cm from the center of the plate (Supplementary Fig. 1) and incubated at 4 °C for 2 d prior to germination. Seeds were then germinated at 22 °C under continuous light (approximately 100 μE s^{-1} m^{-2}) in half strength Murashige and Skoog (1/2 MS) medium [1/2 MS basal salt mixture (092623020, MP Biomedicals), 1% (w/v) sucrose and 0.5% (w/v) MES-KOH (pH 5.7)] containing 0.4% (w/v) Gellan Gum (Wako, Japan).

2.2 | MeJA treatment

The liquid of MeJA (500 nmol/plate) (Sigma-Aldrich) was applied to a filter paper pad left in the cap of an Eppendorf tube (1.5 mL) for evaporation and positioned in the center of a plate (φ = 9 cm) (Supplementary Fig. 1). The evaporated MeJA gradually and uniformly diffused into the enclosed air space of the plate. The distance between the plants and the source of MeJA was important, and the arrangement ensured uniform and equal exposure of all seedlings to MeJA. Leaf

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samples for each replicate were randomly collected, frozen in liquid nitrogen, and stored at –80 °C for subsequent molecular analyses.

2.3 | Binocular and confocal microscope

A binocular fluorescence microscope (Zeiss SteREO V12) was used to observe fluorescent proteins in whole leaves. The images were captured using a CCD camera (Zeiss AxioCam MRc5). A confocal laser scanning microscope (LSM880, Carl Zeiss, Jena, Germany) was used to observe fluorescent proteins.

2.4 | RNA isolation and Quantitative RT-PCR

Total RNA was extracted from the aerial parts (aboveground) of 12-d-old seedlings using the TRI Reagent (Molecular Research Center, Cincinnati, USA). After dissolving the RNA in distilled water and digesting contaminated genomic DNA with DNase I (Sigma-Aldrich), the first cDNA strand was generated from 1 μg of the RNA using Ready-to-Go RT-PCR beads (GE Healthcare) and random oligomers. PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA) was used for the quantification of cDNA of *UBIQUITIN 10* (*UBQ10*), *TGG1*, *TGG2*, *FAMA*, *VSP2*, and *BGLU18* using an instrument (QuantStudio 12K Flex, Thermo Fisher Scientific). Gene-specific primer sets were generated using the Primer3Plus software. The *UBQ10* gene was used as a reference gene because of its stable and constitutive expression in all Arabidopsis tissues and treatments (Czechowski et al., 2005). Relative expression of the target genes was normalized to that of *UBQ10*.

2.5 | SDS-PAGE and Immunoblot Analysis

Total proteins were extracted from 50 mg of leaves with 200 μL of 2× sample buffer (20 mM Tris-HCl buffer, pH 6.8, 40% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS), and 2% (v/v) 2-mercaptoethanol). The homogenate was centrifuged at 12,000 × *g* for 5 min to remove debris. Extracts (10 μL) were electrophoresed on SDS-polyacrylamide gels. After separation, the proteins were transferred onto a nylon membrane and subjected to immunoblotting. The anti-TGG1 and anti-TGG2 antibodies were diluted 5000-fold for each treatment (Ueda et al., 2006; Shirakawa et al., 2014). The proteins were stained with Coomassie Brilliant Blue R-250.

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3 | Results

3.1 | Long-term exposure to airborne MeJA increases the expression level of *TGG1* and *TGG2* in Arabidopsis rosette leaves

JA treatment inhibits true leaf and cotyledon growth (Zhang and Turner, 2008), elongation (Chen et al., 2013), and adventitious root development (Gutierrez et al., 2012). We treated 7-d-old *Arabidopsis thaliana* seedlings with vaporized MeJA for 5 d and found that airborne MeJA treatment reduced plant size, as previously reported (Zhang and Turner, 2008; Chen et al. 2013; Gutierrez et al., 2012). Next, we examined the expression levels of two myrosinase genes, *TGG1* and *TGG2*, after airborne MeJA treatment to determine whether JA induces the expression of these genes. Reverse transcription-quantitative PCR (RT-qPCR) analysis of shoots revealed that the expression levels of both genes were significantly increased after 5 d of airborne MeJA treatment, but not in the mock control treatment (Fig. 1b). In addition, immunoblot analysis showed that exposure to airborne MeJA for 5 d significantly increased TGG1 and TGG2 protein levels in the shoots (Fig. 1c). To monitor the activity of the *TGG2* promoter in each rosette leaf, we used a transgenic plant containing the *TGG2* promoter and *Venus-2sc* reporter construct (*pTGG2:Venus-2sc*), in which Venus fluorescence was observed mainly in myrosin cells and to some extent in the stomata guard cells (Fig. 1d) (Shirakawa et al., 2016). We measured the myrosin cell area in the images based on Venus fluorescence. The myrosin cell area was not dramatically changed by MeJA treatment in the first, second, and third leaves but was significantly increased in the fourth leaves of MeJA-treated plants (Fig. 1e, f; Supplementary Fig. 2a). We also measured the myrosin cell area of the eleventh leaves after very long-term MeJA treatment. The myrosin cell area of these young leaves tended to increase after a very long MeJA treatment (Supplementary Fig. 2b), suggesting that MeJA promoted myrosin cell area expansion in young leaves at certain plant ages or leaf orders under our experimental conditions. Venus fluorescence intensity in myrosin cells did not change significantly in the first, second, third, and fourth leaves of MeJA-treated plants compared to those in the mock-treated plants (Supplementary Fig. 3). As a different experiment, we started MeJA treatment in earlier day (2 days after germination) when the first leaf is emerging, for examining if MeJA treatment enhances myrosin cell differentiation in the first leaf. However, the myrosin cell number was not increased by MeJA treatment in the emerging first leaf (Fig. 1g).

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As TGG2 is known to accumulate in stomatal guard cells, we counted the number of stomatal cells in MeJA-treated leaves. No significant changes in stomatal density were observed in the first and second leaves, but stomatal density was reduced in the third and fourth leaves of MeJA-treated plants (Supplementary Fig. 4).

3.2 | Long-term airborne MeJA treatment increases *TGG1* and *TGG2* expression in the canonical JA-insensitive *coi1-16* and *myc2,3,4* mutants

COI1 is the JA receptor that forms a co-receptor complex with JAZ proteins, and MYC2, MYC3, and MYC4 are key regulators that control the expression of JA response genes (Sheard et al., 2010; Fernandez-Calvo et al., 2011). Therefore, we examined the expression of *TGG1* and *TGG2* in Arabidopsis *coi1* and *myc2,3,4* deficient mutants. We used the *coi1-16* mutant allele because it is fertile at 16 °C, unlike other *coi1* mutant alleles, and can be maintained as a pure homozygous line (Ellis and Turner, 2002). We found that 1 d of airborne MeJA treatment tended to enhance *TGG2* expression in wild-type plants, but not in *coi1-16* and *myc2,3,4* mutants. However, long-term airborne MeJA treatment led to a significant increase in *TGG1* and *TGG2* expression levels in *coi1-16* and *myc2,3,4* mutants, as well as in the wild-type plants (Fig. 2b, c). We examined TGG1 and TGG2 protein levels by immunoblot analysis, and the results showed that long-term (5 d) MeJA treatment increased TGG1 and TGG2 protein levels in wild type, *coi1-16* and *myc2,3,4* plants (Fig. 2d, e), which was consistent with *TGG1* and *TGG2* expression levels. Taken together, our results suggest that long-term MeJA treatment induces *TGG* genes independently of the canonical JA signaling pathway components (COI1 and MYC2/3/4).

3.3 | Long-term MeJA treatment does not influence the *FAMA* expression

Because FAMA is a regulator of myrosin cell differentiation and *TGGs* expression (Li and Sack, 2014; Shirakawa et al., 2014; Feng et al., 2021), we further investigated the possible role of MeJA in increasing myrosin cell differentiation by monitoring the expression of *FAMA*. However, long-term MeJA treatment did not significantly upregulate the expression of *FAMA* in wild-type plants, which was also the case in *coi1-16* and *myc2,3,4* mutants (Fig. 3a). We also used *pFAMA:GFP* transgenic plants (Shirakawa and Hara-Nishimura, 2018) to validate the expression pattern of *FAMA* and did not observe any obvious difference in the expression pattern of GFP between the

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emerging first and second leaves of mock- and MeJA-treated plants (Fig. 3b), suggesting that MeJA treatment does not promote myrosin cell differentiation.

3.4 | Airborne MeJA treatment inhibits the plant growth in the wild type but not in *coi1-16* and *myc2,3,4* mutants

As the *coi1-16* and *myc2,3,4* mutants have been described to have a leaky JA response (Ellis and Turner, 2002; Song et al., 2017), and the extent of the JA response in these mutants was not known in the long-term airborne MeJA treatment, we examined the plant growth phenotype of these mutants in the long-term airborne MeJA treatment as the same experimental condition as in Fig. 2. Airborne MeJA treatment significantly reduced shoot growth (Fig. 4a) and shoot fresh weight (Fig. 4b) in the wild type, but not in the *coi1-16* and *myc2,3,4* mutants. The wild-type plants showed stunted growth, smaller leaves, and altered leaf shape in the MeJA treatment, whereas the *coi1-16* and *myc2,3,4* mutants did not show such differences between the treated and untreated plants. The roots of wild-type seedlings avoided growing towards the MeJA source (center of the plate in Fig. 4a), whereas the roots of *myc2,3,4* and especially *coi1-16* mutants tended to grow in divergent directions, including towards the MeJA source. These results indicate that *coi1-16* and *myc2,3,4* mutants reduced or eliminated the MeJA response under our experimental conditions.

3.5 | *VSP2* and *BGLU18* gene expression was strongly reduced in *coi1-16* and *myc2,3,4* mutants following short and long-term exposure to airborne MeJA

To further investigate the response of *coi1-16* and *myc2,3,4* mutants to airborne MeJA treatment, we examined the expression levels of two JA-inducible genes, *VSP2* and *BGLU18*. The expression levels of *VSP2* and *BGLU18* were significantly increased after short-term (1 d) airborne MeJA treatment, and these levels were further increased by long-term (5 d) airborne MeJA treatment in wild-type plants. In contrast, *coi1-16* and *myc2,3,4* mutant seedlings showed strongly reduced expression of *VSP2* and *BGLU18* under the same treatment (Fig. 5). These results indicate that the expression of *VSP2* and *BGLU18* is exclusively dependent on COI1 and MYC2/3/4, and suggest that the pathway regulating the expression of *TGG1* and *TGG2* is distinct from the pathway regulating the *VSP2* and *BGLU18* genes in long-term MeJA treatment.

4 | Discussion

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In nature, plants are constantly subjected to stresses such as wounding or herbivore attack, which induce JA biosynthesis and subsequently activate the JA signaling pathway (McConn et al., 1997; Halitschke and Baldwin, 2004). Exposure of plants to exogenous MeJA elicits stress responses by activating JA-dependent signaling pathways (Jiang et al., 2017). In this study, we found that airborne MeJA signals induce defense-related myrosinase genes, namely *TGG1* and *TGG2*, in Arabidopsis plants. However, we unexpectedly found that long-term MeJA treatment induced the expression of *TGG1* and *TGG2* independent of the canonical JA signaling pathway using COI1 and MYC2/3/4.

It has been previously reported that a 1 d MeJA treatment induces the expression of *TGG1* but reduces the expression of *TGG2* (Feng et al., 2021). Under our experimental conditions, we did not observe any significant changes in the expression of *TGG1* after 1 d of MeJA treatment. This discrepancy may be due to the method of MeJA treatment; in this study slowly evaporating MeJA was applied to the plants through the air inside the plates, whereas MeJA was directly applied to the plants in the study by Feng et al. (2021). We found the slight induction of *TGG2* in the 1 d JA treatment in a COI1-dependent manner, which may be consistent with the findings of Feng et al. (2021) because they show the expression of *TGGs* in a COI1-dependent manner.

Long-term airborne MeJA treatment induces *TGG1* and *TGG2*, but not through COI1 and MYC2/3/4 signaling pathways and transcription factors. In response to long-term MeJA treatment, *TGG1* and *TGG2* genes were expressed in both the *coi1-16* and *myc2,3,4* mutants, but typical JA responses, such as *VSP2* and *BGLU18* gene expressions (Liu & Timko, 2021; Schweizer et al., 2013; Stefanik et al., 2020) and the induction of growth inhibition (Zhang & Turner, 2008) were impaired in these mutants. Therefore, the regulatory systems of *TGG1* and *TGG2* in response to long-term MeJA treatment appear unique. There are a few examples of COI1-independent JA analog responses in plants. For example, the plant pathogenic bacterium *Pseudomonas syringae* produces a chemical called coronatine, which functions as a COI1 ligand and induces the JA response in plants. Coronation induces stomatal pore opening through COI1 for bacterial invasion into plant tissues (Melotto et al., 2006), but a recent finding indicated the existence of an additional COI1-independent signaling pathway for the stomatal pore opening response in Arabidopsis (Ueda et al., 2017). In the bryophyte *Marchantia polymorpha*, the COI1 homolog (MpCOI1) is a receptor for dinor-12-oxo-10,15(Z)-phytodienoic acid (dn-OPDA), which acts as a JA analog. However, dn-OPDA enhances heat stress resistance independent of MpCOI1 in *M. polymorpha*, suggesting

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that the ligand can activate another signaling pathway independent of MpCOI1 (Monte et al., 2020). Although the detailed molecular mechanism remains controversial, the collective findings from their study and ours suggest the existence of a COI1-independent ligand signaling pathway in land plants. It is still obscure the TFs involved in the regulation of *TGG1* and *TGG2* expression after long-term MeJA treatment. FAMA is a key transcription factor for myrosin and stomatal guard cell differentiation, and *TGG1* and *TGG2* expression (Li and Sack, 2014; Shirakawa et al., 2014; Feng et al., 2021). However, we did not observe a significant effect on the levels or tissue distribution of the *FAMA* expression, indicating that the induction of *TGG1* and *TGG2* after long-term MeJA treatment cannot be explained by *FAMA* expression. MYC5 belongs to another subfamily that is classified along with MYC2/3/4 in Arabidopsis. Although MYC5 is involved in male fertility (Figueroa and Browse, 2015), it functions redundantly with MYC2/3/4 to inhibit JA-induced root growth (Song et al., 2017). Although we do not exclude the possibility that MYC5 regulates *TGG1* and *TGG2* expression in the *myc2,3,4* mutants, considering that MYC5 is an MYC2/3/4 homolog that is included in the COI1-dependent pathway, it is reasonable to infer that an alternative TF distinct from MYC2/3/4/5 is responsible for the regulation of *TGG1* and *TGG2* expression in response to long-term MeJA treatment.

We found a positive impact of long-term MeJA treatment on the myrosin cell area in young leaves at certain plant ages, indicating that this phenomenon positively correlates with the upregulation of *TGG1* and *TGG2* expression in shoots. Myrosin cell area expansion can be explained by two different mechanisms: one is by promoting myrosin cell differentiation to increase these cell numbers and the other is by promoting myrosin cell enlargement. However, MeJA treatment did not enhance myrosin cell numbers in young leaves (Fig. 1g), and there was no significant difference in *FAMA* expression between MeJA-treated and untreated leaves (Fig. 3), suggesting that the increase in myrosin cell area was not a consequence of differentiation of myrosin and stomatal guard cells. Therefore, we speculate that long-term exposure to MeJA enhances myrosine cell expansion, along with *TGG1* and *TGG2* expression at certain developmental stages or leaf orders. Supporting this hypothesis, Noir et al. (2013) reported that MeJA inhibits the mitotic cycle in the G1 phase before the S-phase transition in leaf and root cells, resulting in an increase in cell size but a decrease in cell number. Furthermore, a similar effect of JA was observed on the number of stomata; long-term MeJA treatment decreased stomatal numbers in young leaves in our experiment.

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In conclusion, this study highlights the complexity of MeJA signaling in plant defense. The expression patterns of *TGG1* and *TGG2* in *coi1-16* and *myc2,3,4* mutants suggest that multiple gene regulatory pathways are involved and that long-term MeJA treatment induces an additional pathway. Our analysis highlights the importance of the MeJA treatment method and duration of treatment in gene expression changes. Our study provides insight into the mechanisms underlying the regulation of myrosinases in response to MeJA.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

Author Contributions

M.M. and K.Y. designed the study. M.M. and A.P. performed the experiments. M.M. and K.Y. analyzed the data. M.M., K.E., E.D., and K.Y. wrote the manuscript.

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Figure legends

Figure 1. Long-term MeJA treatment increases the expression of *TGG1* and *TGG2* in wild-type rosette leaves.

a) The schemes show the experimental setup of mock and MeJA treatment. MeJA treatment was started 7 d after germination, and plants were sampled 12 d after germination. **b, c)** The relative expression levels of *TGG1* and *TGG2* (b), and the protein amount of TGG1 and TGG2 (c) in the shoots of mock or long-term MeJA treated Arabidopsis wild type (Col-0) plants. Error bars denote the standard error of three biological replications. Double asterisks denote $p < 0.01$ based on the student's *t*-test. **d)** Confocal microscopic images of the first leaves of 12-d-old transgenic plants harboring *pTGG2:Venus-2sc*. Myrosin cells (arrows) and stomata guard cells (arrowheads) are recognized with Venus fluorescence but have different shapes. **e, f)** The ratio of myrosin cell areas per leaf area in the first and second (e) or fourth (f) leaves, which is calculated from confocal images. Error bars denote the standard error of seven biological replications. *** denotes $p < 0.001$ and ns denotes no significance based on the student's *t*-test. **g)** Confocal microscopic images of the emerging first leaves of 6-d-old transgenic plants harboring *pTGG2:Venus-2sc*. MeJA treatment was started 2 d after germination. Green shows Venus fluorescence in myrosin cells, and red shows propidium iodide fluorescence in cell walls and nuclei.

Figure 2. Long-term MeJA treatment increases the expression of TGG1 and TGG2 in *coi1-16* and *myc2,3,4* mutants.

a) The schemes show the experimental setup of mock, short-term and long-term MeJA treatments. MeJA treatment was started 7 (for the long-term MeJA treatment) or 11 d (for the short-term MeJA treatment) after germination, and plants were sampled 12 d after germination. **b-e)** The relative expression levels of *TGG1* (b) and *TGG2* (c), and the protein amount of TGG1 (d) and TGG2 (e) in the shoots of mock, short- and long-term MeJA treated Arabidopsis wild type (Col-0), *coi1-16* and *myc2,3,4* mutants. Error bars denote the standard error of three biological replications. Double asterisks denote $p < 0.01$ based on the student's *t*-test.

Figure 3. Long-term MeJA treatment does not change the expression of *FAMA*.

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a) The relative expression levels of *FAMA* in the rosette leaves of 12-d-old Arabidopsis wild-type (Col-0) plants. Error bars denote the standard error of three biological replications. ns, no significance based on the student's *t*-test. **b)** Epifluorescence microscope images of the first leaves of 10-d-old transgenic plants harboring *pFAMA:GFP*. Long-term MeJA treatment was started 5 d after germination.

Figure 4. MeJA treatment does not reduce plant growth in *coi1-16* and *myc2,3,4* mutants.
a) Images of mock or long-term MeJA treated 12-d-old-plants. Arrows show root growth toward the MeJA source (asterisks). **b)** Shoot fresh weight of plants used in (a). Error bars denote the standard error of ten plants. Asterisk denotes $p < 0.05$, and ns denotes no significance based on the student's *t*-test.

Figure 5. Canonical MeJA-responsive genes do not respond in *coi1-16* and *myc2,3,4* mutants.
a, b) The relative expression levels of *VSP2* (a) and *BGLU18* (b) in the shoots of mock, short- and long-term MeJA-treated Arabidopsis wild type (Col-0), *coi1-16* and *myc2,3,4* mutants. Error bars denote the standard error of three biological replications. Double asterisks denote $p < 0.01$ based on the student's *t*-test.

Supplementary Figure 1. Airborne MeJA treatment method.
The liquid of MeJA was poured onto a small paper pad in the cap of Eppendorf tube and positioned at the center of a plate (φ = 9 cm), and plants were grown 4 cm away from the MeJA source. Distilled water was used instead of MeJA in the mock treatment.

Supplementary Figure 2. Long-term MeJA treatment enhances myrosin cell area in younger leaves but not in older leaves
a) The ratio of myrosin cell areas per leaf area in the third leaf was calculated from confocal microscope images of *pTGG2:Venus-2sc* transgenic plants. Error bars denote the standard error of seven biological replications. The MeJA treatment was conducted in the same way as in Figure 1a. **b)** The chart shows the ratio of myrosin cell areas per leaf area in the 11th leaves, but the MeJA treatment was prolonged for 19 d. Error bars denote the standard error of seven biological replications. * denotes $p < 0.05$, and ns denotes no significance based on the Student's *t*-test.

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Supplementary Figure 3. Long-term MeJA treatment does not change Venus fluorescence in myrosin cells.

a) The schemes show the experimental setup of mock and long-term MeJA treatment. The treatment was conducted in the same way as in Figure 1a; MeJA treatment was started 7 d after germination, and plants were examined 12 d after germination. **b)** The fluorescent intensity in myrosin cells was calculated from the confocal microscope images of *pTGG2:Venus-2sc* transgenic plants. Error bars denote the standard error of seven biological replications. ns denotes no significance based on the Student's *t*-test.

Supplementary Figure 4. Long-term MeJA treatment reduces stomatal density in younger leaves.

a) The schemes show the experimental setup of mock and long-term MeJA treatment. The treatment was conducted in the same way as in Figure 1a; MeJA treatment was started 7 d after germination, and plants were examined 12 d after germination. **b)** Stomatal density was calculated from the confocal microscope images of *pTGG2:Venus-2sc* transgenic plants. Error bars denote the standard error of seven biological replications. ** denotes $p < 0.01$, and ns denotes no significance based on the Student's *t*-test.

Supplementary Table 1. List of primers used in this study.

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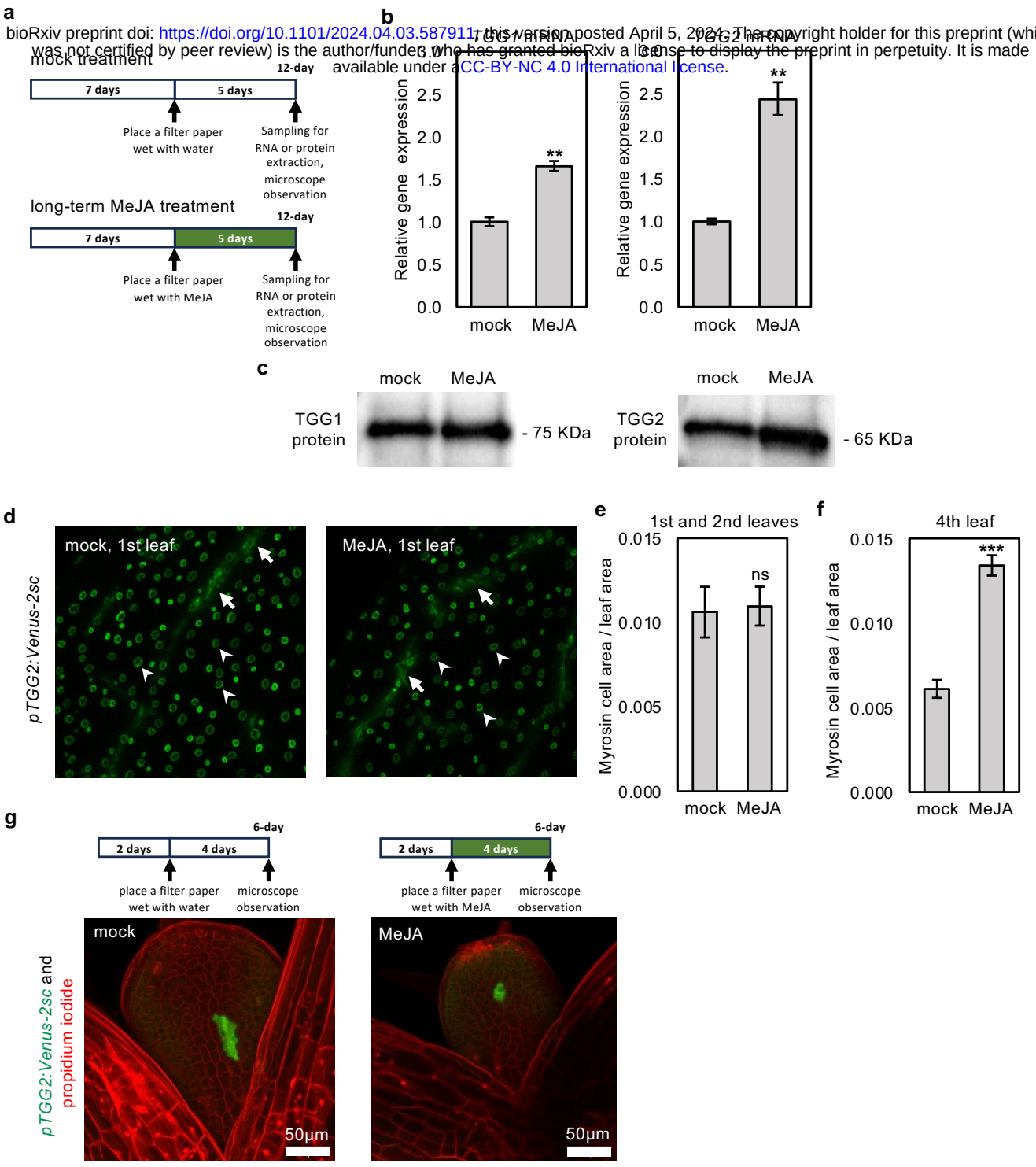

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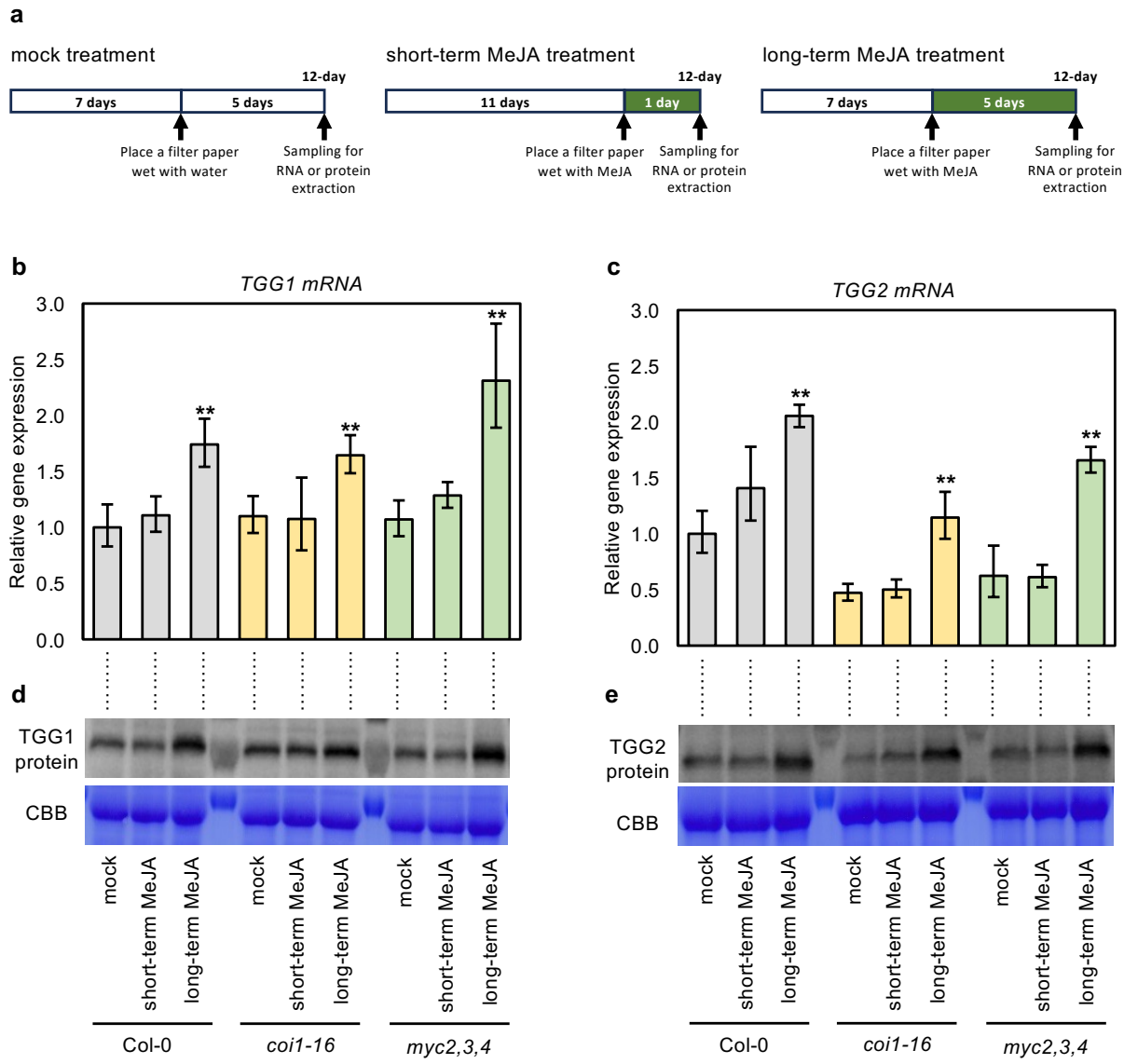

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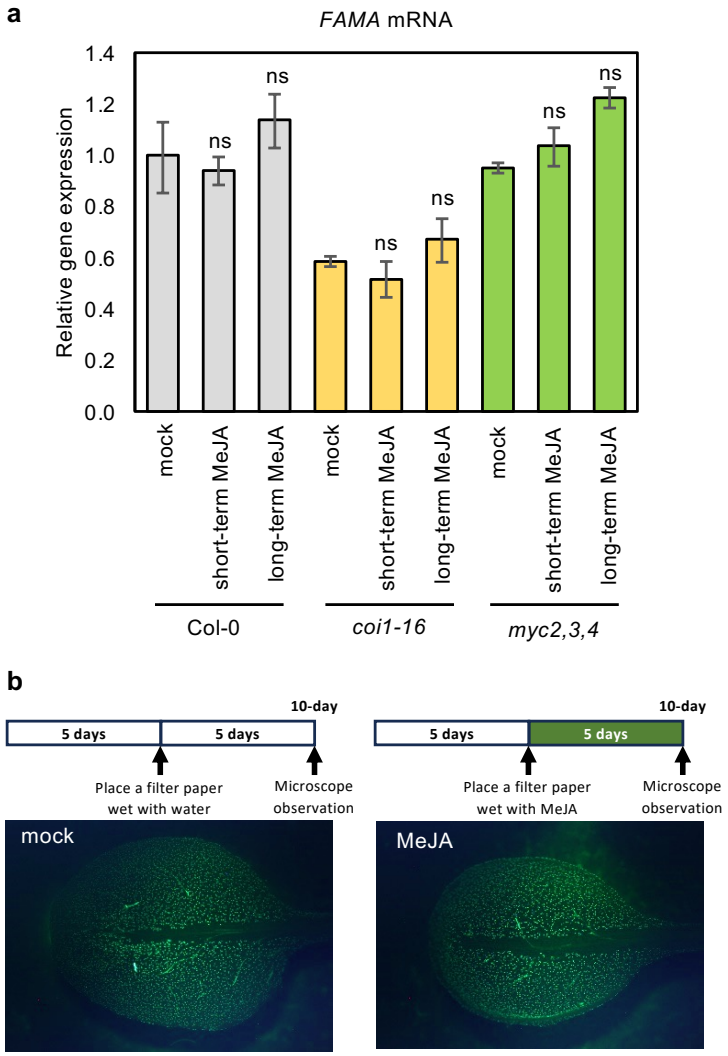

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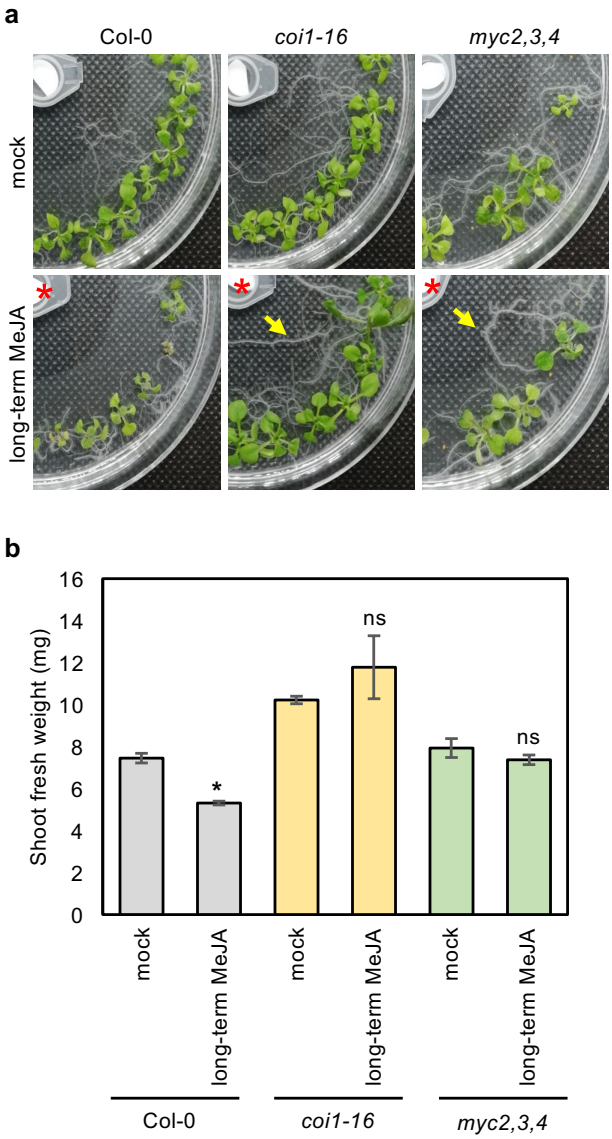

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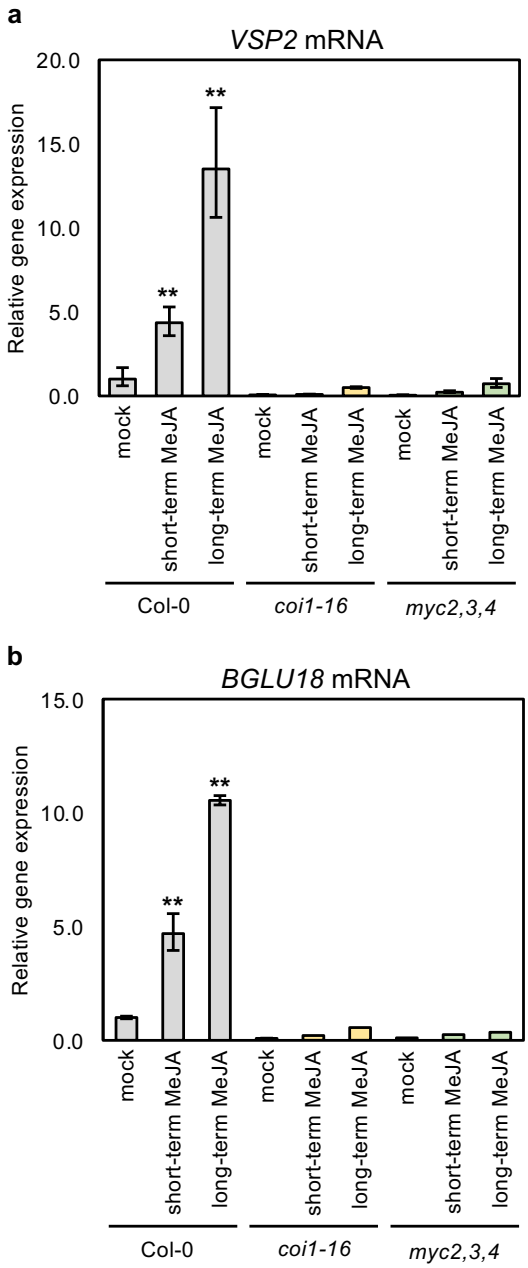

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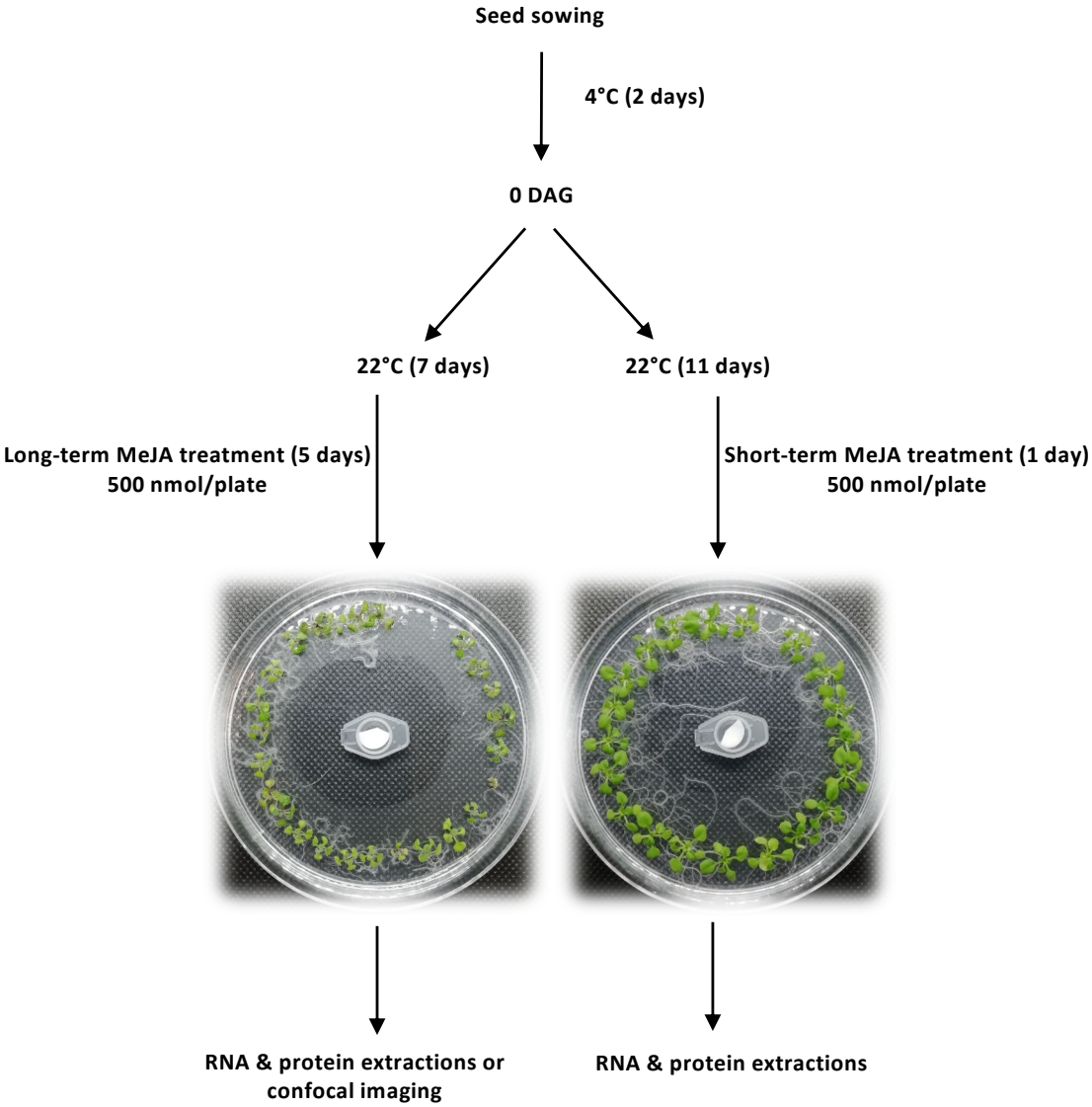

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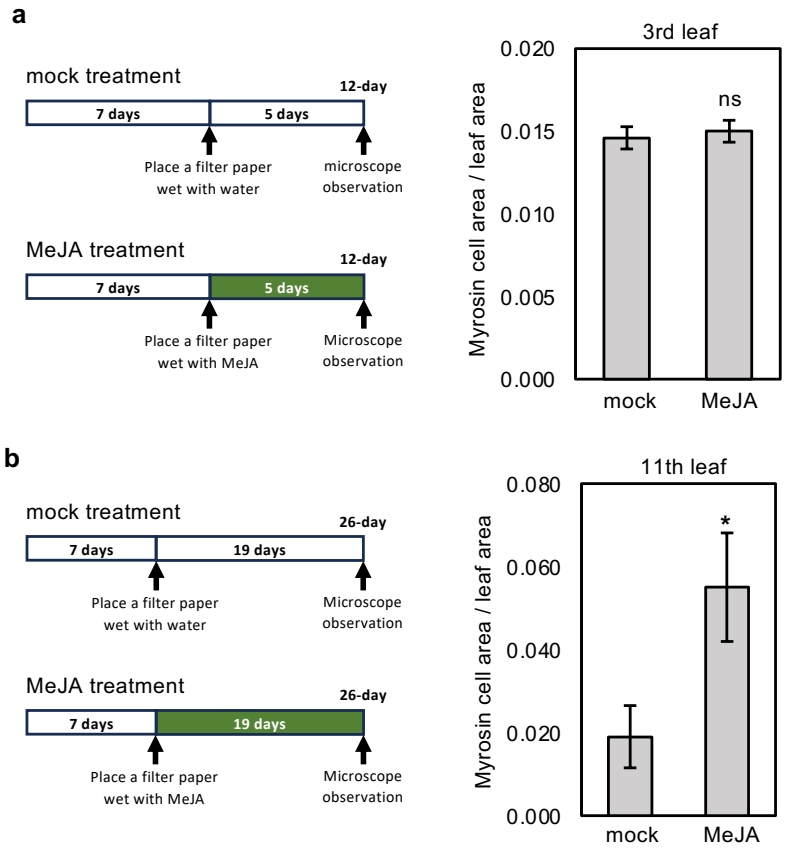

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a) The ratio of myrosin cell areas per leaf area in the third leaf is calculated from confocal microscope images of *pTGG2:Venus-2sc* transgenic plants. Error bars denote the standard error of seven biological replications. The MeJA treatment was conducted in the same way as in Figure 1a. **b)** The chart shows the ratio of myrosin cell areas per leaf area in the 11th leaves, but the MeJA treatment was prolonged for 19 d. Error bars denote the standard error of seven biological replications. * denotes $p < 0.05$, and ns denotes no significance based on the Student's *t*-test.

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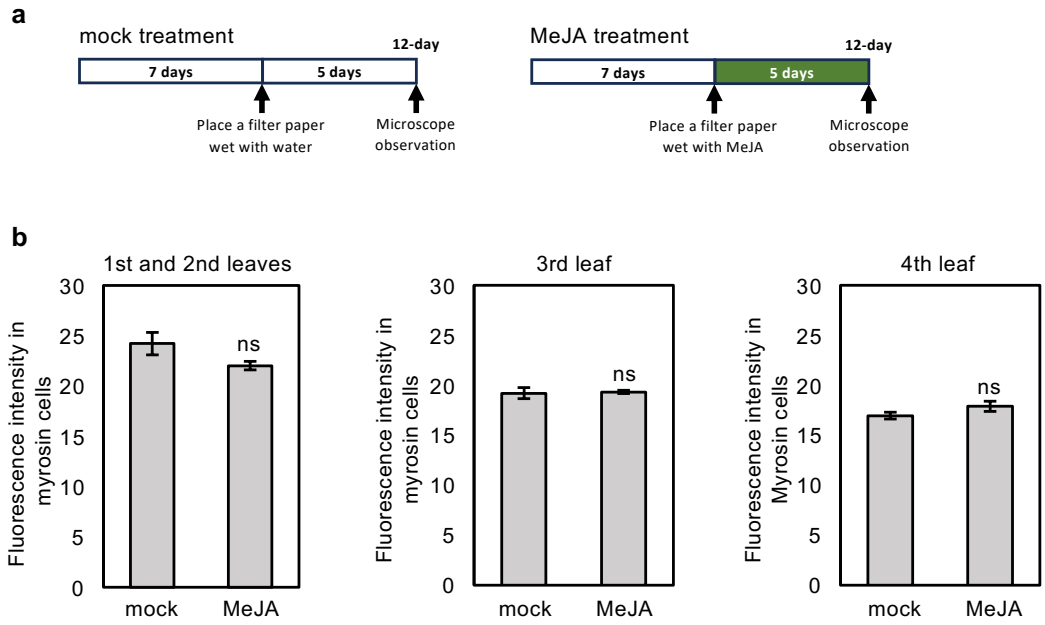

Supplementary Figure 3. Long-term MeJA treatment does not change Venus fluorescence in myrosin cells.

a) The schemes show the experimental setup of mock and long-term MeJA treatment. The treatment was conducted in the same way as in Figure 1a; MeJA treatment was started 7 d after germination, and plants were examined 12 d after germination. **b)** The fluorescent intensity in myrosin cells was calculated from the confocal microscope images of *pTGG2:Venus-2sc* transgenic plants. Error bars denote the standard error of seven biological replications. ns denotes no significance based on the Student's *t*-test.

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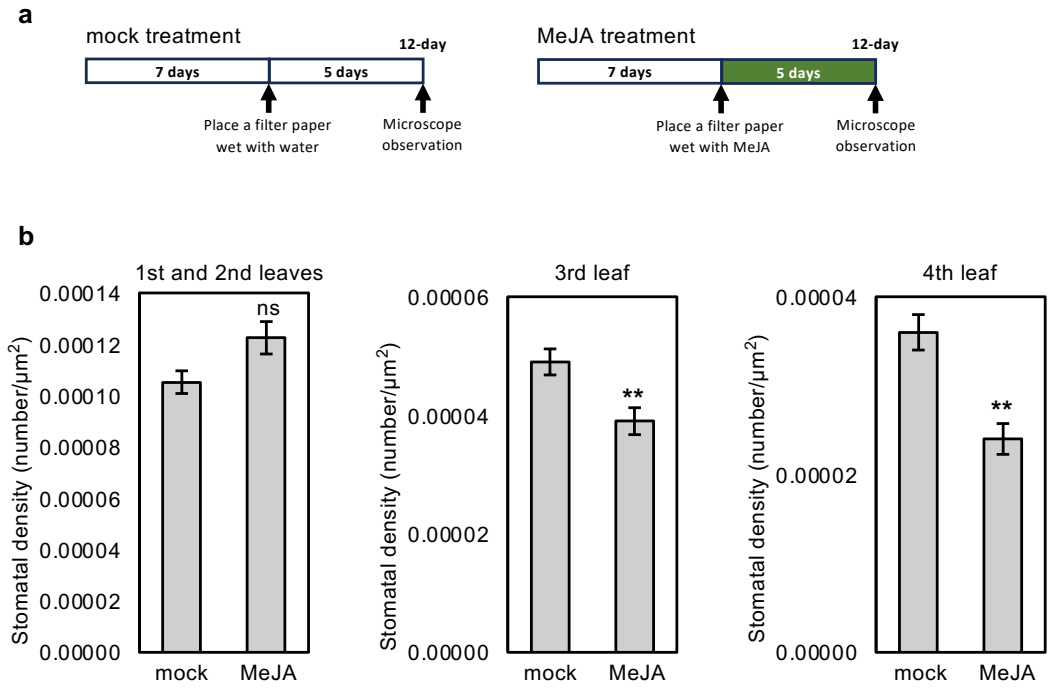

Supplementary Figure 4. Long-term MeJA treatment reduces stomatal density in younger leaves.

a) The schemes show the experimental setup of mock and long-term MeJA treatment. The treatment was conducted in the same way as in Figure 1a; MeJA treatment was started 7 d after germination, and plants were examined 12 d after germination. **b)** Stomatal density was calculated from the confocal microscope images of *pTGG2:Venus-2sc* transgenic plants. Error bars denote the standard error of seven biological replications. ** denotes $p < 0.01$, and ns denotes no significance based on the Student's *t*-test.

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Supplementary Table 1. List of primers used in this study

Name	Sequence (5' to 3')
qPCR_TGG1-FP	GCAGCGGCCGTTGATGTTTA
qPCR_TGG1-RP	TAGCCCGCTCAGTTGCATCT
qPCR_TGG2-FP	TGGAGCCGCTAACAAAGGGT
qPCR_TGG2-RP	AGGCGAGCTTCTGTGCTGTT
qPCR_FAMA-FP	ACTACGGTAGCGAACCAAGC
qPCR_FAMA-RP	CGACTTGTTCTCCGCAGTCT
qPCR_BGLU18-FP	TGAGTGGCAAGATGGGTACA
qPCR_BGLU18-RP	TCAGCTTGGAGGTTGGAAAC
qPCR_VSP2-FP	CGTCGATTCGAAAACCATCT
qPCR_VSP2-RP	GGCACCGTGTCGAAGTCTAT
UBQ10-Q2-F	GAAGTGGAAAGCTCCGACAC
UBQ10-Q2-R	TTAGAAACCACCACGAAGACG