Jasmonate Primes Plant Responses to Extracellular ATP through Purinoceptor P2K1

Jeremy B. Jewell[a,c], Ashleigh Carlton[b], Laura E. Bartley[a], Kiwamu Tanaka[c]*
[a] Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164
[b] School of Molecular Biosciences, Washington State University, Pullman, Washington 99164
[c] Department of Plant Pathology, Washington State University, Pullman, Washington 99164
*Correspondence: kiwamu.tanaka@wsu.edu

ABSTRACT

Extracellular ATP (eATP) signaling in *Arabidopsis thaliana* is mediated by the purinoceptor P2K1. Previous studies have clarified that the downstream transcriptional responses to eATP involve jasmonate (JA)-based signaling components such as the JA receptor (COI1) and JA-responsive bHLH transcription factors (MYCs). However, the specific contributions of JA signaling itself on eATP signaling are unexplored. Here, we report that JA primes plant responses to eATP through P2K1. Our findings show that JA treatment significantly upregulates P2K1 transcription, corroborating our observation that JA facilitates eATP-induced cytosolic calcium elevation and transcriptional reprogramming in a JA signaling-dependent manner. Additionally, we find that salicylic acid represses eATP-induced plant response. These results suggest that JA accumulation during biotic or abiotic stresses may potentiate eATP signaling, enabling plants to better cope with subsequent stress events.

INTRODUCTION

ATP is present in high concentrations within the cell (~mM) but is typically found at much low levels (~nM) in the extracellular space, where it functions as a potent signaling molecule essential for cellular communication **(Watt et al 1998, Weerasinghe et al 2000)**. This purinergic signaling is well-documented across eukaryotic systems **(Burnstock and Verkhratsky 2009)**. In animals, extracellular ATP (eATP) has been extensively studied since the 1970s for its roles in neurotransmission, cellular physiology, and pathophysiology **(Burnstock 2012)**. More recently, purinergic signaling in plants has gained considerable attention, particularly following the identification of the first plant purinoceptor, the receptor kinase P2K1. This receptor was discovered through Arabidopsis *dorn1* mutants that do not respond to exogenous ATP

treatment with the typical increase in cytosolic calcium levels ($[Ca^{2+}]_{cyt}$)—a characteristic of purinergic signaling **(Choi et al. 2014).**

An emerging theme of these studies is the regulation of plant growth and defense mechanisms by P2K1 and eATP, especially under biotic and abiotic stresses **(Chen et al. 2017, Kumar er al. 2020, Jewell et al. 2022, Myers et al. 2022, Kim et al. 2023a, Sowders et al. 2023, Sowders et al. 2024)**. Thus, eATP is recognized as a damage-associated molecular patterns (DAMP) in plants, triggering defense responses upon cellular injury (**Tanaka et al. 2014; Tanaka and Heil 2021**).

Transcriptional responses to eATP show remarkable similarity to those induced by wounding or treatment with the plant hormone jasmonic acid (JA) **(Choi et al. 2014; Jewell et al. 2019)**, an essential regulator of plant responses to various stresses **(Zhang et al. 2008, Browse 2009, Howe et al. 2018, Pascual et al. 2023)**. Similarly, eATP is also implicated in systemic responses to leaf wounding **(Myers et al. 2022)**, a process largely mediated by JA signaling **(Reymond et al. 2000, Devoto et al. 2005).** Notably, molecular components of the JA signaling pathway, including the receptor COI1 and bHLH transcription factors MYC2, MYC3, and MYC4, are required for eATP-induced transcriptional responses, indicating a significant interaction between eATP and JA signaling pathways **(Jewell et al. 2019)**.

While JA signaling components are essential for eATP responses, the influence of JA on eATP-mediated processes remains unexplored. Since eATP signaling depends on JA pathway components, understanding how JA affects eATP-induced responses is critical. Here, we investigate this relationship by examining how JA modulates eATP responses in *Arabidopsis thaliana.* Our findings indicate that JA primes the plant for eATP signaling by upregulating the *P2K1* receptor gene, suggesting a functional interplay in which JA signaling enhances plant sensitivity to eATP.

RESULTS

JAZ1 protein stability is not involved in eATP signaling

It has been demonstrated that eATP acts through JA signaling, as many eATP-responsive genes require the JA receptor COI1 for their expression **(Jewell et al., 2019)**. To investigate whether JA-signaling-dependent eATP responses also involve JAZ proteins—COI1's co-receptors—we tested the effect of eATP on JAZ protein stability. To this end, Arabidopsis

plants constitutively expressing the JAZ1-β-GLUCURONIDASE (JAZ1-GUS) fusion protein **(Thines et al. 2007)** with 2 µM methyl jasmonate (MeJA) or 1000 µM ATP for 30 minutes and JAZ1-GUS protein levels were quantified using a biochemical GUS activity assay **(Figure 1)**. In the three independent trials (not statistically different; *P*=0.13, F-test), MeJA treatment reduced JAZ1-GUS levels by approximately 48% (*P*<1e-7, Tukey HSD), while ATP treatment had no detectable effects (*P*=0.99, Tukey HSD). Similarly, ATP treatment did not affect JAZ1-GUS stability in the *aos* JA biosynthesis mutant (*P*=0.97, Tukey HSD), whereas MeJA still reduced GUS levels by about 49% (*P*=5.3e-3, Tukey HSD; **Supplemental Figure S1**). These findings suggest that while eATP signaling depends on COI1, it is independent of JAZ protein stability, indicating a distinct mechanism of JA-signaling-mediated eATP response.

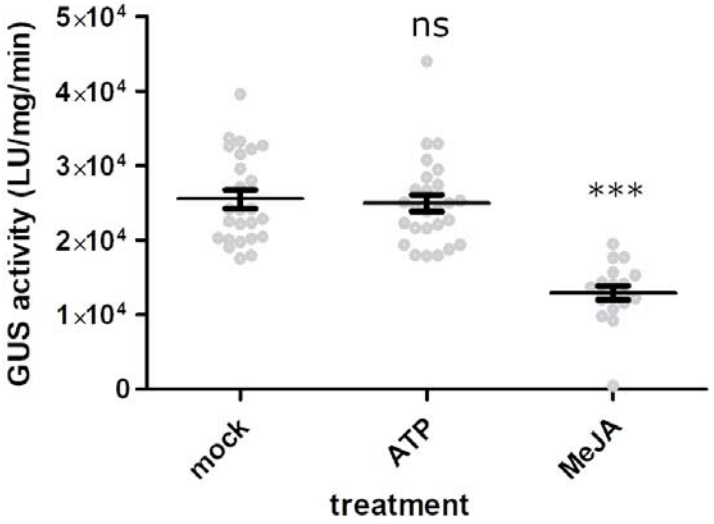

Figure 1. Exogenous ATP treatment does not reduce JAZ1 protein stability in *Arabidopsis* seedlings, while methyl jasmonate does. WT seedlings expressing JAZ1-GUS were treated with 2 µM MeJA or 1 mM ATP for 30 minutes and processed for quantitative GUS activity measurements to assess the JAZ1 protein stability, as described in Methods. Statistical comparisons to mock-treated seedlings are indicated by *** and ns for $P < 0.001$ and $P > 0.99$, respectively (Fisher's LSD). Error bars indicate SEM, with n = 19-27 replicates per treatment across 3 trials.

JA pre-treatment enhances eATP-induced cytosolic calcium response through a P2K1-dependent pathway

Given that eATP signaling integrates with JA pathways, we next explored whether JA itself modulates plant responses to eATP. To test this, we pre-treated transgenic Arabidopsis seedling expressing the calcium-sensitive AEQUORIN protein (hereafter AEQ seedlings)

overnight with 50 µM MeJA or a mock solution before treating them with 100 µM exogenous ATP and recorded luminescence as an indication of cytosolic calcium levels ($[Ca^{2+}]_{cyt}$). Without MeJA pre-treatment, ATP induced the typically transient $[Ca^{2+}]_{cyt}$ response **(Tanaka et al. 2010)**, peaking around ~30-45 seconds and tapering reduction thereafter **(Figure 2a)**. However, MeJA pre-treatment resulted in a notably earlier and enhanced responses to eATP, approximately 50% higher than in mock-treated samples over the course of 2 minutes **(Figure 2)**. In constant, AEQ seedling in the *dorn1-3* knockout mutant background (lacking functional P2K1) did not show a $[Ca^{2+}]_{cyt}$ response to ATP, regardless of MeJA pre-treatment, confirming that the JA-enhanced plant response to ATP is P2K1-dependent.

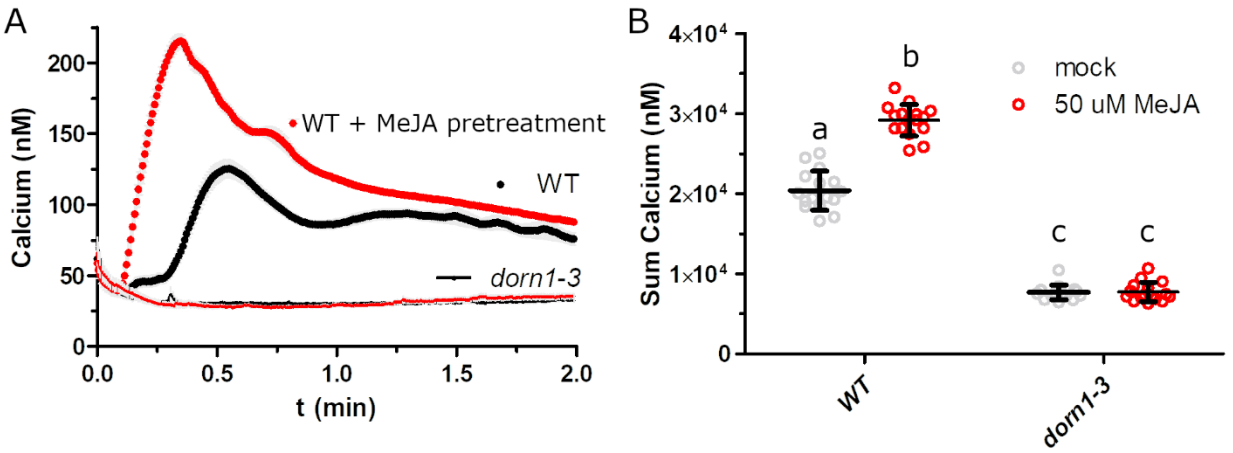

Figure 2. JA pre-treatment increases ATP-induced cytosolic calcium response. A, Seven-day-old aequorin-expressing seedlings were incubated overnight in reconstitution buffer and treated with (red symbols) or without (black symbols) 50 µM MeJA, followed by treatment with 100 µM ATP. Aequorin luminescence was recorded for 2 minutes to assess the cytosolic calcium levels (mean ± SEM, n = 16 seedlings per treatment). **B,** Summed calcium values from panel A. Different letters indicate statistically significant differences (P < 0.05, Tukey HSD). Mean, individual values, and SEM are shown, with n = 16 seedlings per treatment.

Next, we asked whether shorter MeJA pre-treatment durations could also enhance the ATP-induced $[Ca^{2+}]_{cyt}$ response. Replacing the medium with MeJA-containing solution at 18, 6, or 3 hours prior to ATP treatment revealed that even the shortest duration (3 hours) reliably increased the ATP-induced $[Ca^{2+}]_{cyt}$ response, with longer pre-treatment durations showing progressively larger effects **(Figure 3a,b**). Testing MeJA concentrations from 6.25 to 100 µM revealed a dose-dependent enhancement, where even the lowest concentration (6.25 µM) significantly increased ATP-induced $[Ca^{2+}]_{cyt}$ levels, with higher MeJA doses resulting in greater responses **(Figure 3c,d)**. One hour after wounding, Arabidopsis leaves contain JA at about 1-3

nanomoles per gram of fresh weight, or roughly micromolar concentration **(Kimberlin et al. 2022)**. Thus, these MeJA treatment dosages and durations are of the same magnitude of physiological JA levels after wounding, suggesting that JA priming may naturally augment eATP responses in wounded plants.

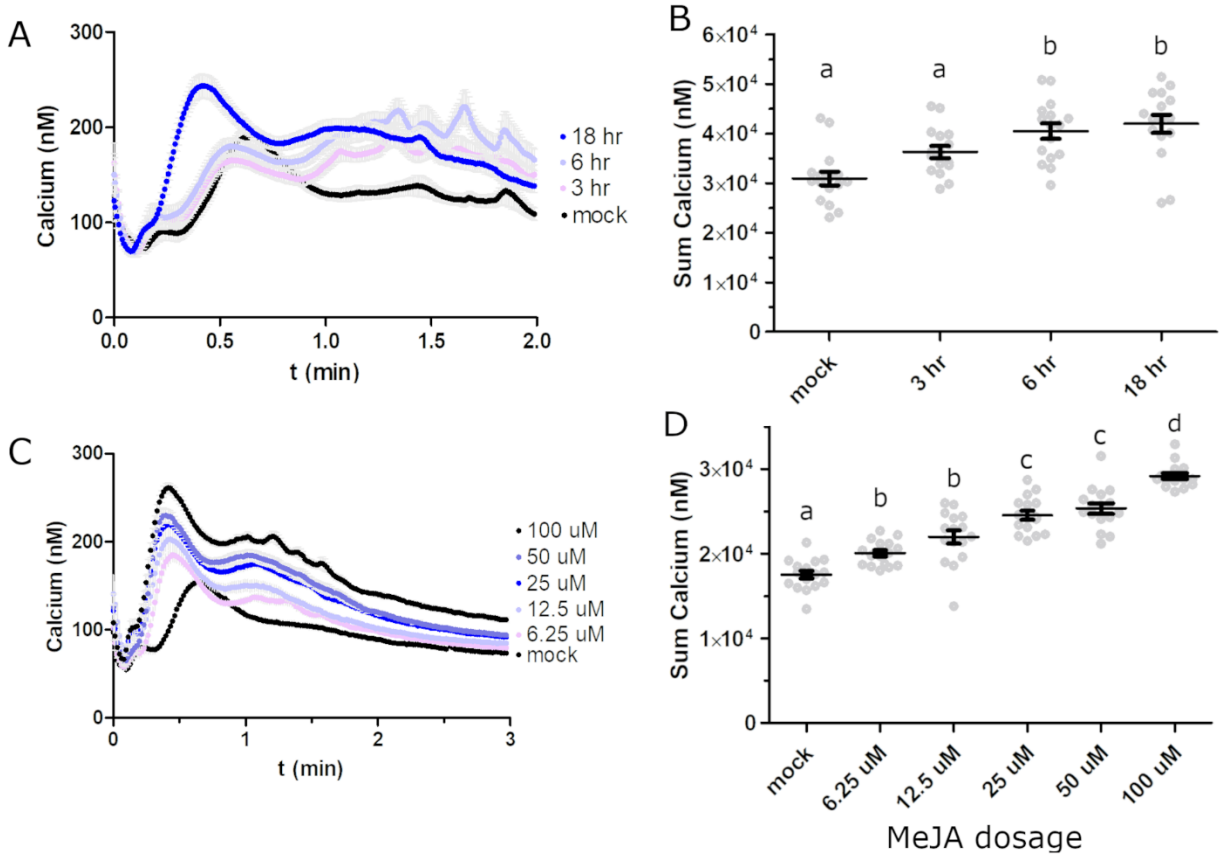

Figure 3. Effects of JA pre-treatment dosage and duration on ATP-induced calcium response. A, Seedlings were treated as in Figure 1, with MeJA added to the reconstitution buffer 50 µM for 3, 6, or 18 hours, followed by recording of ATP-induced aequorin luminescence. **B,** Summed calcium values from panel A. **C,** Seedlings were treated as in Figure 1, with the reconstitution buffer containing varying MeJA concentrations. **D,** Summed calcium values from panel C. All graphs show mean ± SEM (n = 16), with statistically significant differences indicated by different letters ($P < 0.05$, Tukey HSD).

Differential effects of plant stress hormones on ATP-Induced cytosolic calcium response: enhancement by JA and suppression by SA

To examine whether other plant stress hormones impact eATP-induced $[Ca^{2+}]_{cyt}$ responses, we pre-treated AEQ seedlings overnight with either 50 µM MeJA, 300 µM salicylic acid (SA), 50 µM of the ethylene precursor aminocyclopropane-carboxylic acid (ACC), or 50 µM

abscisic acid (ABA) prior to the ATP treatment. Consistent with previous results, MeJA pre-treatment significantly increased the ATP-induced $[Ca^{2+}]_{cyt}$ response by approximately 50% **(Figure 4)**. In contrast, the SA pre-treatment suppressed the ATP-induced $[Ca^{2+}]_{cyt}$ response by ~40% relative to mock-treated controls, while neither ABA nor ACC pre-treatment had a significant effect **(Figure 4)**. These findings suggest a unique role for JA in priming eATP signaling, while SA may act antagonistically modulating ATP-induced plant response in a distinct manner.

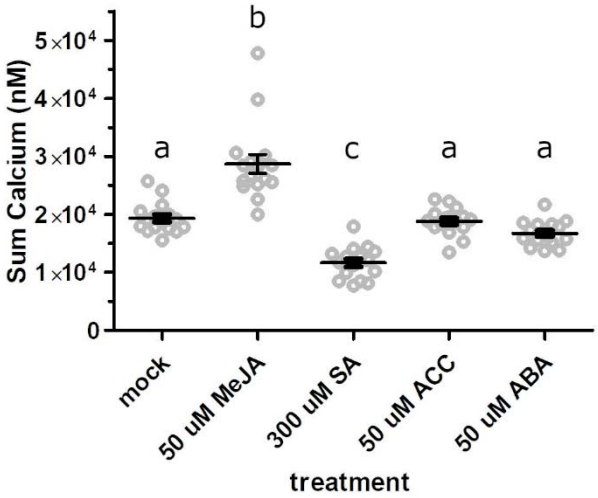

Figure 4. SA reduces ATP-induced calcium response, while JA increases it, but ABA and ACC have no effect. Aequorin-expressing seedlings were treated as in Figure 1, with overnight pre-treatment in reconstitution buffer containing 50 µM MeJA, 300 µM SA, 50 µM ACC, or 50 µM ABA, followed by recording ATP-induced luminescence. Data indicates summed calcium values over 2 minutes (mean ± SEM, n = 16 seedlings per treatment). Different letters indicate statistically significant differences ($P < 0.05$, Tukey HSD).

COI1 is essential for JA-primed eATP-induced cytosolic calcium response

Previous research on the interaction of JA and eATP signaling has primarily focused on transcriptional responses, without examining calcium signaling **(Jewell et al. 2019)**. To examine whether the ATP-induced $[Ca^{2+}]_{cyt}$ response relies on JA signaling, we crossed AEQ-expressing plants with *coi1-1* mutants to generate F3 lines segregating for the *coi1-1* mutant allele while maintaining AEQ expression. This segregating progeny approach is necessary given that homozygous *coi1* null mutants are male sterile **(Xie et al. 1998)**. AEQ seeds with *COI1-1*$^{+/-}$ segregants were additionally exposed to 50 µM MeJA to differentiate *coi1-1* mutants from those with a WT *COI1* allele. After 7 days, these 3 genotypes were incubated overnight in

coelenterazine reconstitution buffer with (all 3 genotypes) or without (WT) 50 µM MeJA and subsequently treated with 100 µM ATP. Consistent with our hypothesis, WT and *COI1*$^{+/-}$ seedlings exhibited a robust ATP-induced $[Ca^{2+}]_{cyt}$ response following MeJA treatment **(Figure 5)**. In contrast, the *coi1-1* mutants showed a significantly reduced ATP-induced $[Ca^{2+}]_{cyt}$ response, even after MeJA priming. These results indicate that COI1 is essential both for MeJA-induced priming of eATP-induced calcium response and for basal WT-like responses to eATP.

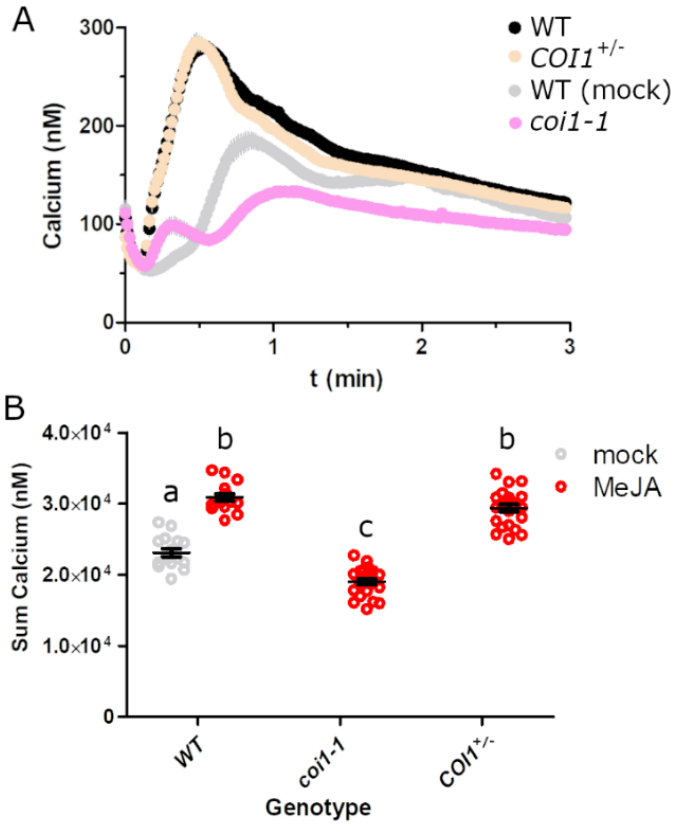

Figure 5. COI1 is required for both basal and MeJA-stimulated ATP-induced calcium response. A, Seedlings of the indicated genotypes were treated overnight with 50 µM MeJA and, ATP-induced luminescence was recorded (mean ± SEM, *n* = 16-24). **B,** Summed calcium values from panel A. Different letters indicate statistically significant differences ($P < 0.05$, Tukey HSD).

P2K1 gene expression is induced by JA treatment in a COI1-dependent manner

The eATP receptor P2K1 is known to be upregulated in response to wounding, with expression rising 2- to 3-fold in adult Arabidopsis within 1 hour and persisting for up to 24 hours **(Kilian et al. 2007). Similarly**, treatment with 10 µM MeJA induces a ~5-fold increase in *P2K1* transcript in seedlings within an hour **(Supplemental Figure S2a, Goda et al. 2008**;

https://bar.utoronto.ca/efp/), and the bacterial JA-Ile mimic coronatine has been shown to elevate P2K1 expression in mature plants over 24 hours **(Attaran et al. 2014)**. Additionally, the JA-responsive transcription factors MYC2 and MYC3 are known to bind to the presumptive *P2K1* promoter region **(Supplemental Figure S2b, Zander et al. 2020; http://neomorph.salk.edu/MYC2)**, suggesting that COI1 may be required for JA-induced *P2K1* gene expression. To test this idea, we grew WT plants, *COI1*$^{+/-}$ segregants, and the *coi1-1* mutants for four weeks, treated leaves 50 µM MeJA, and then quantified *P2K1* gene expression levels using RT-qPCR. As shown in **Figure 6**, MeJA treatment significantly increased *P2K1* expression by ~6-fold in both WT and *COI1*$^{+/-}$ plants, whereas no increase was observed in the *coi1-1* mutants. This confirms that COI1 is required for *P2K1* induction in response to JA.

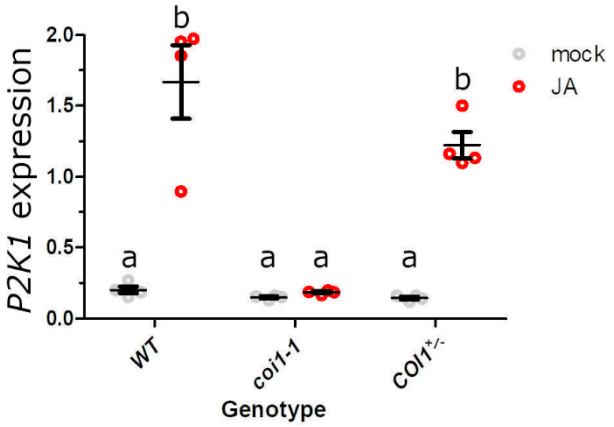

Figure 6. JA induces *P2K1* gene expression in a COI1-dependent manner. *P2K1* expression was measured in 4-week-old aequorin expressing seedlings of the indicated genotypes either 1 hour after a 50 µM MeJA spray or without MeJA treatment. Gene expression was normalized by *PP2A.* Mean, SEM, and individual values are shown (n = 4 leaves per treatment). Different letters indicate statistically significant differences ($P < 0.05$, Tukey HSD).

JA primes eATP-responsive gene expression

To further evaluate the physiological impact of JA priming on ATP responses, we tested whether MeJA pre-treatment enhances ATP-responsive gene expression. Given that many ATP-responsive genes also respond to JA **(Choi et al. 2014, Jewell et al. 2019),** we treated plants with MeJA, then replaced the media to allow a 24 hour recovery period, thereby enabling JA-responsive genes to return to basal levels prior to the ATP treatment (see Materials and Methods). We selected eight genes with previously reported subtle ATP-induced expression changes, reasoning that an exaggeration of this marginal induction could be easier to detect.

Across these genes, there were significant effects due to MeJA, ATP, and their interaction (*P*<2e-16, *P*=1.1e-10, and *P*=2.6e-3, respectively, F-test). Of these, six genes showed significant MeJA priming of ATP responsiveness (MeJA by ATP interaction, P<0.05, F-test): *LIPOXYGENASE 3* (*LOX3;* **Figure 7a**), *CALCIUM-DEPENDENT PROTEIN KINASE 28* (*CPK28*, **Figure 7b),** *RESPIRATORY BURST OXIDASE HOMOLOGUE D* (*RBOHD*, **Figure 7c)**, *P2K1* (**Figure 7d),** *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*, **Supplemental Figure S3a)**, and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 6* (*ACS6*, **Supplemental Figure S3b).** The remaining two genes, *RESPONSIVE TO DESSICATION 20* (*RD20,* **Supplemental Figure S3c)** and *MITOGEN-ACTIVATED PROTEIN KINASE 3* (*MPK3*, **Supplemental Figure S3d)**, did not show statistically significant MeJA priming. These findings suggest that JA priming enhances transcriptional responses to eATP, particularly for genes involved in stress and signaling pathways.

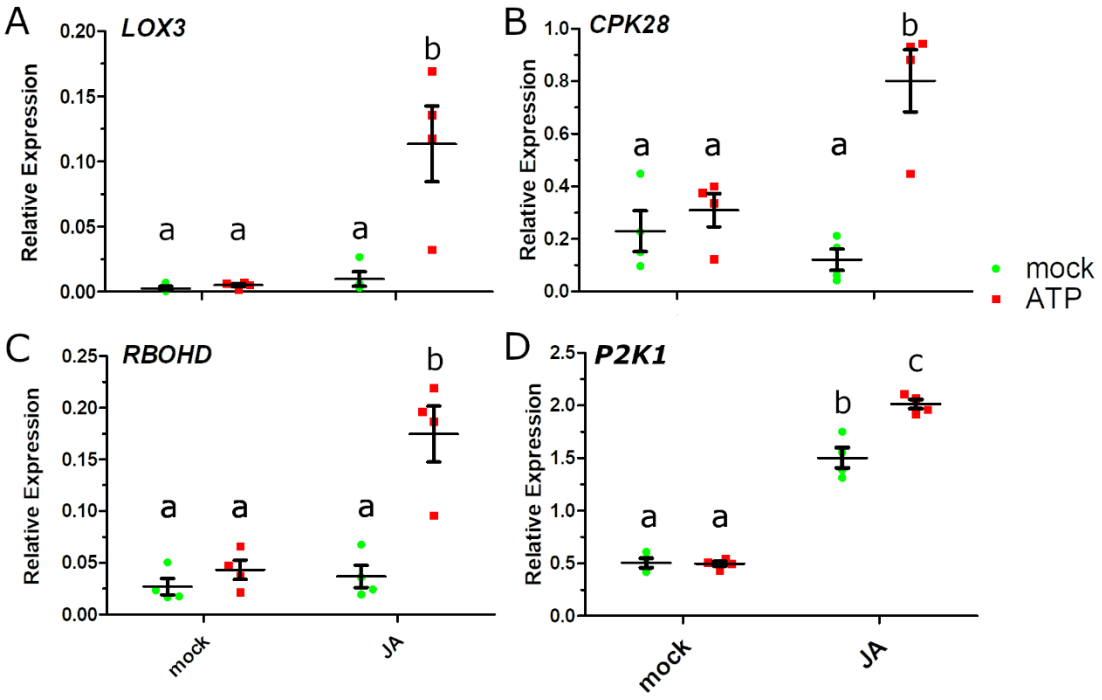

Figure 7. JA treatment potentiates eATP-induced transcriptional responses. Wild-type seedlings were pre-treated with or without 20 µM MeJA, followed by treatment with 0.5 mM ATP, as described in Materials and Methods. Gene expression was evaluated by RT-qPCR for **A,** *LOX3*, **B,** *CPK28,* **C,** *RBOHD,* and **D,** *P2K1*. Mean ± SEM and individual values are shown, with different letters indicating statistically significant differences in gene expression (n = 4, P < 0.05, Tukey HSD).

DISCUSSION

In this study, we report that JA potentiates Arabidopsis responses to eATP through mechanisms dependent on the JA receptor COI1 and the eATP receptor P2K1. Our findings further indicate that JA treatment induces *P2K1* gene expression in a COI1-dependent manner, suggesting that JA-primed eATP response acts by transcriptional upregulation of *P2K1*. Supporting this, published ChIP-seq data **(Zander et al. 2020)** demonstrate specific binding of MYC2 and MYC3 transcription factors to the *P2K1* promoter, suggesting that JA induction of *P2K1* relies on the canonical JA signaling pathway involving bHLH MYC transcription factors. Importantly, the priming effect was observed even at low JA concentrations, consistent with endogenous JA levels after wounding **(Kimberlin et al. 2022)**, hinting at the physiological relevance of this JA-eATP signaling interaction.

Since the initial report of ATP-induced degradation of JAZ1 in 2018 **(Tripathi et al.)**, no studies have corroborated or explained this phenomenon. While JAZ1 degradation is not central to our conclusions, its irreproducibility has implications for understanding the full mechanism by which eATP activates JA-responsive genes. It remains possible that other JAZ proteins could play a role in eATP-induced activation of JA-responsive genes. For example, JAZ8, which lacks a canonical degradation mechanism within JA signaling (**Shyu et al. 2012**) regulates both JA-responsive and JA biosynthesis genes in response to stress-induced $[Ca^{2+}]_{cyt}$ elevations (**Yan et al. 2018)**, a hallmark of eATP response. Despite the uncertainty surrounding JAZs, activation of JA-responsive genes in this pathway clearly relies on the P2K1 receptor and the MYC and CAMTA3 transcription factors **(Jewell et al. 2019)**, with upstream signaling involving the P2K1-interacting and -phosphorylated INTEGRIN-LINKED KINASE 5 (ILK5, **Kim et al, 2023b**). Recently, it was suggested that phosphorylation of ZmMYC2 transcription factors by the maize mitogen-activated protein kinase (MAPK) ZmMPK4 enhances transcriptional activation activity of ZmMYC2s **(Li et al, 2024)**. Given that MPK phosphorylation is a characteristic feature of eATP responses **(Choi et al, 2014; Cho et al, 2022)** and is reduced in *ilk5* Arabidopsis mutants **(Kim et al, 2023b)**, these cross-species comparisons suggest that eATP may activate MYC2 through a MAPK cascade. Experimental evaluation of this model should be an interesting direction for future research. Collectively, these findings suggest that JA and eATP pathways amplify each other's signaling as part of a defense priming mechanisms during stress responses.

Given the roles of JA and eATP signaling in responses to biotic stresses such as pathogens and herbivores **(Browse 2009, Chivasa et al. 2009)**, it is attractive to speculate that

this interaction promotes heightened defense responses after initial injury. However, a novel role for JA signaling in wound healing and tissue regeneration has recently emerged **(Zhang et al. 2019, Zhou et al. 2019).** In addition, the wound-induced tomato elicitor peptide REF1, an orthologue of Arabidopsis PLANT ELICITOR PEPTIDEs (PEPs), dramatically increased wound-induced callus formation and shoot regeneration **(Yang et al. 2024)**, and exogenous ATP treatment induces expression of several PEP-encoding genes (*PROPEP1*, *PROPEP3*, *PROPEP4*, and *PROPEP7*) and two PEP receptor genes in a P2K1-dependent manner **(Jewell et al. 2019).** While Arabidopsis has eight PEPs, to our knowledge none have been investigated for a tissue regeneration activity similar to REF1. This raises the question: does JA potentiation of eATP response contribute not only to defense priming but also to tissue regeneration after wounding? This hypothesis opens exciting avenues for further research. Given the well-documented role of JA in pathogen defense and the more nuanced roles associated with eATP signaling, investigating the potential role of JA-eATP interaction in tissue regeneration may provide new insights into wound response mechanisms.

MATERIALS AND METHODS

Reagents

All chemicals except as otherwise indicated were obtained from Sigma-Aldrich. ATP was prepared as a 0.1 M stock solution in 50 mM MES buffer (pH 5.7) and adjusted to pH 5.7 using sodium hydroxide. Methyl jasmonate (Bedoukian Research), salicylic acid, and abscisic acid were prepared in DMSO as 0.1 M, 0.3 M and 10 mM stock solutions, respectively. 1-aminocyclopropane-1-carboxylic acid was dissolved in distilled deionized water at 0.1 M. Coelenterazine native (NanoLight Technology) was prepared as a 1 mM stock solution in 100% ethanol. All these solutions were stored at –20°C in a sealed, dark container to prevent degradation.

Calcium Assays

Calcium assays were performed as described previously **(Choi et al. 2014)**. Briefly, surface-sterilized and cold-stratified seeds were sewn to square plates containing ½-strength Murashige and Skoog media (pH 5.8) with 0.8% (w/v) sucrose, 0.8% (w/v) agar, and 0.5g/L MES pH-adjusted using potassium hydroxide. After sowing, plates were transferred to a 22°C growth chamber (Conviron) with 12-h light cycle (100-120 µmol photons m^{-2} s^{-1}) and grown

vertically for 7 days. On the morning of the 8th day, individual seedlings were carefully transferred to single wells of white 96-well plates containing 50 µL aequorin reconstitution buffer containing 10 µM coelenterazine (NanoLight Technology), 2 mM MES buffer pH 5.7, and 10 mM $CaCl_2$-heptahydrate and incubated in darkness overnight. Luminescence was monitored after adding 50 µL of 2X treatment solution via autoinjector using the GloMax Navigator (Promega). After elicitor-induced luminescence was recorded, 100 µL of discharge solution (20% ethanol and 2M $CaCl_2$-heptahydrate) was added to each well, and luminescence recorded for an additional 30 s. Luminescence data was converted to $[Ca^{2+}]_{cyt}$ using the formula of **Knight et al. 1996**.

Plant Materials

Arabidopsis (*Arabidopsis thaliana)* WT AEQ (MAQ2.4), *dorn1-3 AEQ*, *35S-JAZ1-GUS, aos 35S-JAZ1-GUS* and *coi1-1* were described previously **(Knight et al. 1991, Choi et al. 2014, Thines et al. 2007, Tripathi et al. 2018, Xie et al. 1998)**. Columbia-0 (CS70000) was obtained from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). Due to the male sterility of *coi1* null alleles, *COI1-1*$^{+-}$ *AEQ* lines were maintained as segregating for the *coi1-1* mutation but fixed for AEQ expression. Segregating lines were identified by crossing WT AEQ and *coi1-1,* selfing F2 plants and selecting F3 seedlings based on MeJA insensitivity and or 25 µg/mL kanamycin resistance. AEQ expression was verified based on luminescence in the AEQ discharge solution **(Choi et al. 2014)**.

Quantitative JAZ1-GUS Stability Assay

The quantitative GUS assay was performed as described **(Jefferson et al. 1987)**. Five to ten 10-day old seedlings of *35S-JAZ1-GUS* **(Thines et al. 2007)** or *aos 35S-JAZ1-GUS* were incubated overnight in buffer (10 mM $CaCl_2$, 2 mM MES, pH5.7). The next day, seedlings were treated with buffer alone (mock), 1 mM ATP, or 2 µM MeJA 30 minutes for in the light, then blotted dry and snap-frozen in liquid nitrogen. The seedlings were ground in 75 µL of GUS extraction buffer (GEB; 50 mM sodium phosphate pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% triton X-100, 0.1% sarkosyl) and centrifuged at 4°C for 15 minutes at 15,000 rcf to remove insoluble debris. Supernatant (65 µL) was collected to separate tubes on ice and protein quantity estimated using Bradford reagent. For each sample, equal amounts of protein (~9 µg) were added to individual wells of a white 96-well plate in a final volume of 100 µL GEB containing 1 mM 4-Methylumbelliferyl-β-D-glucuronide hydrate (MUG) prewarmed to 37°C after which plate was stored in a dark 37°C incubator. At 60 minutes, 60 µL aliquots were added

to 60 µL of 0.2 M Na_2CO_3 stop buffer in a separate white 96-well plate. Fluorescence from MU released in each well was measured in a plate reader with excitation at 365 nm and emission at 455 nm, and rate was calculated as light units/mg/min. In preliminary experiments, signal was not reliably detected at 15 minutes incubation, but activity was linear between 30 to 90 minutes.

RT-qPCR

For MeJA-primed eATP-induced gene expression experiments, Col-0 seedlings were grown as described above. On the 7th day, 20 seedlings were transferred to 6-well plates containing 2 mL of ½-strength sterile MS liquid media (without sucrose and agar) with or without 20 µM MeJA and returned to the growth chamber. The next day, the MeJA-containing liquid media was removed, and 2 mL of fresh liquid media was added to each well. Finally, on the 9th day, 2 mL of liquid media with or without 0.5 mM ATP was added, and the plates were returned to the growth chamber for 30 minutes. Thereafter, seedlings were removed from the wells, gently blotted on tissue paper, flash frozen in liquid nitrogen, and stored for the subsequent RT-qPCR experiments. All media changes were performed in a sterile laminar flow hood.

For the MeJA-induced *P2K1* gene expression studies, WT AEQ, and AEQ lines segregating for the *coi1-1* mutation were initially grown as above for 7 days, then 8 (WT) or ~60 (*COI1*$^{+/-}$) seedlings were transferred to autoclave-sterilized soil in a growth room (22°C, 12 h light, 100-120 µmol photons m^{-2} s^{-1}) and grown for an additional 3 weeks. A single leaf from each plant was excised with a scalpel and snap frozen and stored. Immediately after initial harvest, all plants were uniformly sprayed with 50 µM MeJA in 0.1% Tween-20 until they were dripping. After 1 hour, another set of single leaves were harvested, snap-frozen, and stored at -80°C. Thereafter, the same plants were sprayed twice over 2 weeks with 500 µM MeJA in 0.1% Tween-20, which induced anthocyanin accumulation in plants with a functional *COI1* allele. After flowering, sterile plants (a hallmark of *coi1* null mutants) were noted. Sterile plants not accumulating anthocyanin were recorded as *coi1-1* mutants. Leaves of four plants from each genotype (AEQ, *coi1-1,* and *COI*$^{+/-}$) were selected for subsequent RT-qPCR experiments.

Frozen plant tissue was disrupted with a bead beater (MIni-Beadbeater-96, BioSpec Products) and RNA was isolated using the Quick-RNA Miniprep Kit (Zymo Research), including the on-column DNase I treatment as per the manufacturer's instructions, which we have found is critical for reproducible RT-qPCR results with low-expressed eATP responsive genes.

After RNA isolation, 1 µg of RNA was used in a 20-µL reverse transcription reaction according to the manufacturer's instructions (iScript; Bio-Rad) followed by a 5-fold dilution with

ultrapure water. RT-qPCR was performed using 2 uL of diluted cDNA in a 20-µL reaction with SYBR Green dye polymerase mix (SsoAdvanced; Bio-Rad) in white-walled plates in a CFX96 thermocycler (Bio-Rad). Gene expression was normalized to the reference gene *PP2A* (AT1G13320) as described **(Czechowski et al. 2005, Rieu and Powers 2009)**. Primers used are listed in **Supplemental Table S1.**

Statistical Analyses

Figures were created using GraphPad Prism 5 (GraphPad Software). Statistical analyses were performed in R version 4.3.1 **(R CoreTeam, 2020)**, using ANOVA with Tukey's HSD post-hoc testing where appropriate.

ACKNOWLEDGEMENTS

Thank you to John Browse for the *35S-JAZ1-GUS* seeds, the *coi1-1* seeds, the *aos* seeds, leadership by example, and support and encouragement throughout the years. Thank you to Marc Knight for the MAQ2.4 seeds. Thank you to Nathan Havko for insightful discussion. This work was funded by the NSF (no. IOS-1557813, IOS-2048410) and USDA NIFA (Hatch project no. 1015621). Funding was also provided by the Center for Bioenergy Innovation (CBI) led by Oak Ridge National Laboratory (ORNL). CBI is funded as a DOE Bioenergy Research Center supported by the BER Program in the DOE Office of Science under FWP ERKP886. ORNL is managed by UT-Battelle, LLC, for the DOE under contract no. DE-AC05-00OR22725.

ACCESSION NUMBERS

Sequence data and gene information from this article can be found at The Arabidopsis Information Resource (https://www.arabidopsis.org/).

AUTHOR NOTES

JBJ designed experiments with input from KT. JBJ and AC performed experiments. JBJ performed statistical analyses of data. JBJ and AC wrote the paper with suggestions and approval of KT and LB.

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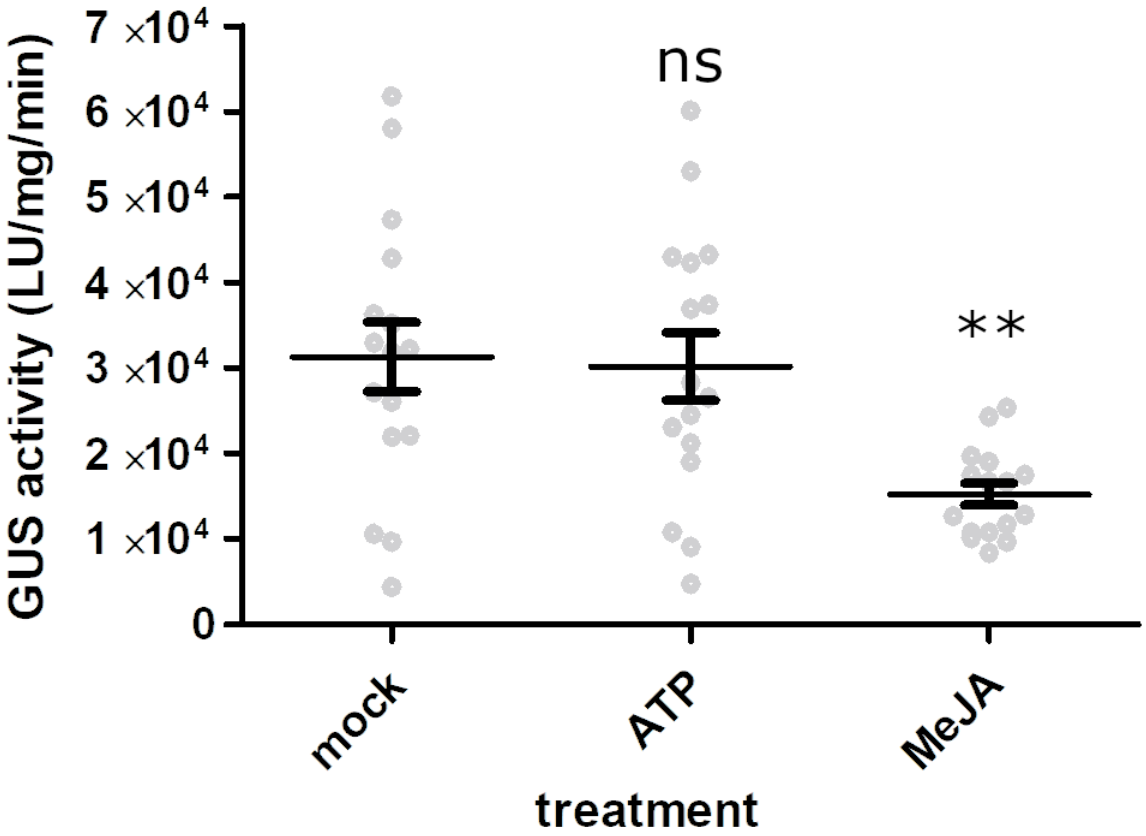
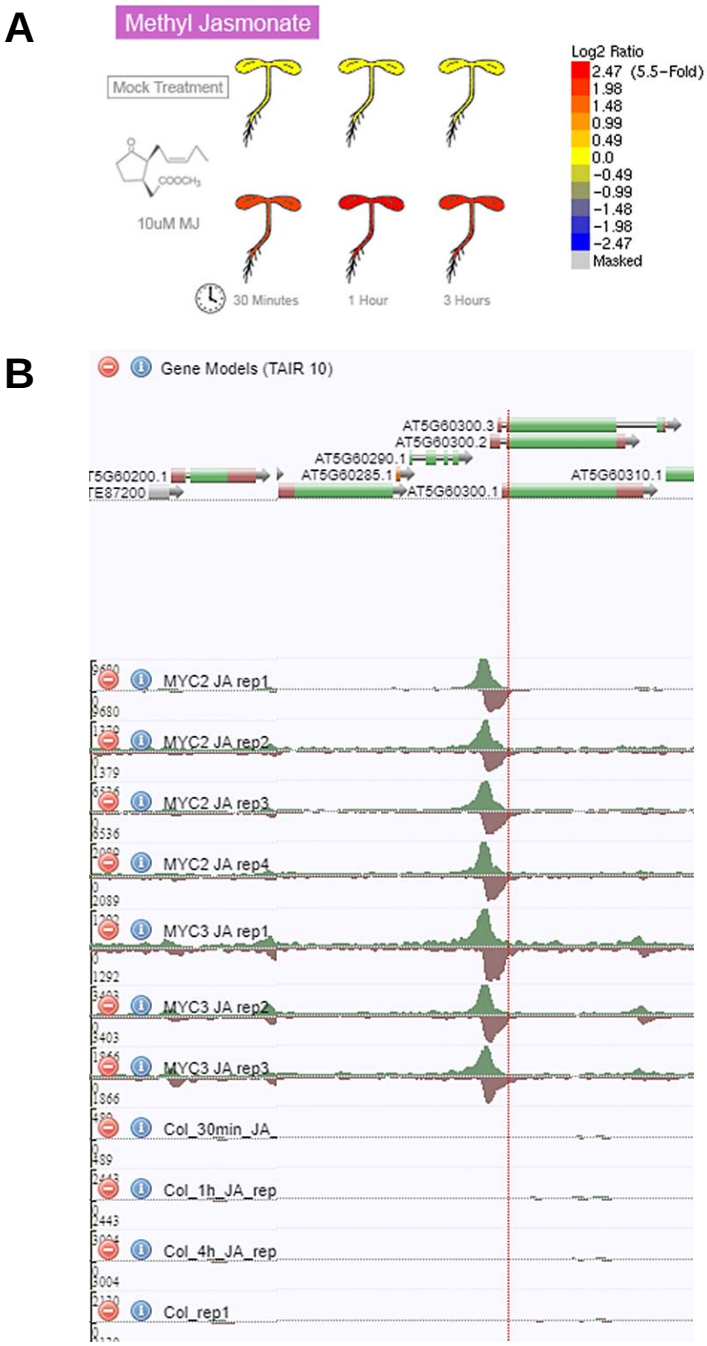
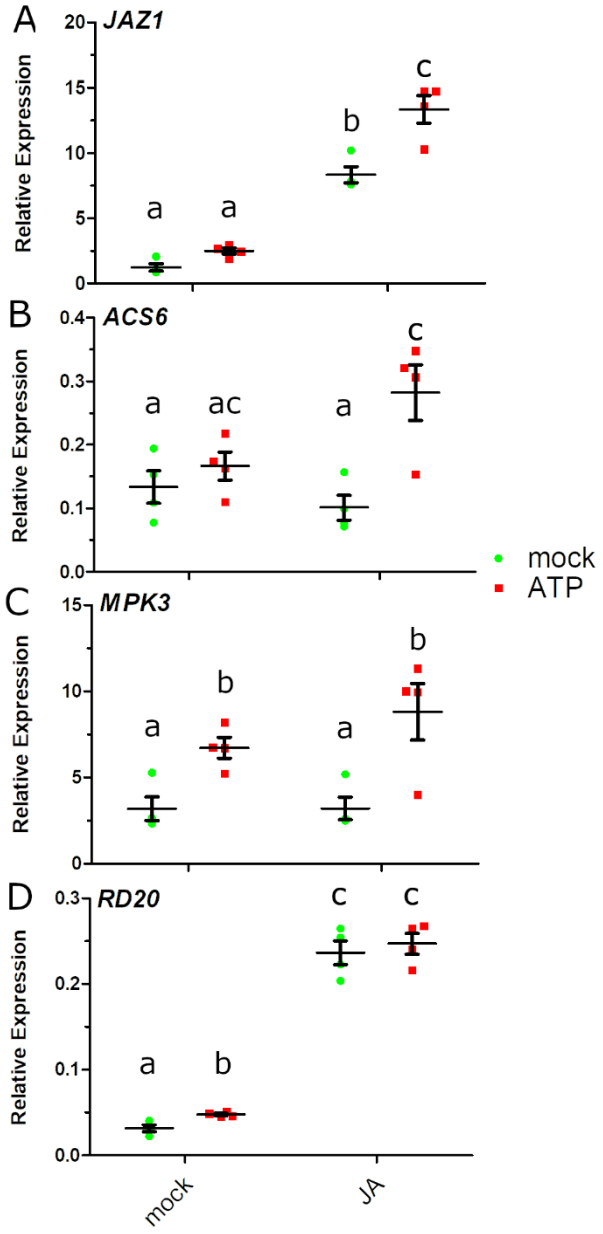


Supplemental Figure S1. Exogenous ATP treatment does not reduce JAZ1 protein stability in *aos* mutant seedlings, while MeJA does. Mutant *aos* seedlings expressing JAZ1-GUS were treated with 2 µM MeJA or 1 mM ATP for 30 minutes, and processed for quantitative GUS activity measurements to assess JAZ1 protein stability as described in Methods. Statistical comparisons to mock-treated seedlings are indicated by ** ($P < 0.01$) and ns ($P = 0.97$, Fisher's LSD). Error bars indicate SEM, n=16 replicates per treatment across 2 trials.



Supplemental Figure S2. JA-mediated induction of P2K1 by MYC transcription factors. A. Data from the Arabidopsis eFP Browser (Winter et al., 2007) shows strong *P2K1* upregulation by MeJA treatment, visualized at http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi. **B.** ChIP-seq data (Zander et al., 2020) demonstrate specific MYC2 and MYC3 binding to the promoter region of *P2K1* (AT5G60300), visualized at http://neomorph.salk.edu/MYC2.



Supplemental Figure S3. JA-primed eATP-responsive gene expression. Wild-type seedlings were pre-treated with or without 20 µM MeJA, followed by 0.5 mM ATP treatment as described in Materials and Methods. Gene expression was evaluated by RT-qPCR for **A,** *JAZ1*, **B,** *ACS6,* **C,** *RD20*, and **D,** *MPK3*. Mean ± SEM and individual values are shown with different letters indicating statistically significant differences in gene expression (n = 4, P < 0.05, Tukey HSD).

Supplemental Table S1

Primer	Sequence 5'-3'	Reference
qJAZ1 f	GAGCAAAGGCACCGCTAATA	Grunewald et al. 2009
qJAZ1 r	TGCGATAGTAGCGATGTTGC	
qMPK3 f	GCTTGGCACACCGACAGAATC	Choi et al. 2014
qMPK3 r	CGTGGGAAGTTGGGAAGTTGC	
qP2K1 f	TGGAGTTTGTCAGGTCCATCG	Choi et al. 2014
qP2K1 r	CTGAGGATCTTCTGCAGGCAA	
qPP2A f	TAACGTGGCCAAAATGATGC	Czechowski et al. 2005
qPP2A r	GTTCTCCACAACCGCTTGGT	
qRD20 f	TGACACCGAAGGAAGGTATGTCC	Choi et al. 2014
qRD20 r	CTTTAACCGTTAGCGCGTATTTGC	
qCPK28 f	ACCCACGAGCACGGCTAA	Choi et al. 2014
qCPK28 r	TTCTCTAACCCACGCATGTGAT	
qRBOHD f	CATGCGGGTGCCCATTT	Choi et al. 2014
qRBOHD r	ATCCGCGGCAATTAAACG	
qLOX3 f	GCTCGCTAAAGCCCACGTTAGTTC	Choi et al. 2014
qLOX3 r	AGCATGCATGTGTCCGTAACCAG	
qACS6 f	CAGCAACGTTTGATTCGGAAA	Choi et al. 2014
qACS6 r	CGTTGAGCTTCACTTGGTGAAC	

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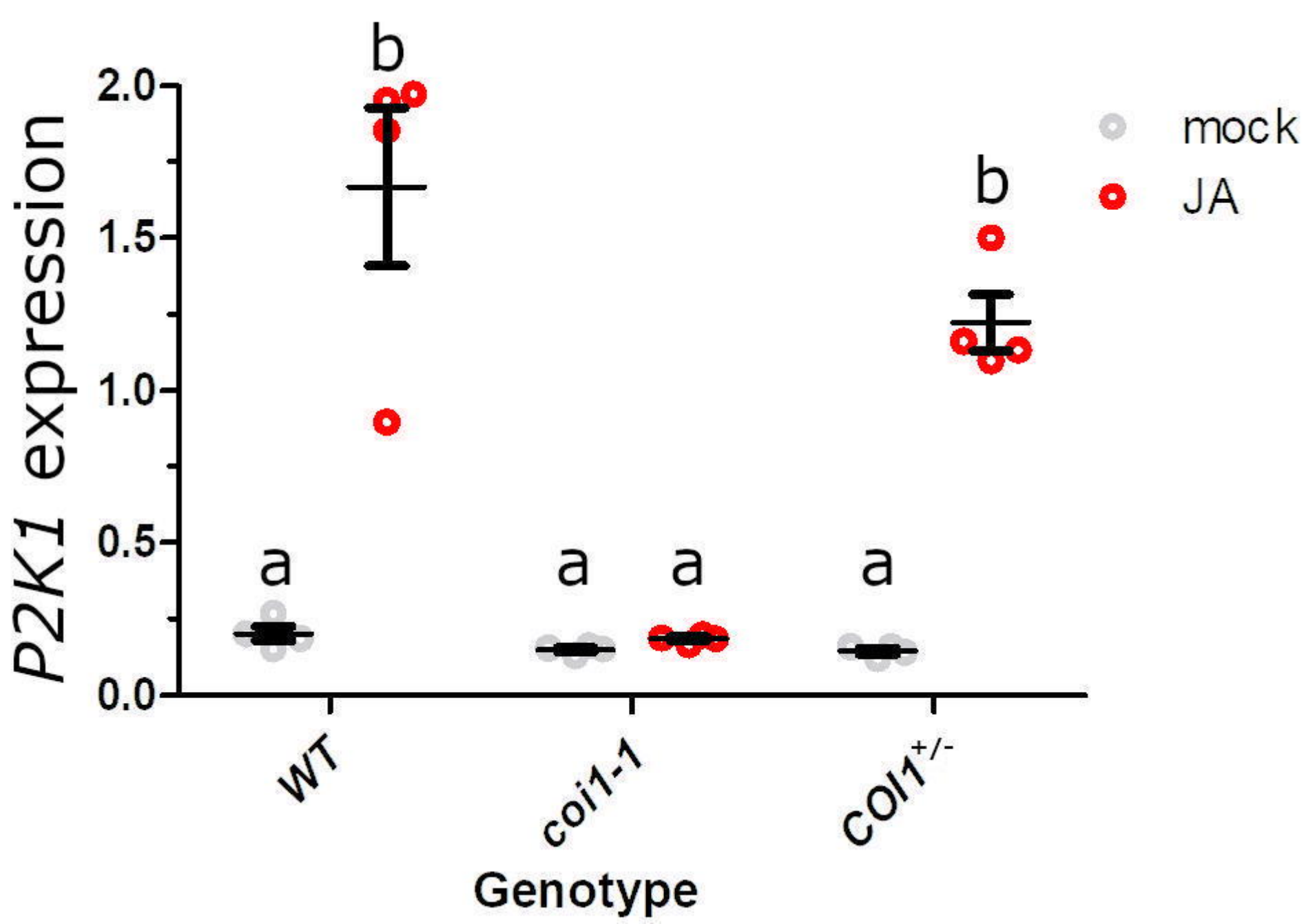
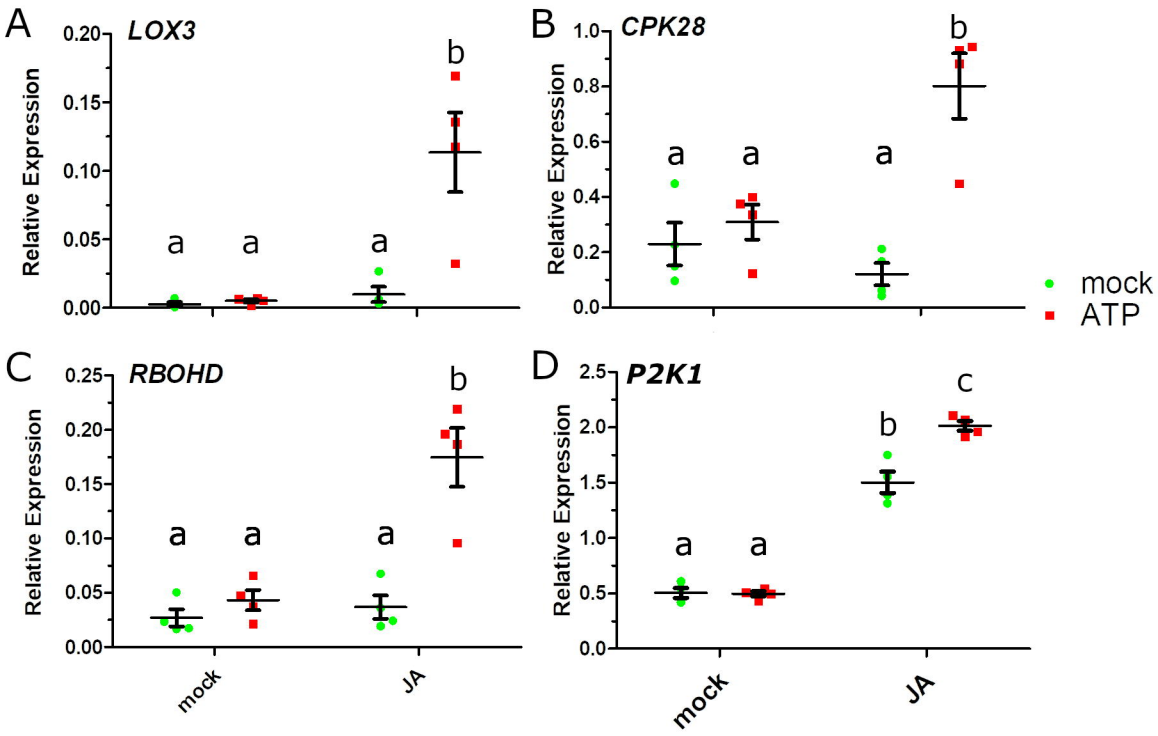
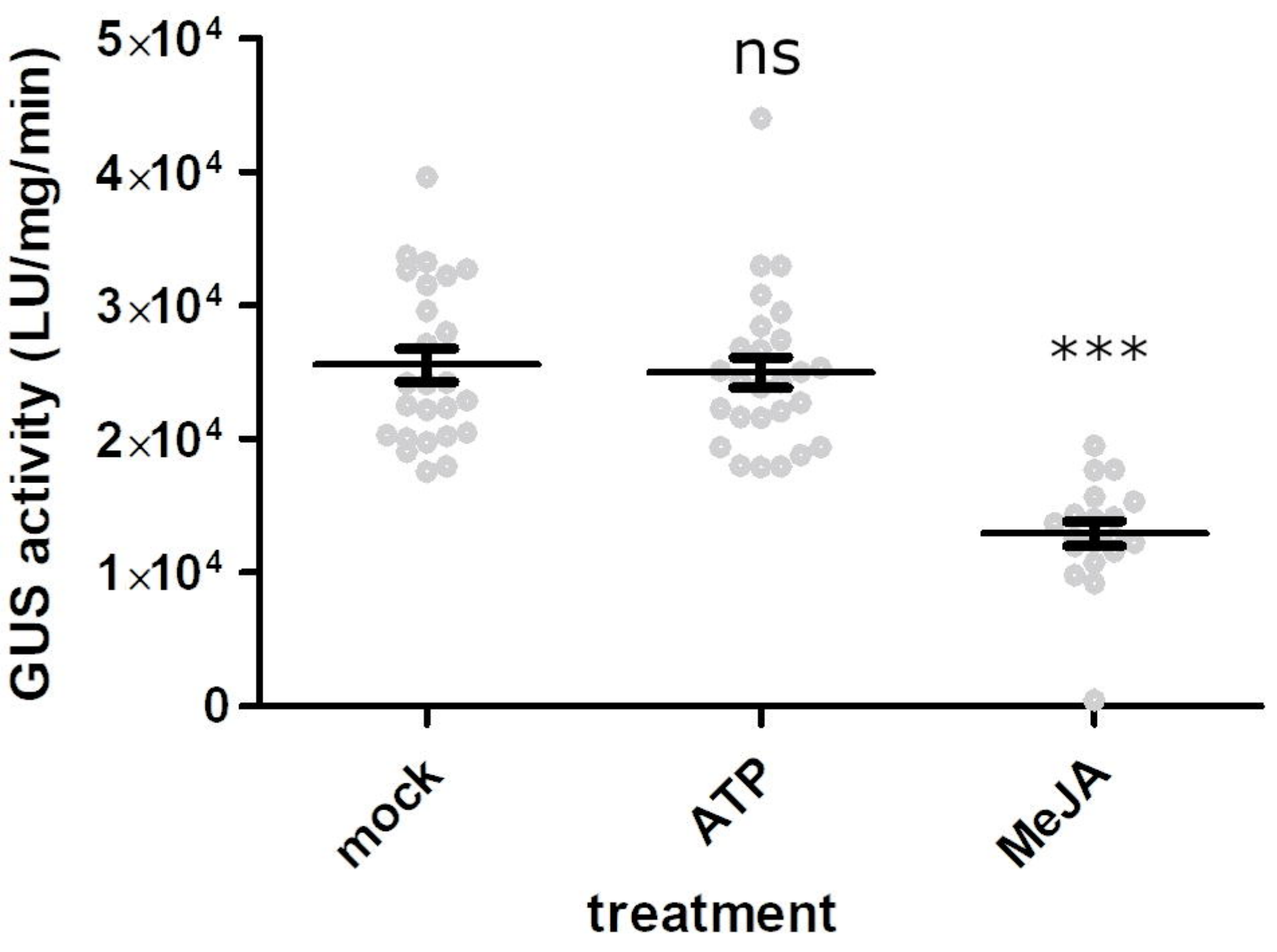
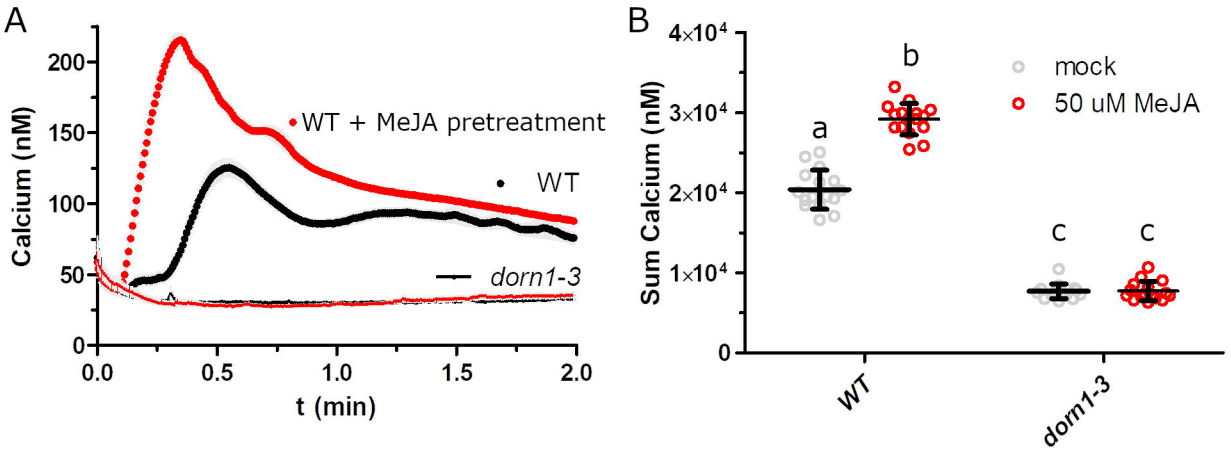
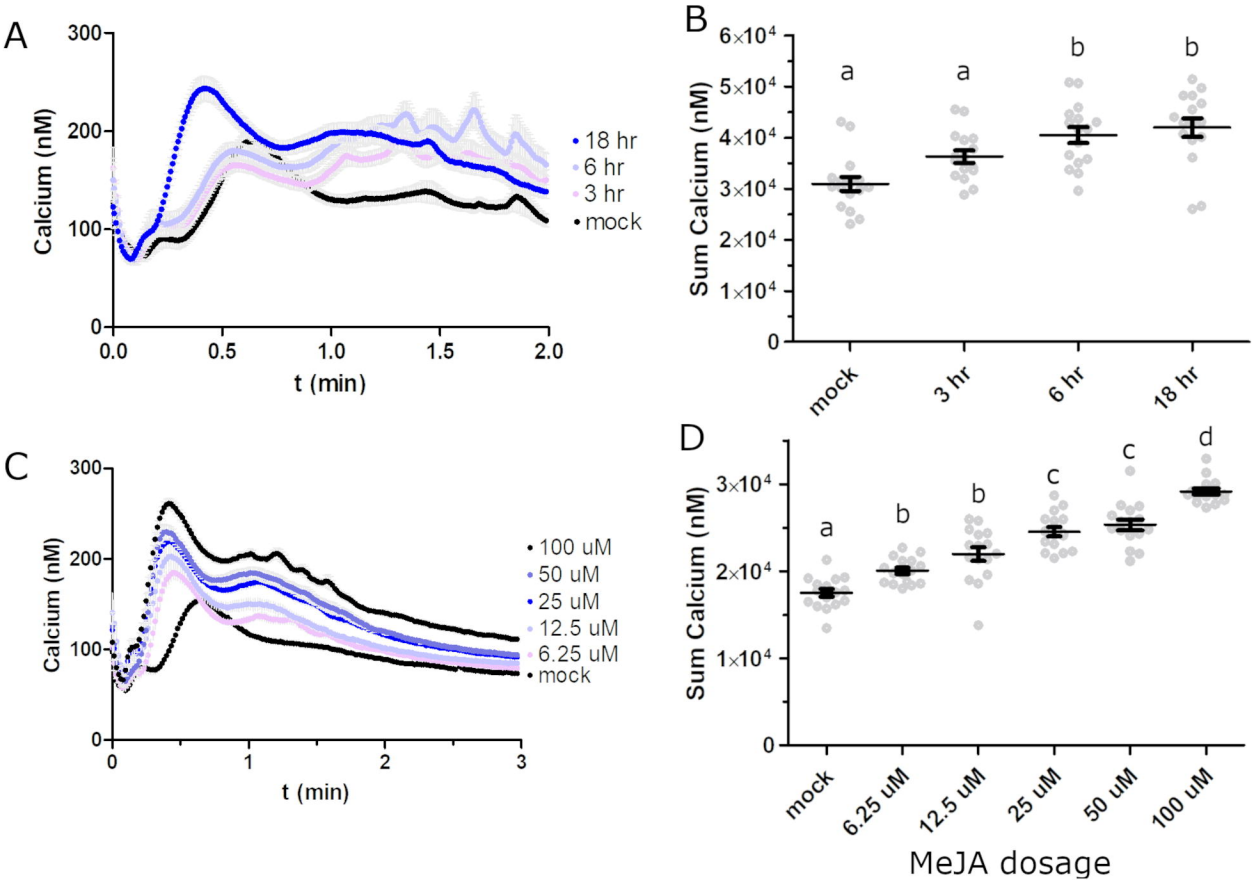
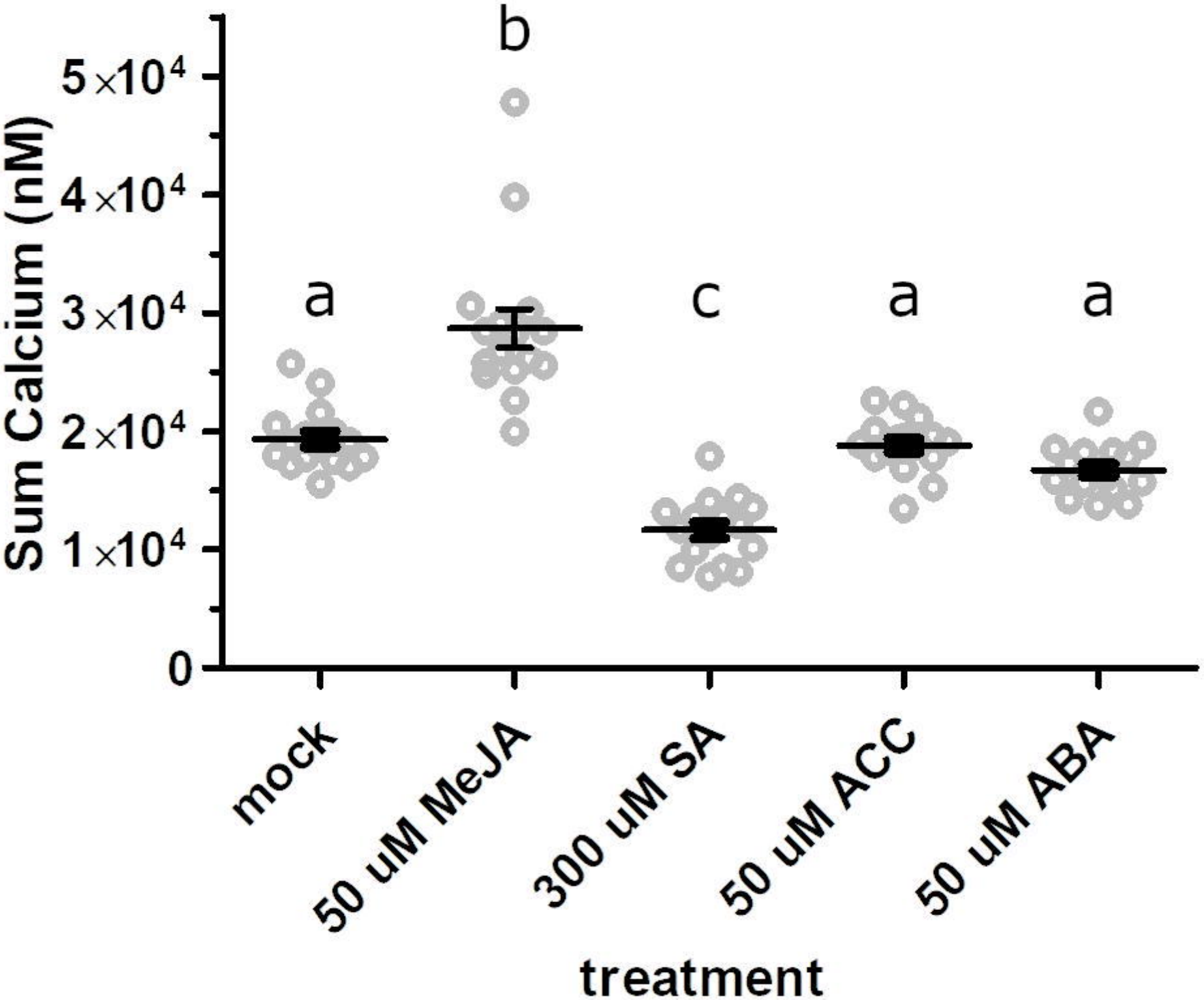

P2K1 expression
2.0
1.5
1.0
0.5
0.0
b
b
a
a
a
a
mock
JA
WT
coi1-1
COI1+/-
Genotype

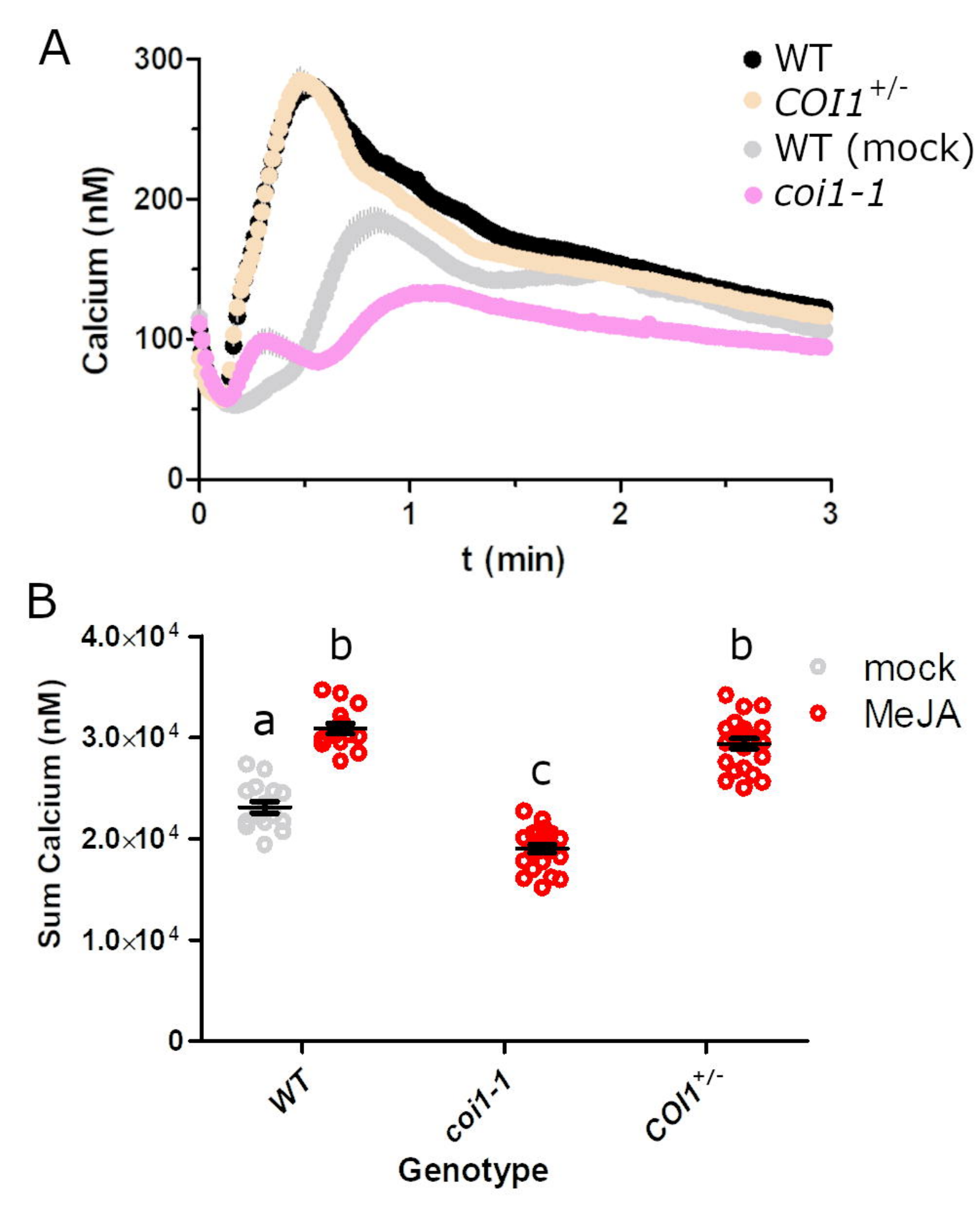

A
LOX3
Relative Expression
0.20
0.15
0.10
0.05
0.00
a
a
a
b
B
CPK28
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
a
a
a
b
mock
ATP
C
RBOHD
Relative Expression
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
b
mock
JA
D
P2K1
Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
c
mock
JA


GUS activity (LU/mg/min)
5×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
ns
***
mock
ATP
MeJA
treatment


A
Calcium (nM)
200
150
100
50
0
0.0
0.5
1.0
1.5
2.0
t (min)
WT + MeJA pretreatment
WT
dorn1-3
B
Sum Calcium (nM)
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
a
b
c
c
mock
50 uM MeJA
WT
dorn1-3


A
Calcium (nM)
t (min)
18 hr
6 hr
3 hr
mock
B
Sum Calcium (nM)
mock
3 hr
6 hr
18 hr
C
Calcium (nM)
t (min)
100 uM
50 uM
25 uM
12.5 uM
6.25 uM
mock
D
Sum Calcium (nM)
mock
6.25 uM
12.5 uM
25 uM
50 uM
100 uM
MeJA dosage


Sum Calcium (nM)
5×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
a
b
c
a
a
mock
50 uM MeJA
300 uM SA
50 uM ACC
50 uM ABA
treatment


A
300
200
100
0
Calcium (nM)
0
1
2
3
t (min)
WT
COI1+/-
WT (mock)
coi1-1
B
4.0×10⁴
3.0×10⁴
2.0×10⁴
1.0×10⁴
0
Sum Calcium (nM)
a
b
c
b
mock
MeJA
WT
coi1-1
COI1+/-
Genotype