# Jasmonate Primes Plant Responses to Extracellular ATP

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## SUMMARY

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 pretreatment 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.

## SIGNIFICANCE STATEMENT

Plant hormone jasmonate (JA) enhances plant responses to extracellular ATP (eATP) in *Arabidopsis thaliana* through a mechanism dependent on the JA receptor COI1 and the eATP receptor P2K1. The reciprocal amplification of these signals provides a mechanistic explanation for how plants effectively respond to different stress events.

## INTRODUCTION

ATP is present in high concentrations within the cell (~mM) but is typically found at much lower 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 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; Kim et al., 2023b; Kim et al., 2023c; Sowders et al., 2023; Sowders et al.; 2024)**. Thus, eATP is recognized as a damage-associated molecular pattern (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 has also been 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

64 **JAZ1 protein stability is not affected by eATP signaling**

65 It has been demonstrated that eATP acts through JA signaling, as many eATP-responsive
66 genes require the JA receptor COI1 for their expression **(Jewell et al., 2019)**. It has also been
67 reported that eATP stimulus promotes the degradation of a jasmonate co-receptor protein (JAZ1)
68 in a jasmonate-independent manner **(Tripathi et al., 2018)**. The mechanism of this surprising
69 phenomenon has not been investigated in subsequent reports; therefore, we re-tested the effect
70 of eATP on JAZ1 protein stability. Arabidopsis plants constitutively expressing the JAZ1-β-
71 GLUCURONIDASE (JAZ1-GUS) fusion protein **(Thines et al., 2007)** were treated with 2 μM
72 methyl jasmonate (MeJA) or 1 mM ATP for 30 minutes and JAZ1-GUS protein levels were
73 quantified using a biochemical GUS activity assay **(Figure 1)**. Across three independent and not
74 statistically different trials ($P$=0.13, one factor ANOVA F-test), MeJA treatment reduced JAZ1-
75 GUS levels by approximately 48% ($P$<1e-7, Tukey HSD), while ATP treatment had no detectable
76 effects ($P$=0.99, Tukey HSD). Similarly, ATP treatment did not affect JAZ1-GUS stability in the
77 *aos* JA biosynthesis mutant ($P$=0.97, Tukey HSD), whereas MeJA still reduced GUS levels by
78 about 49% ($P$=5.3e-3, Tukey HSD; **Supplemental Figure S1**). These findings suggest that while
79 eATP signaling depends on COI1, it is independent of JAZ protein stability, indicating a distinct
80 mechanism of JA-signaling-mediated eATP response, and suggesting that a model of eATP-
81 stimulated JAZ1 degradation should be critically reevaluated.

82

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

85 Given that eATP signaling integrates with JA pathways, we next explored whether JA itself
86 modulates plant responses to eATP. To test this, we pre-treated transgenic Arabidopsis seedling
87 expressing the calcium reporter apoaequorin protein (hereafter AEQ seedlings) overnight with 50
88 μM MeJA or a mock solution before adding 100 μM exogenous ATP to the same wells and
89 recorded luminescence as an indication of cytosolic calcium levels ($[Ca^{2+}]_{cyt}$) as previously
90 described (Choi et al., 2014). Without MeJA pre-treatment, ATP induced the typically transient
91 $[Ca^{2+}]_{cyt}$ response **(Tanaka et al., 2010)**, peaking around ~30-45 seconds and tapering thereafter
92 **(Figure 2A)**. However, MeJA pre-treatment resulted in enhanced response to eATP,
93 approximately 50% higher than in mock-treated samples over the course of 3 minutes and with a
94 significantly earlier peak calcium level **(Figure 2, Supplemental Figure S2)**. We note that
95 overnight pretreatment with 0.05% DMSO (the MeJA carrier) resulted in ~18% reduced $[Ca^{2+}]_{cyt}$
96 response to ATP treatment **(Supplemental Figure S3)**, so the MeJA-primed eATP-induced

calcium values are an underestimate. We also note that neither DMSO nor 50 μM MeJA treatment differed in changes to $[Ca^{2+}]_{cyt}$ relative to water **(Supplemental Figure S4)**. In contrast to WT seedlings, the *p2k1-3* mutant (lacking functional P2K1) did not show the typical $[Ca^{2+}]_{cyt}$ response to ATP **(Figure 2)**, regardless of MeJA pre-treatment, confirming that the JA-enhanced plant response to ATP is P2K1-dependent and the previously described second eATP receptor, P2K2 (Pham et al., 2020), is insufficient to allow for detectable changes to $[Ca^{2+}]_{cyt}$ in response to ATP. Interestingly, we were also able to detect MeJA-stimulated release of ATP into media surrounding leaf discs 1 hour and 16 hours after treatment, though not after 15 minutes **(Supplemental Figure S5)**. The mechanism and significance of this observation remains to be determined.

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.

**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. These

130 contrasting effects of MeJA and SA are consistent with extensive previous literature **(Van der**
131 **Does et al., 2013).**

132

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

134 Previous research on the interaction of JA and eATP signaling has primarily focused on
135 transcriptional responses, without examining calcium signaling **(Jewell et al., 2019)**. To examine
136 whether the ATP-induced $[Ca^{2+}]_{cyt}$ response relies on JA signaling, we crossed AEQ-expressing
137 plants with *coi1-1* mutants to generate F3 lines segregating for the *coi1-1* mutant allele while
138 maintaining AEQ expression. This segregating progeny approach is necessary given that
139 homozygous *coi1* null mutants are male sterile **(Xie et al., 1998)**. AEQ seeds with *COI1-1*$^{+/-}$
140 segregants were additionally exposed to 50 μM MeJA to differentiate *coi1-1* mutants from those
141 with a WT *COI1* allele. After 7 days, these 3 genotypes were incubated overnight in coelenterazine
142 reconstitution buffer with (all 3 genotypes) or without (WT) 50 μM MeJA and subsequently treated
143 with 100 μM ATP. Consistent with our hypothesis, WT and *COI1*$^{+/-}$ seedlings exhibited a robust
144 ATP-induced $[Ca^{2+}]_{cyt}$ response following MeJA treatment **(Figure 5A, B)**. In contrast, the *coi1-1*
145 mutant seedlings showed a significantly reduced ATP-induced $[Ca^{2+}]_{cyt}$ response, even after
146 MeJA priming. To test the effects of COI1 mutation on basal and MeJA-primed eATP-responsive
147 changes to $[Ca^{2+}]_{cyt}$ (i.e. without the MeJA pretreatment which is a necessity for selecting
148 seedlings for treatment given the male sterility of COI1 null mutants), we used leaf discs from 4-
149 week-old plants, *COI1*$^{+/-}$ or *coi1-1*. Changes to $[Ca^{2+}]_{cyt}$ after ATP treatment were much lower in
150 leaf discs relative to seedlings, and were not reliably observed in the absence of MeJA treatment
151 **(Figure 5C,D).** In addition, MeJA was unable to prime eATP calcium responses in the *coi1-1*
152 mutant. These results indicate that COI1 is essential both for MeJA-induced priming of eATP-
153 induced calcium response in seedlings and mature leaves, and for basal WT-like responses to
154 eATP in seedlings.

155

156 ***P2K1* gene expression is induced by JA treatment and wounding in a COI1-dependent**
157 **manner**

158 The eATP receptor P2K1 is known to be upregulated in response to wounding, with
159 expression rising 2- to 3-fold in adult Arabidopsis within 1 hour and persisting for up to 24 hours
160 **(Kilian et al., 2007).** Similarly, treatment with 10 μM MeJA induces a ~5-fold increase in *P2K1*
161 transcript in seedlings within an hour **(Supplemental Figure S6A, Goda et al., 2008**;

162 https://bar.utoronto.ca/efp/), and the bacterial JA-Ile mimic coronatine has been shown to elevate
163 P2K1 expression in mature plants over the course of 24 hours **(Attaran et al., 2014)**. Additionally,
164 chromatin immunoprecipitation sequencing experiments revealed that the JA-responsive
165 transcription factors MYC2 and MYC3 bind to the presumptive *P2K1* promoter region
166 **(Supplemental Figure S6B, Zander et al., 2020;** http://neomorph.salk.edu/MYC2**)**. Furthermore,
167 the *P2K1* transcript is induced significantly less (~30% and 80% after 30 and 120 minutes,
168 respectively) in the *myc2* mutant relative to WT after ~5 μM gaseous MeJA treatment
169 (**Supplemental Table S1**, Zander et al., 2020). Altogether, these previous data suggest that
170 canonical JA signaling is likely required for JA-induced *P2K1* gene expression. To test this idea,
171 we grew WT plants, *COI1*$^{+/-}$ segregants, and the *coi1-1* mutants for four weeks, treated leaves 50
172 μM MeJA, and then quantified *P2K1* gene expression levels using RT-qPCR. As shown in **Figure**
173 **6**, MeJA treatment significantly increased *P2K1* expression by ~6-fold in both WT and *COI1*$^{+/-}$
174 plants, whereas no increase was observed in the *coi1-1* mutants. Similarly, 1 hr following
175 wounding *P2K1* transcript levels significantly increased by ~4-fold in WT and *COI1*$^{+/-}$ plants but
176 not *coi1-1* **(Figure 6B).** This confirms that COI1 is required for *P2K1* induction in response to JA
177 and wounding, correlating with the MeJA-potentiated and COI1-dependent increased $[Ca2+]_{cyt}$ in
178 response to treatment with ATP.

179

180 **JA primes eATP-responsive gene expression**

181 To further evaluate the physiological impact of JA priming on ATP responses, we tested
182 whether MeJA pre-treatment enhances ATP-responsive gene expression. Given that many ATP-
183 responsive genes also respond to JA **(Choi et al., 2014; Jewell et al., 2019),** we treated plants
184 with MeJA, then replaced the media to allow a 24-hour recovery period, thereby enabling JA-
185 responsive genes to return to basal levels prior to the ATP treatment (see Materials and Methods).
186 We selected eight genes with previously reported subtle ATP-induced expression changes,
187 reasoning that an exaggeration of this marginal induction could be easier to detect. Across these
188 genes, there were significant effects due to MeJA, ATP, and their interaction ($P$<2e-16, $P$=1.1e-
189 10, and $P$=2.6e-3, respectively, two factor ANOVA F-test). Of these, six genes showed significant
190 MeJA priming of ATP responsiveness (MeJA by ATP interaction, P<0.05, two factor ANOVA F-
191 test): *LIPOXYGENASE 3* (*LOX3;* **Figure 7A**), *CALCIUM-DEPENDENT PROTEIN KINASE 28*
192 (*CPK28*, **Figure 7B),** *RESPIRATORY BURST OXIDASE HOMOLOGUE D* (*RBOHD*, **Figure 7C)**,
193 *P2K1* (**Figure 7D),** *JASMONATE-ZIM-DOMAIN PROTEIN 1* (*JAZ1*, **Supplemental Figure S7A)**,
194 and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE 6* (*ACS6*, **Supplemental**

195 **Figure S7B).** The remaining two genes, *RESPONSIVE TO DESSICATION 20* (*RD20,*
196 **Supplemental Figure S7C)** and *MITOGEN-ACTIVATED PROTEIN KINASE 3* (*MPK3*,
197 **Supplemental Figure S7D)**, did not show statistically significant MeJA priming. These findings
198 suggest that JA priming enhances transcriptional responses to eATP, particularly for genes
199 involved in stress and signaling pathways.

200

201 **DISCUSSION**

202 In this study, we report that JA potentiates two Arabidopsis responses to eATP through
203 mechanisms dependent on the JA receptor COI1 and the eATP receptor P2K1. Our findings
204 further indicate that JA treatment induces *P2K1* gene expression in a COI1-dependent manner,
205 suggesting that JA-primed eATP response acts by transcriptional upregulation of *P2K1*.
206 Supporting this, published ChIP-seq data **(Zander et al., 2020)** demonstrate specific binding of
207 MYC2 and MYC3 transcription factors to the *P2K1* promoter, suggesting that JA induction of *P2K1*
208 relies on the canonical JA signaling pathway involving bHLH MYC transcription factors.
209 Importantly, the priming effect was observed even at low JA concentrations, consistent with
210 endogenous JA levels after wounding **(Kimberlin et al., 2022)**, hinting at the physiological
211 relevance of this JA-eATP signaling interaction. Interestingly, we also observed that MeJA
212 treatment of Arabidopsis leaf discs induced ATP release into surrounding media. Though the
213 physiological relevance and mechanistic basis of this observation is unknown, it suggests multiple
214 levels to the JA stimulation of eATP signaling.

215 Since the initial report of ATP-induced degradation of JAZ1 in 2018 **(Tripathi et al.)**, no
216 studies have corroborated or explained this phenomenon. While JAZ1 degradation is not central
217 to our conclusions, its irreproducibility has implications for understanding the full mechanism by
218 which eATP activates JA-responsive genes. It remains possible that other JAZ proteins could play
219 a role in eATP-induced activation of JA-responsive genes. For example, JAZ8, which lacks a
220 canonical degradation mechanism within JA signaling (**Shyu et al., 2012**) regulates both JA-
221 responsive and JA biosynthesis genes in response to stress-induced $[Ca^{2+}]_{cyt}$ elevations (**Yan et**
222 **al., 2018)**, a hallmark of eATP response. Despite the uncertainty surrounding JAZs, activation of
223 JA-responsive genes in this pathway clearly relies on the P2K1 receptor and the MYC and
224 CAMTA3 transcription factors **(Jewell et al., 2019)**, with upstream signaling involving the P2K1-
225 interacting and -phosphorylated INTEGRIN-LINKED KINASE 5 (ILK5, **Kim et al, 2023b**).
226 Recently, it was suggested that phosphorylation of ZmMYC2 transcription factors by the maize

227 mitogen-activated protein kinase (MAPK) ZmMPK4 enhances transcriptional activation activity of
228 ZmMYC2s **(Li et al, 2024)**. Given that MPK phosphorylation is a characteristic feature of eATP
229 responses **(Choi et al, 2014; Cho et al, 2022)** and is reduced in *ilk5* Arabidopsis mutants **(Kim**
230 **et al, 2023b)**, these cross-species comparisons suggest that eATP may activate MYC2 through
231 a MAPK cascade. Experimental evaluation of this model should be an interesting direction for
232 future research. Collectively, these findings suggest that JA and eATP pathways amplify each
233 other's signaling as part of a defense priming mechanisms during stress responses.

234 Given the roles of JA and eATP signaling in responses to biotic stresses such as
235 pathogens and herbivores **(Browse 2009; Chivasa et al., 2009)**, it is attractive to speculate that
236 this interaction promotes heightened defense responses after initial injury. However, a novel role
237 for JA signaling in wound healing and tissue regeneration has recently emerged **(Zhang et al.,**
238 **2019; Zhou et al., 2019).** In addition, the wound-induced tomato elicitor peptide REF1, an
239 orthologue of Arabidopsis PLANT ELICITOR PEPTIDEs (PEPs), dramatically increased wound-
240 induced callus formation and shoot regeneration **(Yang et al., 2024)**, and exogenous ATP
241 treatment induces expression of several PEP-encoding genes (*PROPEP1*, *PROPEP3*,
242 *PROPEP4*, and *PROPEP7*) and two PEP receptor genes in a P2K1-dependent manner **(Jewell**
243 **et al., 2019).** While Arabidopsis has eight PEPs, to our knowledge none have been investigated
244 for a tissue regeneration activity similar to REF1. This raises the question: does JA potentiation
245 of eATP response contribute not only to defense priming but also to tissue regeneration after
246 wounding? This hypothesis opens exciting avenues for further research. Given the well-
247 documented role of JA in pathogen defense and the more nuanced roles associated with eATP
248 signaling, investigating the potential role of JA-eATP interaction in tissue regeneration may
249 provide new insights into wound response mechanisms.

250

251 **MATERIALS AND METHODS**

252 **Plant Materials**

253 Arabidopsis (*Arabidopsis thaliana)* WT AEQ (pMAQ2.4), *p2k1-3 AEQ*, *35S-JAZ1-GUS,*
254 *aos 35S-JAZ1-GUS* and *coi1-1* were described previously **(Knight et al., 1991; Choi et al., 2014;**
255 **Thines et al., 2007; Tripathi et al., 2018; Xie et al., 1998)**. Columbia-0 (CS70000) was obtained
256 from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). Due to the male sterility
257 of *coi1* null alleles, *COI1-1*$^{+-}$ *AEQ* lines were maintained as segregating for the *coi1-1* mutation
258 but fixed for AEQ expression. These were identified by crossing WT AEQ and *coi1-1,* selfing F2

259 plants and selecting F3 lines based on segregating MeJA insensitivity andfixed 25 µg/mL
260 kanamycin resistance. AEQ expression was verified based on luminescence in the AEQ
261 discharge solution **(Choi et al., 2014)**.

262 **Reagents**

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

271 **Assays to Monitor Changes in Cytosolic Calcium**

272 Assays to monitor changes in $[Ca^{2+}]_{cyt}$ were performed as described previously **(Choi et**
273 **al., 2014)**. Briefly, surface-sterilized and cold-stratified seeds were sewn to square plates
274 containing ½-strength Murashige and Skoog (MS) media (pH 5.8) with or without 50 µM MeJA
275 and with 0.8% (w/v) sucrose, 0.8% (w/v) agar, and 0.5g/L MES pH-adjusted using potassium
276 hydroxide. After sowing, plates were transferred to a 22°C growth chamber (Conviron) with 12-h
277 light cycle (100-120 µmol photons $m^{-2}$ $s^{-1}$) and grown vertically for 7 days. On the morning of the
278 $8^{th}$ day, individual seedlings were carefully transferred to single wells of white 96-well plates
279 containing 50 µL aequorin reconstitution buffer containing 10 µM coelenterazine (NanoLight
280 Technology) with or without various hormones as described in the Results section, 2 mM MES
281 buffer pH 5.7, and 10 mM $CaCl_2$-heptahydrate and incubated in darkness overnight. For 50 µM
282 MeJA treatments, the final concentration of DMSO in the treatment solution was 0.05%.
283 Luminescence was monitored after adding 50 µL of 2X treatment solution via autoinjector using
284 the GloMax Navigator (Promega). After elicitor-induced luminescence was recorded, 100 µL of
285 discharge solution (20% ethanol (v/v) and 2M $CaCl_2$-heptahydrate) was added to each well, and
286 luminescence recorded for an additional 30 s. Luminescence data were converted to $[Ca^{2+}]_{cyt}$
287 using the formula of **Knight et al., 1996**. Summed calcium data in figures were calculated by
288 adding $[Ca^{2+}]_{cyt}$ values at each timepoint.

289 To test cytosolic calcium responses to eATP of *COI1*$^{+/-}$ versus *coi1-1* absent MeJA
290 pretreatment, seedlings were grown and selected as above and then transferred to soil in a 22°C

291 growth chamber (Conviron) with 12-h light cycle (100-120 µmol photons $m^{-2}$ $s^{-1}$). After ~4 weeks,
292 leaf discs from expanded leaves were collected with a 4 mm biopsy punch (Ted Pella, Inc.) into
293 distilled water in white 96-well plates and allowed to sit under light for 1 hour. Thereafter, the
294 water was removed and 50 µL aequorin reconstitution buffer was added containing either 50 µM
295 MeJA or 0.05% DMSO and plates moved to darkness overnight (~18 hours) after which
296 luminescence was recorded as above.

297 **Quantitative JAZ1-GUS Stability Assay**

298 The quantitative GUS assay was performed as described **(Jefferson et al., 1987)**. Five to
299 ten 10-day old seedlings of *35S-JAZ1-GUS* **(Thines et al., 2007)** or *aos 35S-JAZ1-GUS* were
300 incubated overnight in buffer (10 mM $CaCl_2$, 2 mM MES, pH5.7). The next day, seedlings were
301 treated with buffer alone (mock), 1 mM ATP, or 2 µM MeJA for 30 minutes in the light, then blotted
302 dry and snap-frozen in liquid nitrogen. These concentrations are on the order of concentrations
303 described in the original manuscript (Tripathi et al., 2018). The seedlings were ground in 75 µL of
304 GUS extraction buffer (GEB; 50 mM sodium phosphate pH 7.0, 10 mM EDTA, 10 mM β-
305 mercaptoethanol, 0.1% triton X-100, 0.1% sarkosyl) and centrifuged at 4°C for 15 minutes at
306 15,000 rcf to remove insoluble debris. Supernatant (65 µL) was collected to separate tubes on
307 ice and protein quantity estimated using Bradford reagent. For each sample, equal amounts of
308 protein (~9 µg) were added to individual wells of a white 96-well plate in a final volume of 100 µL
309 GEB containing 1 mM 4-Methylumbelliferyl-β-D-glucuronide hydrate (MUG) prewarmed to 37°C
310 after which the plate was stored in a dark 37°C incubator. At 60 minutes, 60 µL aliquots were
311 added to 60 µL of 0.2 M $Na_2CO_3$ stop buffer in a separate white 96-well plate. Fluorescence from
312 MU released in each well was measured in a plate reader with excitation at 365 nm and emission
313 at 455 nm, and rate was calculated as light units/mg/min. In preliminary experiments, signal was
314 not reliably detected at 15 minutes incubation, but activity was linear between 30 to 90 minutes.

315 **Quantification of Extracellular ATP**

316 Extracellular ATP was quantified as previously described **(Ramachandran et al., 2019)** using
317 Col-0 plants one month after sewing. Individual leaf discs (4 mm, Electron Microscopy Sciences)
318 were collected into 150 µL of distilled deionized water in individual wells of a 96 well plate and the
319 plate transferred to a growth chamber. Two hours later, 50 uL of water, 0.8 M NaCl, 0.2% DMSO,
320 or 200 µM MeJA were added to each well. At 15, 60, and 960 minutes after addition of these
321 solutions, 50 µL was gently removed from each well and immediately flash frozen. Thereafter,
322 these were heated to 96° C for 5 minutes. After allowing to cool, 25 µL was added to wells of a

323 white 96 well plate, and 25 µL of luciferase/luciferin reagent (ENLITEN, Promega) was added to
324 each well by the luminometer's autoinjector and luminescence recorded for 5 seconds. A standard
325 curve was fit by use of 100, 50, 33, 10, 1, 0.1, and 0 nM ATP to calculate ATP concentrations in
326 the wells.

327 **RT-qPCR Analysis**

328 For MeJA-primed eATP-induced gene expression experiments, Col-0 seedlings were
329 grown as described above. On the 7$^{th}$ day, 20 seedlings were transferred to 6-well plates
330 containing 2 mL of ½-strength sterile MS liquid media (without sucrose and agar) with or without
331 20 µM MeJA and returned to the growth chamber. The next day, the MeJA-containing liquid media
332 was removed, and 2 mL of fresh liquid media was added to each well. Finally, on the 9$^{th}$ day, 2
333 mL of liquid media with or without 0.5 mM ATP was added, a concentration optimized previously
334 (Jewell et al., 2019), and the plates were returned to the growth chamber for 30 minutes.
335 Thereafter, seedlings were removed from the wells, gently blotted on tissue paper, flash frozen in
336 liquid nitrogen, and stored for the subsequent RT-qPCR experiments. All media changes were
337 performed in a sterile laminar flow hood.

338 For the MeJA-induced *P2K1* gene expression studies, WT AEQ, and AEQ lines
339 segregating for the *coi1-1* mutation were initially grown as above for 7 days, then 8 (WT) or ~60
340 (*COI1*$^{+/-}$) seedlings were transferred to autoclave-sterilized soil in a growth room (22°C, 12 h light,
341 100-120 µmol photons m$^{-2}$ s$^{-1}$) and grown for an additional 3 weeks. Adult plants were used so
342 that we could reliably determine the genotype of plants without prior MeJA treatment. A single
343 leaf from each plant was excised with a scalpel and snap frozen and stored. Immediately after
344 initial harvest, all plants were uniformly sprayed with 50 µM MeJA in 0.1% Tween-20 until they
345 were dripping. After 1 hour, another set of single leaves were harvested, snap-frozen, and stored
346 at -80°C. Thereafter, the same plants were sprayed twice over 2 weeks with 500 µM MeJA in
347 0.1% Tween-20, which induced anthocyanin accumulation in plants with a functional *COI1* allele.
348 After flowering, sterile plants (a hallmark of *coi1* null mutants) were noted. Sterile plants not
349 accumulating anthocyanin were recorded as *coi1-1* mutants. Leaves of four plants from each
350 genotype (AEQ, *coi1-1,* and *COI*$^{+/-}$) were selected for subsequent RT-qPCR experiments. Plants
351 for wounding and RT-qPCR were grown similarly. Individual leaves of individual plants were
352 wounded twice across the midvein with a hemostat, damaging ~40-50% of leaf area. One hour
353 later, 2 leaves from separate plants (wounded or unwounded) were combined as a replicate with
354 a total of 5 replicates for each genotype/treatment combination.Frozen plant tissue was disrupted
355 with a bead beater (MIni-Beadbeater-96, BioSpec Products) and total RNA was isolated using

356 the Quick-RNA Miniprep Kit (Zymo Research), including the on-column DNase I treatment as per
357 the manufacturer's instructions, which we have found is critical for reproducible RT-qPCR results
358 with low-expressed eATP responsive genes.

359 After RNA isolation, 1 µg of RNA was used in a 20-µL reverse transcription reaction
360 according to the manufacturer's instructions (iScript; Bio-Rad) followed by a 5-fold dilution with
361 ultrapure water. RT-qPCR was performed using 2 µL of diluted cDNA in a 20-µL reaction with
362 SYBR Green dye polymerase mix (SsoAdvanced; Bio-Rad) in white-walled plates in a CFX96
363 thermocycler (Bio-Rad). Gene expression was normalized to the reference gene *PP2A*
364 (AT1G13320) as described **(Czechowski et al., 2005; Rieu and Powers 2009)**. Primers used
365 are listed in **Supplemental Table S2.**

366 **Statistical Analyses**

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

370 **Accession Numbers**

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

373

374 **DATA STATEMENT**

375 Seeds and data underlying the figures are available upon request from Jeremy Jewell
376 (jbjewell@wsu.edu) or Kiwamu Tanaka (kiwamu.tanaka@wsu.edu).

377

378 **ACKNOWLEDGEMENTS**

379 Thank you to John Browse for the *35S-JAZ1-GUS* seeds, the *coi1-1* seeds, the *aos* seeds,
380 leadership by example, and support and encouragement throughout the years. Thank you to Marc
381 Knight for the pMAQ2.4 seeds. Thank you to Nathan Havko for insightful discussion. This work
382 was funded by the NSF (no. IOS-1557813, IOS-2048410) and USDA NIFA (Hatch project no.
383 1015621). Funding was also provided by the Center for Bioenergy Innovation (CBI) led by Oak
384 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.

## AUTHOR CONTRIBUTIONS

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

## SHORT LEGENDS FOR SUPPORTING INFORMATION

**Supplemental Figure S1. Exogenous ATP treatment does not reduce JAZ1 protein stability in *aos* mutant seedlings, while MeJA does.**

**Supplemental Figure S2. The time to maximal ATP-induced $[Ca^{2+}]_{cyt}$ response is reduced after MeJA treatment.**

**Supplemental Figure S3. Effect of overnight DMSO treatment on ATP-induced changes in $[Ca^{2+}]_{cyt}$.**

**Supplemental Figure S4. Effect of water, DMSO, or MeJA on $[Ca^{2+}]_{cyt}$.**

**Supplemental Figure S5. Effect of NaCl or MeJA on ATP release from leaf discs.**

**Supplemental Figure S6 Induction of *P2K1* by JA may be mediated by MYC transcription factors.**

**Supplemental Figure S7. JA-primed eATP-responsive gene expression.**

**Supplemental Table S1. Reduced induction of *P2K1* gene by MeJA treatment in the *myc2* mutant.**

**Supplemental Table S2. Primers used in this study.**

409

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539 **FIGURE LEGENDS**

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

547 **Figure 2. JA pre-treatment increases ATP-induced cytosolic calcium response. (A)** Seven-
548 day-old aequorin-expressing seedlings were incubated overnight in reconstitution buffer and
549 treated with (red symbols) or without (black symbols) 50 μM MeJA, followed by treatment with
550 100 μM ATP. Aequorin luminescence was recorded for 2 minutes to assess the cytosolic calcium
551 levels (mean ± SEM, $n$ = 16 seedlings per treatment). **(B)** Summed calcium values from panel A.
552 Different letters indicate statistically significant differences ($P < 0.05$, Tukey HSD). Mean,
553 individual values, and SEM are shown, with $n$ = 16 seedlings per treatment.

554 **Figure 3. Effects of JA pre-treatment dosage and duration on ATP-induced calcium**
555 **response. (A)** Seedlings were treated as in Figure 1, with MeJA added to the reconstitution buffer

556 50 μM for 3, 6, or 18 hours, followed by recording of ATP-induced aequorin luminescence. **(B)**
557 Summed calcium values from panel A. **(C)** Seedlings were treated as in Figure 1, with the
558 reconstitution buffer containing varying MeJA concentrations. **(D)** Summed calcium values from
559 panel C. All graphs show mean ± SEM ($n$ = 16), with statistically significant differences indicated
560 by different letters ($P$ < 0.05, Tukey HSD).

561 **Figure 4. SA reduces ATP-induced calcium response, while JA increases it, but ABA and**
562 **ACC have no effect.** Aequorin-expressing seedlings were treated as in Figure 1, with overnight
563 pre-treatment in reconstitution buffer containing 50 μM MeJA, 300 μM SA, 50 μM ACC, or 50 μM
564 ABA, followed by recording ATP-induced luminescence. Data indicates summed calcium values
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566 significant differences ($P$ < 0.05, Tukey HSD).

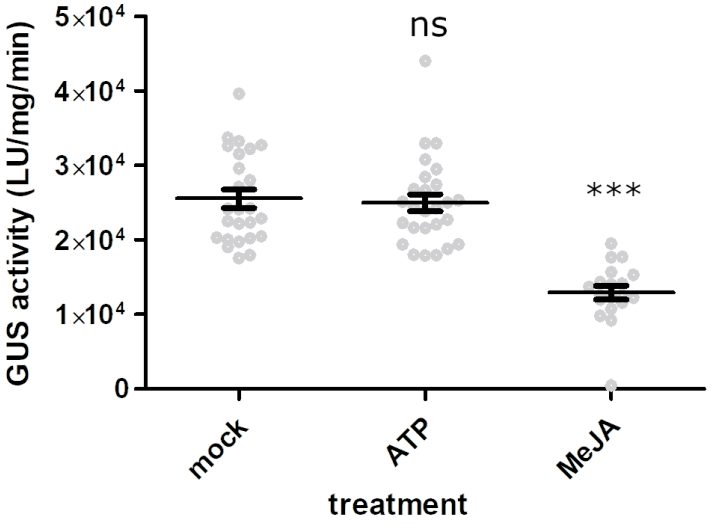
567 **Figure 5. COI1 is required for both basal and MeJA-stimulated ATP-induced calcium**
568 **response. (A)** Seedlings of the indicated genotypes were treated overnight with 50 μM MeJA and
569 100 μM ATP-induced luminescence was recorded (mean ± SEM, $n$ = 16-24). **(B)** Summed calcium
570 values from panel A. Different letters indicate statistically significant differences ($P$ < 0.05, Tukey
571 HSD). **(C)** Leaf discs of the indicated genotype were treated overnight with or without 50 μM MeJA
572 and 100 μM ATP-induced luminescence was recorded (mean ± SEM, $n$ = 16). **(D)** Summed
573 calcium values from panel C. Different letters indicate statistically significant differences ($P$ < 0.05,
574 Tukey HSD).

575 **Figure 6. JA and wounding induces *P2K1* gene expression in a COI1-dependent manner.**
576 *P2K1* expression was measured in 4-week-old aequorin-expressing plants of the indicated
577 genotypes either **(A)** 1 hour after a 50 μM MeJA spray or without MeJA treatment (n=4), or **(B)** 1
578 hour with or without wounding (n=5). Gene expression was normalized by *PP2A.* Mean, SEM,
579 and individual values are shown. Different letters indicate statistically significant differences ($P$ <
580 0.05, Tukey HSD).

581 **Figure 7. JA treatment potentiates eATP-induced transcriptional responses.** Wild-type
582 seedlings were pre-treated with or without 20 μM MeJA, followed by treatment with 0.5 mM ATP,
583 as described in Materials and Methods. Gene expression was evaluated by RT-qPCR for **(A)**
584 *LOX3*, **(B)** *CPK28,* **(C)** *RBOHD,* and **(D)** *P2K1*. Mean ± SEM and individual values are shown,
585 with different letters indicating statistically significant differences in gene expression ($n$ = 4, $P$ <
586 0.05, Tukey HSD).

587

588 **FIGURES**

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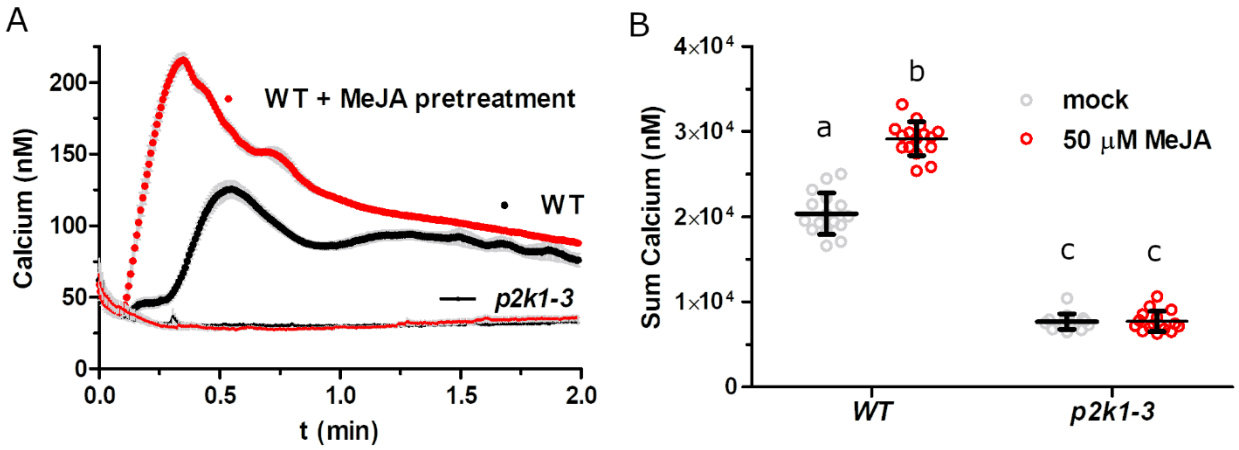


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607 individual values, and SEM are shown, with $n$ = 16 seedlings per treatment.

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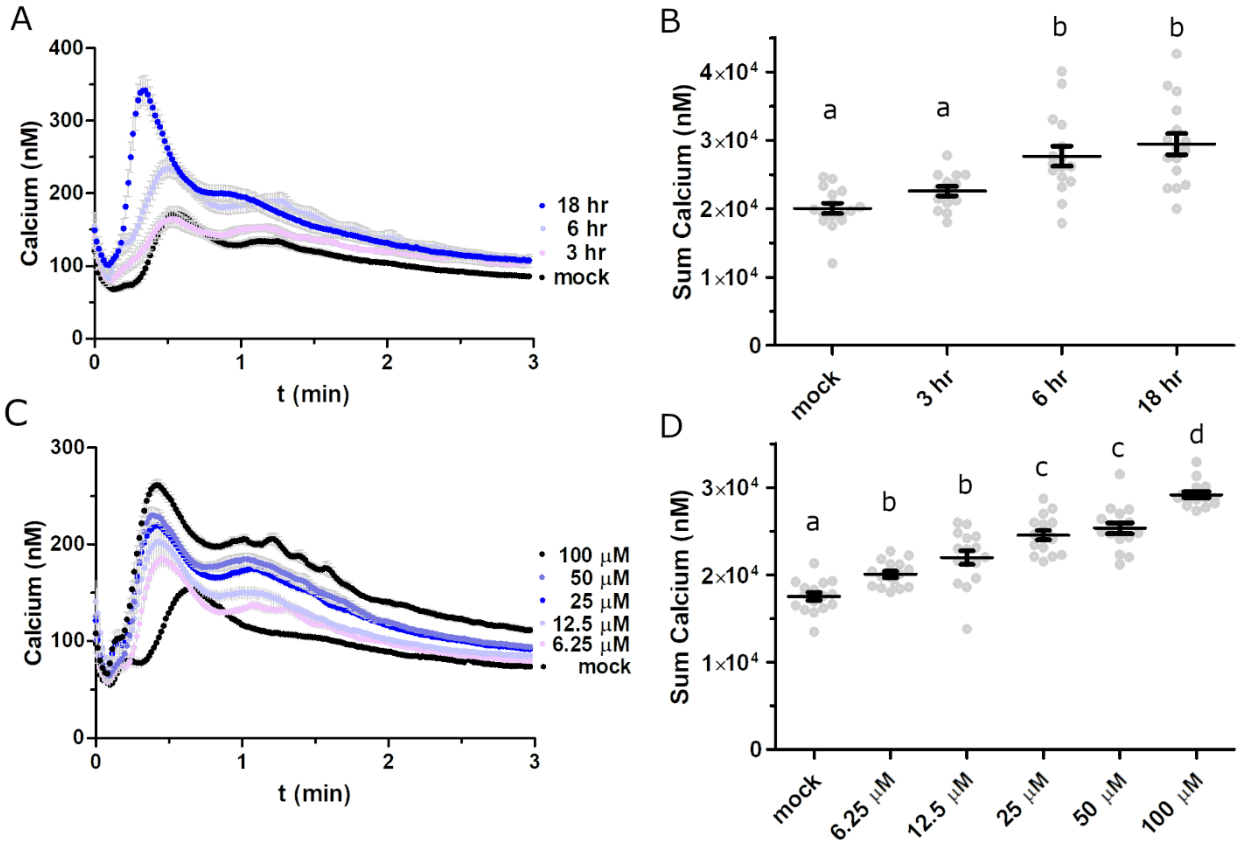


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615 reconstitution buffer containing varying MeJA concentrations. **(D)** Summed calcium values from
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617 indicated by different letters ($P < 0.05$, Tukey HSD).

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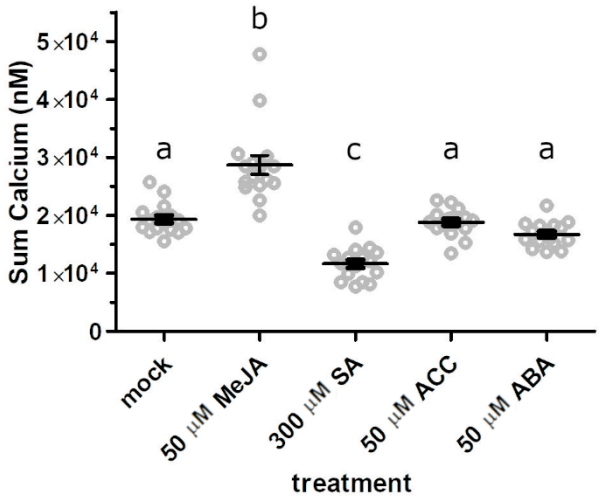


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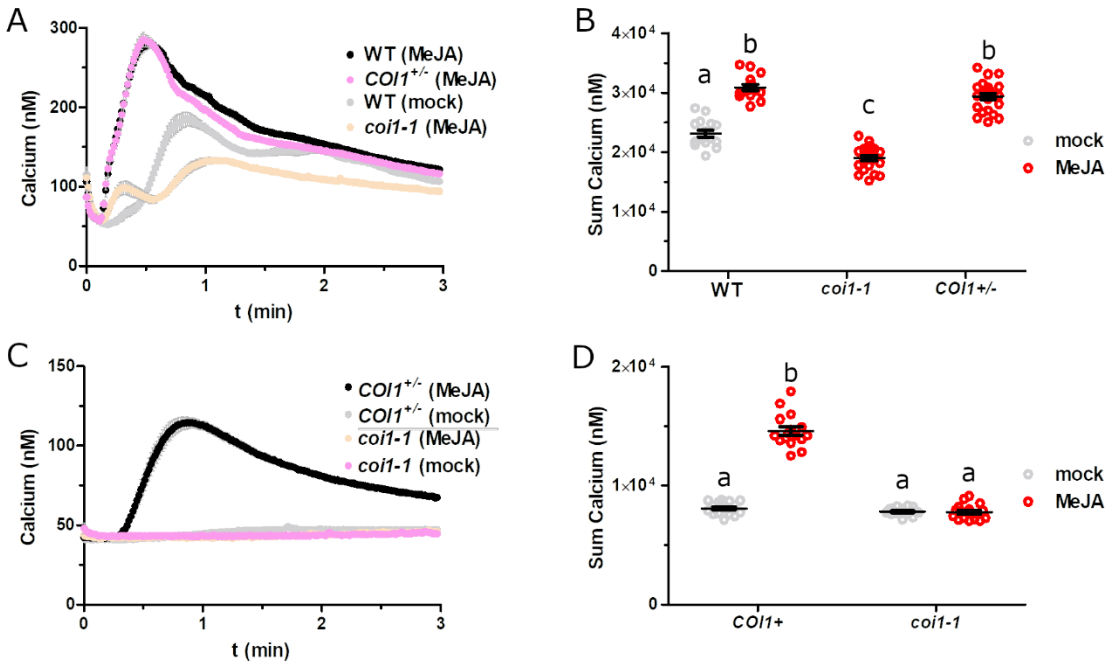


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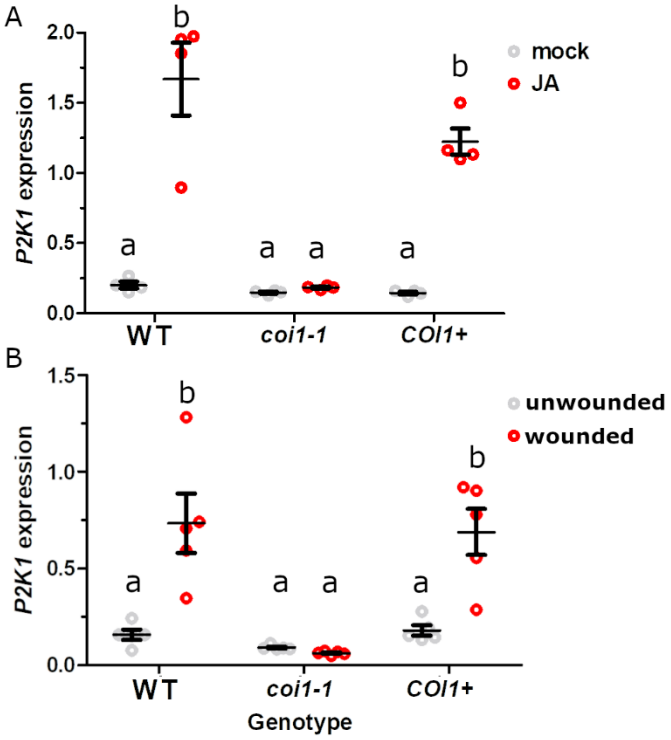


641

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643 *P2K1* expression was measured in 4-week-old aequorin-expressing plants of the indicated
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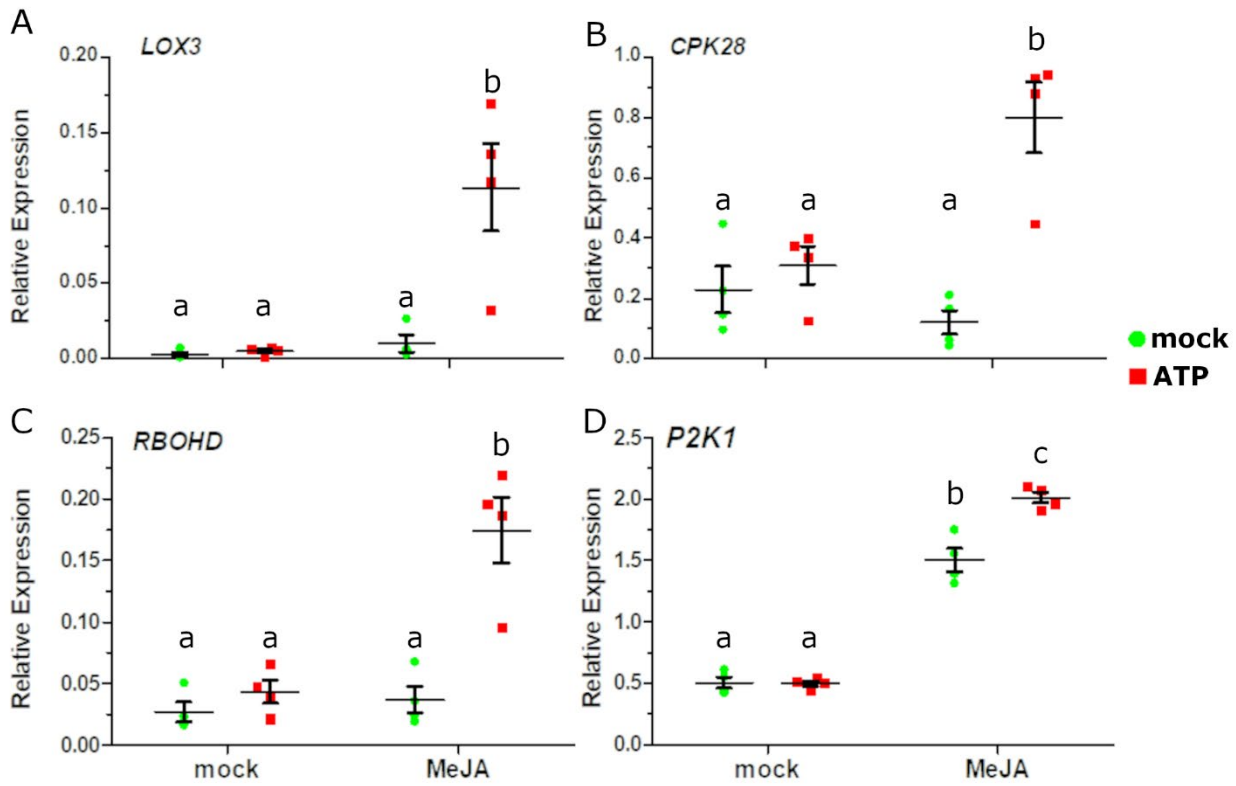


**Figure 7. JA treatment potentiates eATP-induced transcriptional responses.** Wild-type seedlings were pre-treated with or without 20 μM MeJA, followed by treatment with 0.5 mM ATP, as described in Materials and Methods. Gene expression was evaluated by RT-qPCR for **(A)** *LOX3*, **(B)** *CPK28,* **(C)** *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).