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Jasmonic acid and salicylic acid play minor roles in stomatal regulation by CO_2, abscisic acid, darkness, vapor pressure deficit, and ozone

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Summary

Jasmonic acid (JA) and salicylic acid (SA) regulate stomatal closure, preventing pathogen invasion into plants. However, to which extent abscisic acid (ABA), SA, and JA interact and what the roles of SA and JA are in stomatal responses to environmental cues remains unclear. Here, by using intact plant gas-exchange measurements in JA and SA single and double mutants, we show that stomatal responsiveness to CO_2, light intensity, ABA, high vapor pressure deficit, and ozone either did not or for some stimuli only very slightly depended on JA and SA biosynthesis and signaling mutants, including *dde2, sid2, coi1*, *jai1*, *myc2*, and *npr1* alleles. While stomata in the studied mutants clearly responded to ABA, CO_2, light, and ozone, ABA-triggered stomatal closure in *npr1-1* was slightly accelerated comparing with the wild type. Stomatal re-opening after ozone pulses was quicker in the *coi1-16* mutant than in the wild type. In intact Arabidopsis plants, spraying with methyl-JA led to only a modest reduction in stomatal conductance 80 min after the treatments, whereas ABA and CO_2 induced pronounced stomatal closure within minutes. We could not document a reduction of stomatal conductance after spraying with SA. Coronatine-induced stomatal opening was initiated slowly after 1.5-2 h and reached a maximum in 3 h after spraying intact plants. Our results suggest that ABA, CO_2 and light are major regulators of rapid guard cell signaling, whereas JA and SA could play only minor roles in whole-plant stomatal response to environmental cues in Arabidopsis and tomato.

Introduction

Colonization of dry land required vascular plants to reduce excessive water loss from plant tissues. Stomatal pores actively control transpiration as well as the uptake of CO_2 for photosynthesis in mesophyll cells. Guard cells responds to many environmental and endogenous cues and regulates ion channels and solute transporters in the guard cell membranes (Kollist *et al.*, 2014; Assmann and Jegla, 2016; Sussmilch *et al.*, 2019). The resulting reversible changes in the guard cell turgor and volume lead to stomatal opening or closure in accordance with light conditions, intercellular CO_2 concentration, air humidity, and soil water availability. Stomatal closure is also triggered by pathogen-associated molecular patterns and elicitors to prevent an invasion of pathogenic microorganisms into plants (Melotto *et al.*, 2006; Sawinski *et al.*, 2013). As a part of a complex multicellular organism, stomata should be coordinated with processes and events occurring in distant plant organs and tissues. Indeed, guard cells are able to recognize long-distance endogenous stimuli of different nature, including hormones (Marten *et al.*, 1991; Jia and Zhang, 2008). Stomatal responsiveness to plant hormones has been known for a long time (Acharya and Assmann, 2009), although certain aspects of this regulation remain unresolved, e.g. details of the interplay between hormones in stomatal aperture regulation (Murata *et al.*, 2015). Abscisic acid (ABA) efficiently induces fast stomatal closure and modulates stomatal regulation by environmental factors (Merilo *et al.*, 2013; Chater *et al.*, 2015; Brandt *et al.*, 2015; Hsu *et al.*, 2018). In addition, other hormones, including jasmonic acid (JA) and salicylic acid (SA), were suggested to control stomatal apertures (Melotto *et al.*, 2006; Munemasa *et al.*, 2007; Khokon *et al.*, 2010). However, their potential to mediate stomatal regulation in response to changes in the environment requires further research.

Signaling events in guard cells during ABA-induced stomatal closure have been well-characterized (Kim *et al.*, 2010; Munemasa *et al.*, 2015). Binding of ABA by PYR1/PYL/RCAR receptors results in inhibition of type 2C protein phosphatases (PP2C) (Ma *et al.*, 2009; Park *et al.*, 2009). This leads to the activation of the protein kinase OST1 (Park *et al.*, 2009; Vlad *et al.*, 2009; Umezawa *et al.*, 2009; Takahashi *et al.*, 2020), calcium-dependent protein kinases (Geiger et al. 2010; Brandt et al. 2012, 2015), and the receptor-like protein GHR1 (Hua *et al.*, 2012; Sierla *et al.*, 2018), promoting anion currents through the anion channel SLAC1 (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Geiger *et al.*, 2009; Lee *et al.*, 2009). These events trigger the efflux of anions, potassium, and water from guard cells, eventually leading to stomatal closure. Notably, ABA signaling in guard cells is also involved in the regulation of stomatal closure triggered by elevated CO_2, periods of darkness, reduced air

humidity (high vapor pressure deficit, VPD), and ozone (Xue *et al.*, 2011; Chater *et al.*, 2015; Merilo *et al.*, 2013; Merilo *et al.*, 2018; Sierla *et al.*, 2018; Hsu *et al.*, 2018). Low air humidity has been reported to activate ABA biosynthesis in guard cells or whole leaves (Bauer *et al.*, 2013; McAdam *et al.*, 2016), whereas elevated CO_2 concentrations do not trigger rapid ABA accumulation in guard cells (Hsu *et al.*, 2018; Zhang *et al.*, 2020). Furthermore, longer term 24 h and 48 h exposures to elevated CO_2 did not enhance ABA-induced reporter gene expression in guard cells, in contrast to ABA controls (Hsu *et al.*, 2018).

JA and its derivatives regulate vegetative and reproductive plant growth as well as defense responses to abiotic stress and pathogen attack (Katsir, Chung, *et al.*, 2008; Song *et al.*, 2014). Methyl JA (MeJA) was shown to trigger stomatal closure in epidermal peels by activation of slow-type anion channels through a process that requires calcium channels, NO accumulation, and reactive oxygen species (ROS) production by NADPH oxidases (RBOH D and F) (Suhita *et al.*, 2004; Munemasa *et al.*, 2007; Hua *et al.*, 2012; Yan *et al.*, 2015). MeJA-triggered stomatal closure involves the JA receptor CORONATINE INSENSITIVE 1 (COI1) as the stomatal apertures were not reduced by MeJA in the epidermal peels of the *coi1* mutant (Munemasa *et al.*, 2007). However, other research groups could not confirm the MeJA-triggered stomatal closure in Arabidopsis (Montillet *et al.*, 2013) or found that MeJA had a significantly lower potency in promoting stomatal closure compared to ABA or 12-oxo-phytodienoic acid, the precursor of JA (Savchenko *et al.*, 2014). In contrast, yet other research suggested that the pathogen-produced JA mimetic coronatine (COR) opens stomata (Melotto *et al.*, 2006). In JA signaling, MeJA is converted to the biologically active isoleucine-JA conjugate that is bound by the JA co-receptor comprising the JASMONATE ZIM DOMAIN (JAZ) proteins and COI1 (Katsir, Schilmiller, *et al.*, 2008). Isoleucine-JA and COR that mimics isoleucine-JA promote stomatal re-opening and suppress the stomatal closure triggered by pathogen-associated molecular patterns (Melotto *et al.*, 2006; Okada *et al.*, 2009; Toum *et al.*, 2016). Recently, quantification of metabolites in guard cells during high CO_2-induced stomata closure indicated a role for JA in CO_2 signaling. Furthermore, Arabidopsis JA signaling and biosynthesis mutants displayed impaired stomatal responses to elevated CO_2 in mesophyll-free leaf discs (Geng *et al.*, 2016). The involvement of the COI1-dependent JA signaling in the regulation of stomatal apertures and responses to changing environment should be further confirmed in intact plants.

SA has been extensively studied in plant-pathogen interactions, and its role in the regulation of plant development and response to abiotic stress has also been shown (Miura and Tada, 2014). SA is

important for stomatal immunity against pathogens based on impaired pathogen-triggered stomatal closure in mutants defective in SA biosynthesis and signaling (Melotto *et al.*, 2006; Zeng and He, 2010). The ability of SA to induce stomatal closure was directly demonstrated in experiments with SA-treated epidermal peels and detached leaves (Mori *et al.*, 2001; Khokon *et al.*, 2010; Panchal *et al.*, 2016). Furthermore, SA over-accumulating mutants display reduced stomatal aperture and elevated drought tolerance (Miura *et al.*, 2013). SA signaling in guard cells is mediated through the SA receptor NPR1 (Zeng and He, 2010; Ding *et al.*, 2018) and components of ABA signaling, including calcium-dependent protein kinases, but not OST1 (Prodhan *et al.*, 2018). Involvement of ethylene biosynthesis and signaling in SA-induced stomatal closure was recently suggested (Wang *et al.*, 2020). While ABA and MeJA induce ROS production by RBOH D and F in guard cells (Kwak *et al.*, 2003; Suhita *et al.*, 2004), ROS in SA-triggered stomatal closure could be produced by cell wall-bound peroxidases (Mori *et al.*, 2001; Khokon *et al.*, 2010) and, as recently indicated, by RBOH D and F (Wang *et al.*, 2020). SA activates anion currents in guard cells through the slow-type anion channel SLAC1 (Prodhan *et al.*, 2018).

Although it has been demonstrated that ABA signaling is involved in stomatal closure in response to many environmental cues (Merilo *et al.*, 2013; Chater *et al.*, 2015; Hsu *et al.*, 2018), the roles of JA and SA signaling in stomatal responsiveness to environmental stimuli have not been studied thoroughly. Here we used a genetic approach in Arabidopsis and tomato to address the impacts of JA and SA biosynthesis and signaling on stomatal function under changing environmental conditions. Since JA and SA display mutually antagonistic interactions (Bürger and Chory, 2019), we additionally studied double mutants with impaired responses to both JA and SA to explore possible interactions between these hormones in stomatal regulation. These experiments with plants defective in both SA and JA signaling, carried out in two independent labs, highlight that disruption of JA and SA signaling does not considerably modulate stomatal responsiveness to environmental factors. Furthermore, experiments with intact plants treated with increasing concentrations of MeJA and SA indicate that these hormones had a limited effect on stomatal conductance in intact Arabidopsis plants and thus, direct effect of these hormones to induce stomatal closure is significantly lower than that of ABA when these hormones are applied exogenously.

Results and Discussion

Plants defective in JA and SA signaling and biosynthesis display stomatal responses to elevated CO_2, darkness, and low air humidity

Numerous reports have demonstrated the role for ABA signaling in the regulation of stomatal closure in response to low air humidity, darkness, and higher-than-ambient CO_2 (Webb and Hetherington, 1997; Bauer *et al.*, 2013; Merilo *et al.*, 2013; Chater *et al.*, 2015; Merilo *et al.*, 2018; Yaaran *et al.*, 2019; Hsu *et al.*, 2018; Zhang *et al.*, 2020). MeJA and SA, hormones crucial for plant defense responses, have also been reported to affect stomatal aperture (Suhita *et al.*, 2004; Melotto *et al.*, 2006; Munemasa *et al.*, 2007; Khokon *et al.*, 2010; Yan *et al.*, 2015), however, their impact on stomatal responsiveness to environmental cues is less understood. To address this question, we used plant lines with mutations in JA and SA signaling and biosynthesis to monitor their stomatal responses to high/low CO_2, artificial darkness period, and low air humidity.

Throughout the gas-exchange experiments, steady-state whole-plant stomatal conductance in the studied Arabidopsis and tomato mutants did not vary dramatically, although this trait was 20% reduced in the JA-deficient *dde2-2* mutant compared to wild type Col-0 (Figure 1). When the intact rosettes of Arabidopsis plants were subjected to elevated CO_2, effective stomatal closure was observed in wild-type Col-0 as well as in all SA and JA related mutant plants (Figures 2a-d and S1a-d). Elevated CO_2 reduced stomatal conductance in plants with impaired JA and SA biosynthesis (*dde2-2* and *sid2-1*, respectively) or lacking the receptors to JA and SA (*coi1-16* and *npr1-1*, respectively) as well as in the *coi1-16 sid2-1* and *coi1-16 npr1-1*, the double mutants combining impairment of JA and SA signaling and biosynthesis. The magnitudes and rates of high CO_2-induced stomatal closure did not differ between the Col-0 plants and the mutant lines (Figures 2g-j and S1g-j). Stomatal re-opening in the ambient CO_2 was possibly very slightly delayed in *sid2-1*, according to the reduced initial rate of stomatal conductance recovery observed in some experiments (Figure 2i). To confirm these results, we carried out parallel experiments in the laboratory of J.I.S. (UCSD) with another experimental approach where we monitored stomatal conductance of individual Arabidopsis leaves upon changes in CO_2 concentration (Hu *et al.*, 2015). The *myc2-1* mutant with inactive MYC2 transcription factor regulating diverse JA-dependent processes (Lorenzo *et al.*, 2004) and the *coi1-30* mutant without active COI1 were studied. In accordance with the whole-plant responses, intact leaves of the *myc2-1* and *coi1-30* plants effectively closed and opened their stomata under higher and lower than ambient CO_2 concentrations (Figures 2e-f, k-l and S1e-f, k-l). The initial rate of stomatal opening in *coi1-30* was potentially very slightly enhanced compared to Col-0 in some of the experiments (Figure 1k). An intact responsiveness to CO_2 was observed in the top leaves of the tomato *jai1-1* mutant defective in the JA receptor COI1 homolog (Li *et al.*, 2004). This mutant did not differ from the corresponding wild type line in the experiments with high and low CO_2 treatments

(Figures 3 and S2). Thus, the results obtained by two independent laboratories using different experimental setups demonstrate that JA and SA do not play a significant role in high CO_2-induced stomatal closure. Stomatal opening in response to low CO_2 and recovery of stomatal conductance after elevated CO_2 might be only very slightly modulated by JA and SA signaling in Arabidopsis.

A recent study of the guard cell metabolome in *Brassica napus* demonstrated activation of the JA biosynthesis pathway by elevated CO_2 (Geng *et al.*, 2016). This suggested that JA biosynthesis might play a role in CO_2-triggered stomatal closure and further stomatal aperture assays carried out with epidermal peels showed reduced stomatal CO_2 sensitivity in the Arabidopsis JA insensitive mutants (*coi1*, *jar1* and *jin1/myc2*) (Geng *et al.*, 2016). It is possible that the CO_2 insensitivity phenotype that was observed by Geng et al. in the *coi1*, *jar1*, and *jin1/myc2* mutants could be related to the disruption of the contacts between stomata and mesophyll cells in epidermal peels. Involvement of mesophyll-driven signals in CO_2-induced regulation of stomatal apertures was suggested by a number of studies, although the nature of these signals remains elusive (Lawson *et al.*, 2014). Apoplastic malate promotes high CO_2-triggered stomatal closure by enhancement of the activity of the malate-sensitive R-type anion channel QUAC1 (Hedrich and Marten, 1993; Lee *et al.*, 2008; Meyer *et al.*, 2010). Sucrose and glucose in the apoplastic space provide another link between mesophyll and guard cells, connecting photosynthesis with regulation of stomatal apertures (Lawson *et al.*, 2014; Santelia and Lawson, 2016; Flütsch *et al.*, 2020). Rates of high CO_2-triggered stomatal closure can be modulated by a balance between foliar sucrose and malate in ferns and angiosperms (Lima *et al.*, 2019).

Darkness-induced stomatal closure recruits several independent signaling pathways in guard cells. The lack of CO_2 assimilation by photosynthesis in darkness and respiration lead to an increase of intercellular CO_2 that contributes to darkness-induced stomatal closure through activation of CO_2 signaling in guard cells (Roelfsema *et al.*, 2002). Further, the absence of blue light that is a part of the visual spectrum, inactivates phototropins and plasma membrane H^+-ATPases in guard cell membranes (Shimazaki et al., 2007). Darkness-induced stomatal closure depends on ABA signaling as it is partially impaired in the mutants lacking six ABA receptors or OST1 protein kinase (Merilo *et al.*, 2013). The receptor-like protein GHR1 that mediates ABA and CO_2 signaling in guard cells is also important for stomatal closure in darkness (Sierla *et al.*, 2018). In contrast, the mutants with interrupted JA and SA biosynthesis and signaling (*coi1-16*, *dde2-2*, *npr1-1*, *sid2-1*) as well as mutants combining these defects (*coi1-16 sid2-1* and *coi1-16 npr1-1*) demonstrated unaffected

stomatal closure during the artificially imposed darkness periods (Figures S3, S4a-d). Similar to the gas exchange experiments with changing CO_2, *sid2-1* demonstrated slightly slower recovery of stomatal conductance upon re-illumination than the wild-type plants (Figure S4e-h). These experiments indicate that JA and SA are not involved in stomatal closure triggered by darkness, while recovery of stomatal conductance after darkness can depend on SA biosynthesis.

ABA and basal ABA concentrations in guard cells have a paramount role in determining plant overall stomatal conductance (Gonzalez-Guzmann 2012, Merilo et al. 2013, 2018). However, ABA accumulation in guard cells during rapid stomatal closure induced by abrupt changes in vapor pressure deficit (VPD), i.e. reduced air humidity, is still under debate due to conflicting results, likely depending on the experimental setup and species studied. Some reports show that high VPD-induced stomatal closure is controlled by ABA biosynthesis and signaling (Xie *et al.*, 2006; Bauer *et al.*, 2013; Merilo *et al.*, 2013; McAdam *et al.*, 2016). However, a recent study demonstrated that the impaired ABA biosynthesis did not affect high VPD-induced stomatal closure while OST1, one of the central regulators of ABA response in guard cells, was of high importance for stomatal closure in response to reduced air humidity (Xie *et al.*, 2006; Merilo *et al.*, 2018). Apparently, rapid stomatal closure triggered by high VPD is controlled by OST1 which might be activated independently of ABA signaling by Raf-like kinases (Katsuta *et al.*, 2020; Soma *et al.*, 2020). The long-term stomatal adaptation to dry air involves modulation of ABA levels, further decreasing stomatal conductance (Yaaran *et al.*, 2019). In our experiments, single and double JA and SA biosynthesis and signaling mutant plants showed pronounced responses to the increase in VPD (Figure S5). The *sid2-1* mutant demonstrated a slightly lower magnitude of stomatal closure in 15 min and 60 min after the increase in VPD than the wild type Col-0 plants (Figure S6). Our results do not exclude that JA/SA-activated signaling can affect stomatal opening under elevated relative air humidity (>95%) as indicated by previous studies (Panchal *et al.*, 2016; Panchal and Melotto, 2017). Stomatal opening under these conditions is associated with activation of JA signaling and simultaneous down-regulation of SA-responsive genes in guard cells (Panchal *et al.*, 2016; Panchal and Melotto, 2017).

In summary, our results indicate that the SA and JA biosynthesis and signaling mutants maintain stomatal responsiveness to CO_2 levels, light intensity, and low air humidity. At the same time, rates and magnitudes of stomatal movements induced by these stimuli can be partly modulated by JA and SA.

JA and SA biosynthesis and signaling have a minor influence on ABA-induced stomatal closure

Since plant hormones frequently interact in physiological processes, we aimed to study whether JA and SA would influence ABA-induced stomatal closure in intact plants. We sprayed rosettes of wild type plants with 5 μM ABA which induced a fast and robust reduction in stomatal conductance (Figures 4 and S7). Stomata in leaves of the *coi1-16*, *dde2-2*, *npr1-1*, *sid2-1*, *coi1-16 sid2*-1, *coi1-16 npr1-1* mutants displayed rapid stomatal closure in response to 5 μM ABA which was comparable to wild type plants by its magnitude (Figures 4 and S7). Interestingly, stomata in ABA-treated *dde2-2* plants partially re-opened by the end of the experiments and the reduction of stomatal conductance was lower in *dde2-2* than that in the wild type plants 64 min after the ABA spraying (Figure 4e). The JA biosynthesis in *dde2-2* is interrupted before the formation of 12-oxo-phytodienoic acid that is able to induce stomatal closure by itself, most efficiently in combination with ABA (Montillet *et al.*, 2013; Savchenko *et al.*, 2014). Seemingly, the duration of ABA effect on stomata depends on the level of 12-oxo-phytodienoic acid as the *coi1-16* mutant demonstrated the same stomatal responsiveness to ABA as the wild type plants.

The antagonistic interaction between ABA and SA signaling has been suggested in studies of systemic acquired resistance (Yasuda *et al.*, 2008; Ton *et al.*, 2009). We found that the lack of NPR1 in the *npr1-1* mutant resulted in faster ABA-induced stomatal closure than that in the wild type plants, although the magnitude of the reduction in stomatal conductance did not differ between *npr1-1* and Col-0 at 28 min and 64 min after ABA spraying (Figures 4f and S7e). The *sid2-1* mutant displayed the same changes in stomatal conductance as the wild type plants (Figure S7f,i) probably due to a residual level of SA in this mutant (Wildermuth *et al.*, 2001). Thus, our results suggest that NPR1-dependent SA signaling influences ABA-triggered stomatal closure.

Previous research indicated that stomatal closure induced by MeJA and SA depends on both ABA basal levels and ABA signaling. An impaired stomatal responsiveness to MeJA and SA was observed in ABA-deficient mutants (Zeng and He, 2010; Hossain *et al.*, 2011). Studies of ABA insensitive mutants showed that MeJA-induced stomatal closure involves ABI1, ABI2, OST1 and does not require PYR1, PYL1, PYL2 and PYL4 ABA receptors (Munemasa *et al.*, 2007; Hossain *et al.*, 2011; Yin *et al.*, 2016). A recent study using a real time SnRK2/OST1 protein kinase FRET reporter showed strong SnRK2/OST1 activation by abscisic acid, but no activation by MeJA or elevated CO_2 (Zhang *et al.*, 2020). SA was shown to trigger phosphorylation of SLAC1 in guard cells

via calcium-dependent protein kinases, but not by OST1 (Prodhan *et al.*, 2018), possibly this mechanism is involved in the regulation of stomatal closure by pathogens. The dependence of ABA-induced stomatal closure kinetic on JA and SA should be studied additionally.

Disruption of SA and JA biosynthesis and signaling does not influence ozone-triggered stomatal closure

As an air pollutant, ozone damages plants and can lead to substantial losses in crop yield (Ainsworth *et al.*, 2012). Since ozone enters plants through stomata and degrades to reactive oxygen species (ROS) in the apoplast, ozone exposure became instrumental in studies of apoplastic ROS signaling in relation to cell death and stomatal functioning (Kangasjärvi *et al.*, 2005; Kollist *et al.*, 2007; Vahisalu *et al.*, 2010; Xu *et al.*, 2015). Importantly, a short-term ozone pulse induces stomatal closure through activation of ROS-dependent signaling pathways in guard cells (Kollist *et al.*, 2007; Vahisalu *et al.*, 2010). Since the apoplastic ROS formed from ozone resembles the ROS burst during pathogen infection (Vaahtera *et al.*, 2013), we studied whether stomatal responsiveness to apoplastic ROS/ozone depends on JA and SA levels or signaling. The JA and SA biosynthesis and signaling mutants were exposed to 3 min pulse of ~470 nL L^{-1} ozone, and changes in stomatal conductance were monitored. All analyzed mutants effectively closed their stomata after the 3-min ozone pulses, similar to the wild type (Figures 5 and S8). The *coi1-16 sid2-1* double mutant showed slightly reduced magnitude of ozone-induced stomatal closure (Figure S8k). Although recovery of stomatal conductance after ozone-induced stomatal closure has not been studied completely (Moldau *et al.*, 2011), our data indicate that the *coi1-16* mutant plants may show an accelerated stomatal re-opening (Figure 5h). Additionally, the *coi1-16 npr1-1* double mutant demonstrated enhanced rate of stomatal conductance recovery compared with Col-0 (Figures 5l and S8l).

In general, although SA and JA can induce apoplastic ROS formation (Mori *et al.*, 2001; Suhita *et al.*, 2004; Khokon *et al.*, 2010), stomatal responsiveness to apoplastic ROS induced by ozone does not depend on SA and JA levels or signaling. However, the recovery of stomatal conductance after a brief ozone pulse might depend on COI1.

MeJA and SA are considerably less effective to induce stomatal closure than ABA

Stomatal closure induced by MeJA and SA was reported in several studies, typically by measuring stomatal apertures in epidermal peels or detached leaves (Suhita *et al.*, 2004; Melotto *et al.*, 2006;

Munemasa *et al.*, 2007; Khokon *et al.*, 2010; Yan *et al.*, 2015). Here, we compared stomatal responses to MeJA and SA with that to ABA in intact plants. We sprayed whole Arabidopsis plants with ABA, MeJA, or SA and monitored whole-plant stomatal conductance for 23 h (Figures 6a-c and S9a-e). Stomatal conductance in the mock-treated plants was slightly increased in response to sprays and then gradually reduced before the night time according to the diurnal stomatal rhythm (Sierla *et al.*, 2018). As expected, spraying plants with 5 μM ABA resulted in a fast, pronounced, and prolonged stomatal closure (Figures 6a and S9d) which was still clearly detectable after 23 h (Figures S9a,e). At the same time, MeJA and SA in the concentrations up to 200 μM and 1000 μM, respectively, did not induce a prominent reduction of stomatal conductance, which would be comparable to that in plants treated with 5 μM ABA (Figures 6b,c and S9b-e). However, MeJA suppressed stomatal opening induced by brief leaf wetting/high humidity in the chambers (Panchal *et al.*, 2016; Yokoyama *et al.*, 2019) and further induced detectable slight stomatal closure. In 80 min after spraying, stomatal conductance in MeJA-treated plants was significantly reduced by 5-10% of the initial values (Figure S9b,d). Stomatal conductance in SA-treated plants was indistinguishable from that in the corresponding mock plants. These results demonstrate the ability of MeJA to suppress stomatal opening and to induce detectable stomatal closure in the sprayed intact Arabidopsis plants, although this response is not comparable with ABA-induced stomatal closing. Although a role for JA in stomatal opening requires further research, a recent publication demonstrates that JA signaling suppresses high temperature-triggered stomatal opening in tomato plants damaged by insects or mechanical wounding (Havko *et al.*, 2020).

Both MeJA and SA can enter guard cells via diffusion across plasma membranes (Seo *et al.*, 2001; Maruri-López *et al.*, 2019) and specific transporters which still should be identified in guard cells. To confirm that the hormones penetrated the sprayed plants and guard cells, we measured transcript levels of hormone-responsive marker genes (Figure 6d-g). Leaves of MeJA- and SA-treated plants demonstrated accumulation of *JAZ1* and *WRKY38* transcripts (Figure 6d,e), respectively, as it has been shown for comparable plant treatments in other studies (Chung *et al.*, 2008; Kim *et al.*, 2008). Spraying with 5 μM ABA similarly induced elevated ABA-responsive *HAI1* transcripts (Figure S9f). The uptake of the sprayed hormones by guard cells was confirmed by upregulation of *JAZ1* and *WRKY38* in guard cell-enriched epidermal fractions collected from JA- and SA-treated plants, respectively (Figure 6f,g). In another study, concentrations of JA and SA were estimated on the levels of ~9 pg mg^{-1} and ~115 pg mg^{-1} fresh weight, respectively (corresponding to ~40 pmol g^{-1} JA and ~0.83 nmol g^{-1} SA) in guard cell–enriched epidermal peels collected from Arabidopsis plants

(David *et al.*, 2020). Quantification of JA and SA in Arabidopsis leaves revealed similar ranges of the hormones (Forcat *et al.*, 2008; Pan *et al.*, 2010; Trapp *et al.*, 2014). Thus, spraying with the MeJA and SA solutions in the concentrations which were used in this study led to a significant increase of these hormones in guard cells and were efficient regarding the induction of biological effects in guard cells.

As a complementary method to study the role of MeJA and SA in the modulation of stomatal apertures, we collected epidermal peels from Arabidopsis plants and incubated them with ABA, MeJA, or SA for 3 h. Application of 400 and 1000 µM MeJA induced 15% and 26% stomatal closure in epidermal peels, respectively. In these experiments, SA induced noticeable stomatal closure only when applied at higher concentrations (1000 µM; Figure 6h). In comparison, 5 µM ABA triggered a 2.7-fold reduction in stomatal apertures.

Taken together, our gas-exchange results and direct stomatal aperture assays indicate that stomata are much more sensitive to ABA than to MeJA or SA. It is also possible that a long-term accumulation of MeJA or SA in guard cells is required to induce stomatal closure while stomata respond to ABA almost immediately (Figures 4, S7 and 6a).

Coronatine induces stomatal opening in intact Arabidopsis plants

Similar to the endogenous jasmonates, the phytotoxin coronatine (COR) activates JA signaling through COI1 in plants (Katsir, Schilmiller, *et al.*, 2008). COR has been reported to trigger stomatal opening and suppress stomatal closure to promote pathogen invasion (Toum *et al.*, 2016). Since MeJA showed only a weak effect on stomata in intact plants, we decided to investigate whether COR, also acting through COI1, would enhance stomatal conductance under the same conditions. Intact Arabidopsis plants were sprayed with 10 µM COR and stomatal conductance was monitored for 6 h. Similar to other experiments with plant spraying, a transient increase in stomatal conductance was observed during ~90 min after both mock and COR spraying. Although stomatal conductance was not altered after a single spray with 10 µM COR in a preliminary experiment, four consecutive rounds of sprays with 10 µM COR, which provided a long-term treatment for guard cells, resulted in a delayed but significantly increased stomatal conductance compared with mock treatments which were performed in parallel (Figures 7 and S10). Stomatal opening in COR-treated plants started ~110 min after spraying and reached maximal values 3 h after spraying before stomata

closed again. The mock-treated plants displayed a continuous decrease in stomatal conductance, attributed to the diurnal stomatal rhythm.

MeJA and COR have opposing roles in modulating stomatal response. MeJA induces stomatal closure as demonstrated in numerous studies and also this work by using different experimental approaches (Suhita et al., 2004; Munemasa et al., 2007; Hua et al., 2012, also Figures 6b and S9b). At the same time, COR triggers stomatal opening even in low concentrations delivered by spraying plants (Figure 7). The different effects of COR and MeJA on stomatal functioning could be explained by alternative signaling pathways activated by these substances in addition to the canonical COI1-JAZ1 pathway (Devoto *et al.*, 2005; Liu *et al.*, 2009; Zhou *et al.*, 2015). Thus, COR-induced stomatal opening can be mediated by RPM1-INTERACTING PROTEIN4 which activates AHA1 and AHA2 plasma membrane H^+-ATPases (Liu *et al.*, 2009).

Conclusion

The gas exchange experiments with JA and SA biosynthesis and signaling mutants showed that JA and SA did not directly mediate stomatal responses to CO_2, light-darkness transitions, low air humidity, ABA, and ozone in intact plants (Figures 2-5 and S1-S8). Only small differences between the wild type plants and the studied mutants were observed in the rates of stomatal opening and closing. JA signaling tends to restrict stomatal opening as the lack of COI1 accelerated the recovery of stomatal conductance after ozone pulses and very slightly enhanced low CO_2-induced stomatal opening (Figures 2k and 5h). Furthermore, the MeJA-treated plants did not exhibit stomatal opening triggered by high humidity (Figure 6b). At the same time, ABA-induced stomatal closure was accelerated in the SA-insensitive *npr1-1* mutants compared with the wild type. Although the influence of JA and SA on stomatal responsiveness to environmental stimuli was weak, modulation of stomatal opening and closing rates by these hormones deserves further attention.

While stomata respond rapidly and robustly to ABA, CO_2, darkness and ozone, stomatal closure triggered by MeJA and SA is substantially less efficient when the hormones were sprayed on intact plants (Figures 6a-c, S9d). Although the uptake kinetics by guard cells for ABA, MeJA, and SA might be significantly different, all of these hormones were delivered to guard cells by the same spraying method (Figures 6d-g, S9f). The time-resolved analysis of whole-plant stomatal conductance

demonstrates that stomata in intact plants respond to MeJA much slower and in a much smaller extent compared to ABA (Figure 6a,b). Furthermore, the biological effect of COR on guard cells develops with a lag as only a partial stomatal opening was initiated 1.5-2 h and reached the maximum 3 h after spraying with COR (Figure 7), in contrast to the rapid stomatal opening induced by low CO_2 and light. Since spraying with SA did not decrease stomatal conductance, SA-induced stomatal closure apparently requires high concentrations and/or prolonged SA treatments, which can be achieved by soaking epidermal peels in SA solutions (Figure 6h). Our results highlight the different roles of MeJA and SA defense hormones from that of ABA in the regulation of stomatal movements. ABA is a central regulator of many aspects in stomatal function, including stomatal responsiveness to environmental cues (Xue *et al.*, 2011; Merilo *et al.*, 2013; Chater *et al.*, 2015; Hsu *et al.*, 2018). JA and SA, which regulate plant defense against biotic stresses, induce partial stomatal closure as previously reported under specific conditions (e.g. herbivore or pathogen attack) and might modulate rates of stomatal reactions to some environmental cues. Further investigations of stomatal responses under diverse conditions could best be accomplished using gas exchange analyses of whole intact plants and in intact leaves, which enables time-dependent kinetic analysis.

Methods

Plant material and growth conditions

Arabidopsis plants were grown in a soil containing 2:1 (v:v) peat: vermiculite. Arabidopsis plants for whole-plant measurements of stomatal conductance were cultivated in growth chambers (AR-66LX and AR-22L, Percival Scientific, IA, USA) at 12 h photoperiod, 23/18˚C day/night temperature, 150 µmol m^{-2} s^{-1} light, and 70% relative humidity. Soil moisture was kept at ~80% of maximum water capacity. The plants for stomatal aperture assays were grown in growth rooms under the same conditions. For gas-exchange experiments, plants were grown through a hole in a glass plate covering growth pot as described in Kollist et al., (2007). The Arabidopsis plants for studies with individual leaves were grown on soil in a plant growth chamber Conviron E7/2 (Conviron, Canada) at a 12 h photoperiod, 21°C/19°C, relative humidity 70-80%, and photosynthetic photon flux density of ~150 µmol·m^{-2} s^{-1}. The plants were watered twice per week.

JA and SA mutants in this study have been well-characterized before. The following Arabidopsis mutants were used: *dde2-2* (also known as *aos*, allene oxide synthase) and *sid2-1* that fail to synthesize JA and SA, respectively (Wildermuth *et al.*, 2001; Malek *et al.*, 2002), *coi1-16* and *coi1-30* defective in the JA receptor COI1 (Ellis *et al.*, 2002; Xu *et al.*, 2015), and *npr1-1* lacking the SA receptor (Ding *et al.*, 2018). Additionally, we used the *myc2-1* (also known as *jin1*) mutant with inactive MYC2 transcription factor regulating diverse JA-dependent processes (Lorenzo *et al.*, 2004). To acquire more information about interactions of defense hormones in stomatal regulation, we used the double mutants *coi1-16 sid2-1* and *coi1-16 npr1-1* (Xu *et al.*, 2015). All Arabidopsis mutants were in the Col-0 genetic background and their homozygosity was verified routinely.

The tomato *jai1-1* mutant (Li et al., 2004; the *jai1* mutant is equivalent to the *coi1* mutant in Arabidopsis) and the corresponding wild type line (cultivar Castlemart) were germinated on a wet filter paper in darkness and were transferred into the soil at the age of 7-8 days. Plants were grown in the growth chambers at 12 h photoperiod, 23/18˚C temperature, 200 µmol m^{-2} s^{-1} light, and 70% relative humidity. Since the *jai1-1* mutation impairs seed production (Li *et al.*, 2004), homozygous *jai1-1* plants were selected from the progeny of heterozygous *jai1-1* plants (Bosch *et al.*, 2014). At the age of 14-16 days, cotyledons from the tomato plants were collected and used for DNA isolation. The plants were genotyped by using pairs of primers to the genomic DNA with and without the

deletion in the tomato COI1 analog (Table S1). The homozygous *jai1-1* mutants were employed for the gas-exchange experiments at the age of 3-4 weeks.

Gas-exchange experiments

The whole-plant stomatal conductance in the single (*coi1-16*, *sid2-1*, *dde2-2*, *npr1-1*) and double (*coi1-16 sid2-1* and *coi1-16 npr1-1*) Arabidopsis mutants was measured in the 8-chamber gas-exchange devices which were described before (Kollist *et al.*, 2007). Arabidopsis plants at the age of 3-4 weeks were inserted into the device and incubated about 1 h for stabilization of stomatal conductance. The standard conditions in the chambers were as follows: ambient CO_2 (423.9±4.8 μL L^{-1}, here and thereafter average±SD), 150 μmol m^{-2} s^{-1} light, 69.8±2.2% relative air humidity, 24.4±0.15°C. In order to characterize stomatal responses, the following stimuli promoting stomatal closure were applied: elevated CO_2 (798.8±11.5 μL L^{-1} CO_2), light-to-dark transition, spraying with 5 μM ABA, reduction of relative air humidity (from 68.9±1.7% to 27.5±4.85%, increasing vapor pressure deficit from 0.97±0.05 to 2.22±0.16 kPa), 3-min pulse of 467.3±80.7 nL L^{-1} ozone. For ABA treatments, the plants were sprayed with 5 μM ABA (in 0.012% aqueous Silwet L-77 solution) three times from different sides. Stomatal conductance in the JA and SA mutants and the wild type plants was monitored during 1 h. In some experiments, the recovery of stomatal conductance after the treatments was recorded.

Stomatal responses to ABA, MeJA, COR, and SA were also studied in the 8-chamber gas-exchange devices. The applied solutions of these hormones were prepared from 10 mM or 100 mM stock solutions (in 96% ethanol for ABA, MeJA, and SA; in 100% methanol for COR) and were supplemented with 0.012% aqueous Silwet L-77. The working solutions were used during a day. The Arabidopsis Col-0 plants at the age of 3-4 weeks were stabilized for 1 h under the standard conditions in the 8-chamber gas-exchange device. For hormonal treatments or the mock, the plants were taken from the chambers and sprayed three times from different sides. In some experiments, sprays with COR were repeated four times with 12-min intervals. Then, the plants were put back into the chamber and stomatal conductance was recorded every 4 or 16 min. For a mock, a solution with the corresponding concentrations of the solvent and Silwet L-77 were used to spray plants in parallel to the treatments with ABA, MeJA, COR, and SA. All experiments were started at the same time and followed the day/night regime of the plants, if needed.

Photographs of plants were taken before the experiments and leaf areas were calculated using ImageJ 1.37v (National Institutes of Health, USA). Stomatal conductance for water vapor was calculated according to von Caemmerer & Farquhar (1981). The energy budget equation was used to calculate temperatures of the Arabidopsis rosettes in cuvettes of the gas-exchange device (Parkinson, 1985).

Stomatal responsiveness to CO_2 was studied in individual leaves of the *coi1-30* and *myc2-1* Arabidopsis mutants attached to intact plants. Intact leaves of the 4-5-weeks-old plants were analyzed using the LI-6400 infrared (IRGA)-based gas exchange analyzer system with a leaf chamber (LI-6400-40, LI-COR Biosciences, USA). The clamped leaves were equilibrated and stabilized at 150 µmol m^{-2} s^{-1} light intensity (LED light source), 58-65% relative humidity, a temperature of 21°C, 360 ppm CO_2, and an incoming air flow of 200 µmol s^{-1} for 40 minutes. Stomatal conductance was recorded for 30 min at 360 µL L^{-1} CO_2, followed by 60 min at 800 µL L^{-1} CO_2 and 60 min at 100 µL L^{-1} CO_2.

Stomatal conductance in tomato plants was monitored by using a thermostated four-chamber, custom-built, flow-through gas-exchange device (Hõrak *et al.*, 2017). The measurements were performed on 2-3 intact top leaves of the homozygous *jai1-1* mutant and the wild type plants. The leaves were hermetically sealed in the chambers of the device and stabilized at 394.3±20.4 µL L^{-1} CO_2, 150 µmol m^{-2} s^{-1} light, 69.0±9.5% relative air humidity. Stomatal closure and opening were triggered by 740.9±35.6 µL L^{-1} and 93.5±6.0 µL L^{-1} CO_2, respectively.

Measurements of stomatal apertures

Leaves for stomatal aperture assays were collected from 4-5 weeks-old Arabidopsis plants. From the same leaf, abaxial epidermal peels for a mock and a hormonal treatment were collected. The epidermal peels were immediately transferred cuticle-side up into 6-cm Petri dishes filled with 10 mM MES, pH 6.15, 50 mM KCl. The buffer was supplemented with ethanol (mock) or hormones in various concentrations. The Petri dishes were incubated in a thermostated bath at a temperature of 22°C and a light of 150 µmol m^{-2} s^{-1} for 3 h. Stomata in the epidermal peels were examined with a Zeiss Axio Examiner D1 microscope with an x50 objective. Pictures of stomata were collected with VisiView 2.0 software (Visitron) and processed with ImageJ 1.37v to measure stomatal aperture width. For each of the samples, the average width of stomatal apertures was calculated for the treatment and the corresponding mock, based on examination of 15-30 stomata.

Transcripts quantification

To confirm the entry of MeJA and SA into the sprayed leaves, we quantified transcripts that are known to be induced by these hormones. Leaf samples were collected from the hormone-treated plants after the sprays in 2 h for MeJA, in 4 h for SA, and in 2 h for ABA. DNA-free total RNA was isolated from the samples by using the Spectrum Plant Total RNA kit (Sigma) according to the manufacture's recommendations. cDNA was synthesized with the RevertAid Premium Reverse Transcriptase (Thermo Fisher Scientific) and was used for real-time quantitative PCR (qPCR) using conditions described before (Kaurilind and Brosché, 2017). Sequences of the primers used for qPCR are listed in Table S1. Three reference genes *TIP41*, *YLS8*, and *SAND* were used to normalize the qPCR data in qBase 2.0 (Biogazelle). Guard cell-enriched epidermal fractions were collected according to (Jalakas *et al.*, 2017; Bauer *et al.*, 2013).

Statistical analysis

All experiments were repeated at least twice with similar results. One-way ANOVA was used to determine whether there were any statistically significant differences between groups in experiments. If the ANOVA models were statistically significant, post hoc tests were applied to identify the significantly different groups. Tukey's HSD test was used to compare multiple groups of samples (STATISTICA, 7.1). Differences between wild type plants and mutant lines were estimated by using Dunnett's post-hoc test. Effects of hormones on stomatal apertures and stomatal conductance were estimated using paired t-test (STATISTICA, 7.1). All effects were considered significant at $P<0.05$. The ANOVA models, the numbers of biological repeats as well as the results of t-tests and the post-hoc tests are shown in Data S1.

The gas-exchange results are presented in absolute and relative values. To characterize stomatal movements in response to changing environmental conditions and ABA, additional parameters were calculated. Changes in stomatal conductance were computed as a difference between stomatal conductance values before and a certain time after a stimulus was applied. The initial rates of changes in stomatal conductance were calculated as linear slopes of the curve reflecting short-time changes in stomatal conductance after application of a treatment.

Data availability statement

The results of gas exchange measurements and stomatal assays included in this study are available upon request from the corresponding author.

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Author contributions

H.K., M.B., J.I.S., and D.Y. planned the research and designed the experiments. O.Z., S.S., T. A.-S., H.P., J.U., and D.Y. performed experiments. H.K., M.B., J.I.S., D.Y., O.Z., S.S., and T. A.-S. analyzed the data. D.Y. wrote the paper with contributions from H.K., M.B., J.I.S., O.Z., S.S., and T. A.-S.

Conflict of interest statement

The authors declare no conflict of interests

Supporting Information

Figure S1. Changes in stomatal conductance driven by various CO_2 concentrations in Arabidopsis JA and SA biosynthesis and signaling mutants.

Figure S2. Changes in stomatal conductance induced by elevated (a) or reduced (b) CO_2 concentrations in leaves of the *jai1-1* tomato mutant and the corresponding wild type (Castlemart).

Figure S3. Stomatal responsiveness to darkness period in single (a-d, g-j) and double (e-f, k-l) Arabidopsis mutants with disturbed JA and SA biosynthesis and signaling.

Figure S4. Characterization of stomatal movements induced by darkness and re-illumination in JA and SA biosynthesis and signaling mutants.

Figure S5. Reduction of stomatal conductance under elevated vapor pressure deficit in JA and SA biosynthesis and signaling mutants.

Figure S6. Changes in stomatal conductance of the JA and SA biosynthesis and signaling mutants in response to elevated vapor pressure deficit.

Figure S7. ABA-induced reduction of stomatal conductance in JA and SA biosynthesis and signaling mutants.

Figure S8. Ozone-induced changes in stomatal conductance of Arabidopsis plants defective in JA and SA biosynthesis and signaling.

Figure S9. Plant responses to ABA, MeJA, and SA sprays.

Figure S10. Changes in stomatal conductance after spraying with coronatine (COR).

Table S1. Primers used in this work

Supporting data S1. ANOVA tables and results of t-tests and post-hoc tests

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Figure legends

Figure 1. Steady-state stomatal conductance in the studied JA and SA biosynthesis and signaling mutants. Whole-plant stomatal conductance was monitored in Arabidopsis plants at the age of 3-4 weeks (a-b). Stomatal conductance was also measured in individual leaves of intact 4-5-weeks-old Arabidopsis plants (c-d) and in top leaves of tomato plants (e). The boxes extend from the 25th to 75th percentiles with the horizontal lines plotted at the median values. The individual data points are shown as the dots while the whiskers are the min and max values (n=4-10). Asterisks show

significant differences between mutants and wild type (one-way ANOVA followed by Dunnett's post-hoc test; P<0.05).

Figure 2. CO_2-triggered regulation of stomatal conductance in Arabidopsis JA and SA biosynthesis and signaling mutants. Time courses for stomatal conductance in the studied mutants and the corresponding wild type Col-0 plants are shown (average ± SE). Stomatal conductance is shown in relative values calculated from the data presented in Figure S1. At time 0, elevated ~800 µL L^{-1} CO_2 was applied for 60 min, followed by ambient (~420 µL L^{-1}) (a-d) or ~100 µL L^{-1} CO_2 (e,f). Experiments with the intact plants included the single (a-c) and double (d) mutants with impaired JA and SA biosynthesis and signaling (n=6-10). Stomatal responses to elevated and reduced CO_2 were studied in the leaves of the *coi1-30* (e) and *myc2-1* (f) Arabidopsis mutants (n=3-4). Changes in CO_2 concentrations are indicated in the bars above the graphs. (g-j) Initial rates of stomatal closure and stomatal opening were calculated as linear slopes of stomatal conductance curve within 8 min and 20 min after application of high and ambient CO_2, respectively. (k-l) initial rates of stomatal closure and stomatal opening in individual leaves were calculated within 10 min and 20 min after application of high and low CO_2, respectively. The boxes extend from the 25th to 75th percentiles with the horizontal lines plotted at the median values. The individual data points are shown as the dots while the whiskers are the min and max values (n=3-10). Asterisks show significant differences between mutant lines and Col-0 (one-way ANOVA followed by Dunnett's post-hoc test; P<0.05).

Figure 3. Stomatal responsiveness to elevated (a) or reduced (b) CO_2 concentrations in leaves of the *jai1-1* tomato mutant and the corresponding wild type (Castlemart). The treatments were started at time 0. Time courses for stomatal conductance are shown (average ± SE). Stomatal conductance is shown in relative values calculated from the data presented in Figure S2 (n=5 for *jai1-1*, n=7 for Castlemart). (c) Changes in stomatal conductance in leaves of *jai1-1* and Castlemart 80 min and 88 min upon elevated and reduced CO_2, respectively. (d) The rates of stomatal closure and stomatal opening in elevated and low CO_2 were calculated as linear slopes of the stomatal conductance curves within 16 min and 56 min after application of elevated and reduced CO_2, respectively. The boxes extend from the 25th to 75th percentiles with the horizontal lines plotted at the median values. The individual data points are shown as the dots while the whiskers are the min and max values. No statistically significant differences were detected between mutant lines and Castlemart with one-way ANOVA (P<0.05).

Figure 4. Arabidopsis plants impaired in JA and SA biosynthesis and signaling demonstrate pronounced ABA-induced stomatal closure. The 3-4 weeks-old plants were sprayed with 5 μM ABA at time 0 (marked with the arrows). Single mutants with impaired JA (a) and SA (b-c) signaling and biosynthesis as well as double mutants (d) were studied. Time courses for stomatal conductance in the studied mutants and the corresponding wild type Col-0 plants are shown (average ± SE, n=6-10). Stomatal conductance is shown in relative values calculated from the data presented in Figure S7. (e) The reduction of stomatal conductance 28 min and 64 min after ABA spraying. (f) The initial rates of ABA-induced stomatal closure in *npr1-1* and Col-0 were calculated as slopes of stomatal conductance curve within 12 min after ABA spraying. The boxes extend from the 25th to 75th percentiles with the horizontal lines plotted at the median values. The individual data points are shown as the dots while the whiskers are the min and max values (n=6-10). Asterisks show significant differences between mutant lines and wild type (one-way ANOVA followed by Dunnett's post-hoc test; $P<0.05$).

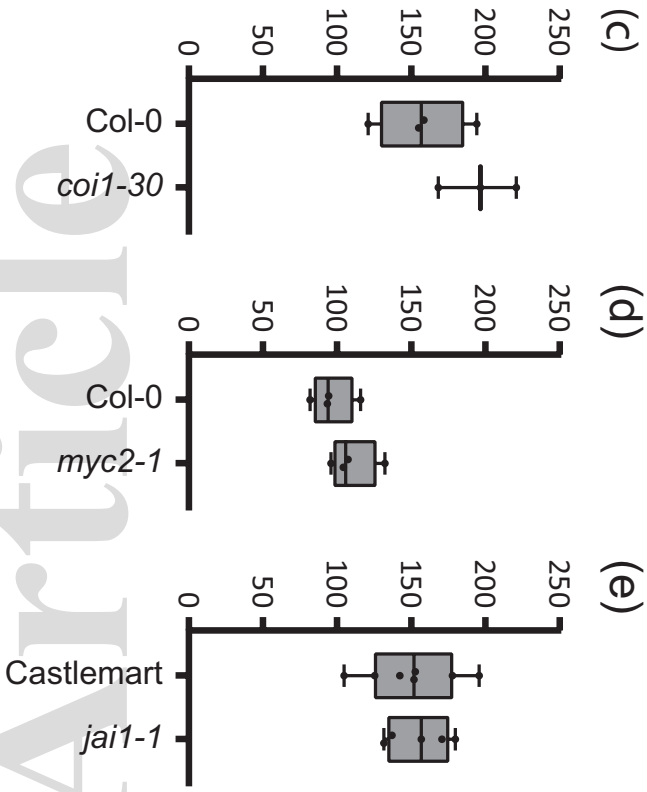
Figure 5. Ozone-induced stomatal closure in Arabidopsis mutants with disrupted JA and SA biosynthesis and signaling. Plants at the age of 3-4 weeks were exposed to ~470 nL L^{-1} ozone pulse for 3 min at time 0. Single mutants with impaired JA (a-b) and SA (c-d) signaling and biosynthesis as well as double mutants (e-f) were studied. Time courses for stomatal conductance in the studied mutants and the corresponding wild type Col-0 plants are shown (average ± SE, n=5-10). Stomatal conductance is shown in relative values calculated from the data presented in Figure S8. (g-j) The rates of stomatal conductance recovery were calculated as the linear slopes of stomatal conductance curve within 23-35 min after ozone exposure. The boxes extend from the 25th to 75th percentiles with the horizontal lines plotted at the median values. The individual data points are shown as the dots while the whiskers are the min and max values. Asterisks show significant differences between mutant lines and wild type (one-way ANOVA followed by Dunnett's post-hoc test; $P<0.05$).

Figure 6. Effects of ABA, MeJA, and SA treatments on stomatal functioning in Arabidopsis.

Arabidopsis plants (Col-0) at the age of 3-4 weeks were sprayed with ABA (a), MeJA (b), or SA (c) in various concentrations. Time courses are shown for stomatal conductance (average ± SE, n=4 for ABA, n=16-24 for MeJA and SA). Relative expression of *JAZ1* and *WRKY38* after MeJA (d) and SA (e) treatments, respectively, in plants analyzed in (b) and (c) (n=3). The same transcripts were quantified in guard-cell enriched epidermal fractions (GC-enriched) collected from MeJA- and SA-treated plants (n=4) (f and g). Asterisks show significant differences between mock and hormonal

treatments (one-way ANOVA followed by Dunnett's post-hoc test; $P<0.05$). (h) ABA demonstrates a significantly higher potency to induce stomatal closure in stomatal assays than MeJA and SA. Epidermal peels were collected from 4-5 weeks old plants and incubated in stomatal opening buffer supplemented with ethanol (mock), ABA, MeJA or SA. The width of stomatal apertures was measured after 3 h exposure (n=9-10 individual plants). The boxes extend from the 25th to 75th percentiles with the horizontal lines plotted at the median values. The individual data points are shown as the dots while the whiskers are the min and max values. Asterisks show statistically significant difference between mock (-) and the hormonal treatment (+) by repeated-measures ANOVA ($P<0.05$).

Figure 7. Stomatal opening induced by coronatine (COR) in intact Arabidopsis plants. (a) Plants at the age of 3-4 weeks were sprayed with 10 μM COR 4 rounds with the interval of 12 min (shown as the gray box). For comparison, other plants were sprayed with mock (0.1% methanol, 0.012% Silwet L-77) in parallel to the COR treatments. Time courses for stomatal conductance are shown (average ± SE, n=4-5). (b) The same data as (a) expressed as relative values to time 0.



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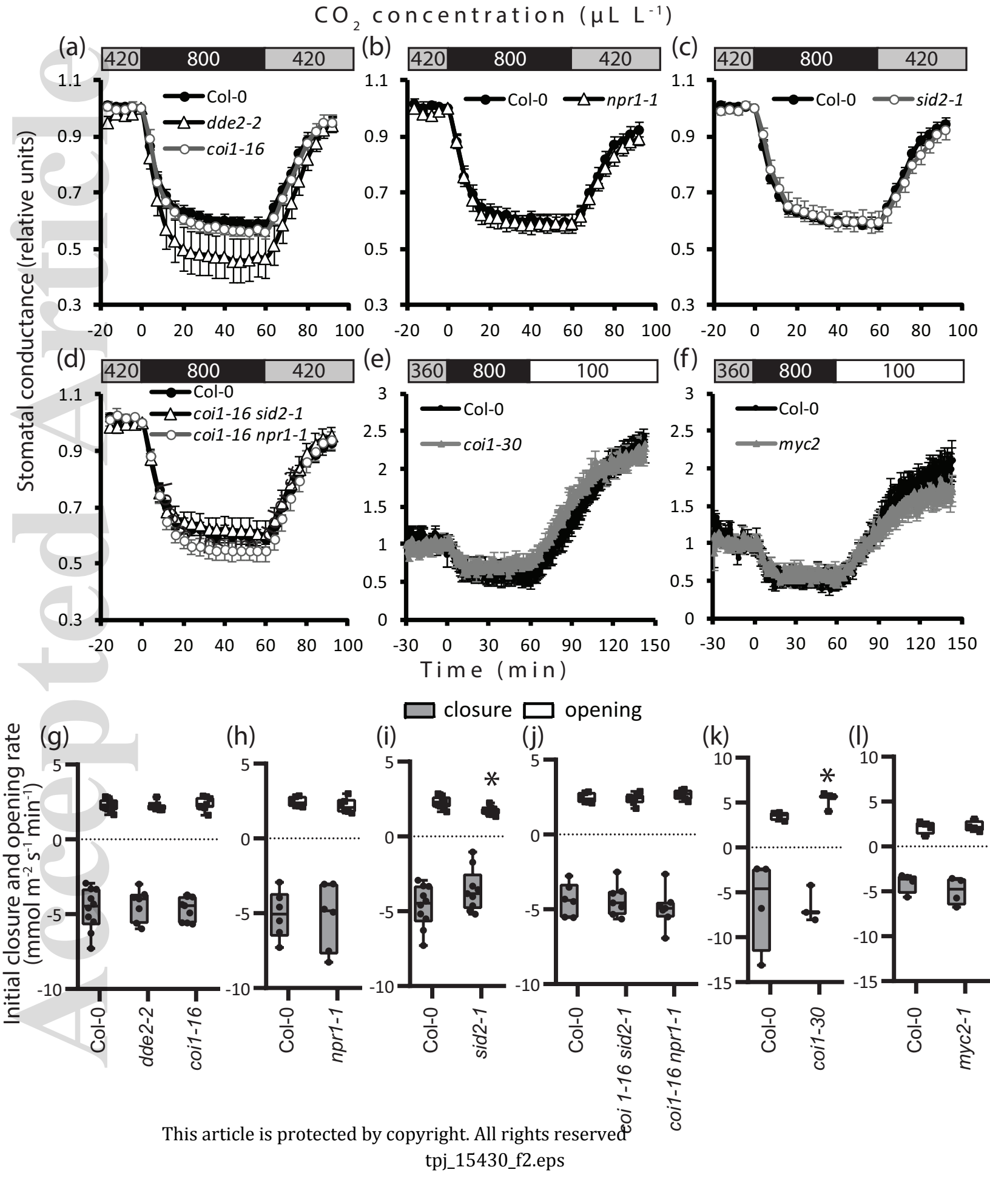

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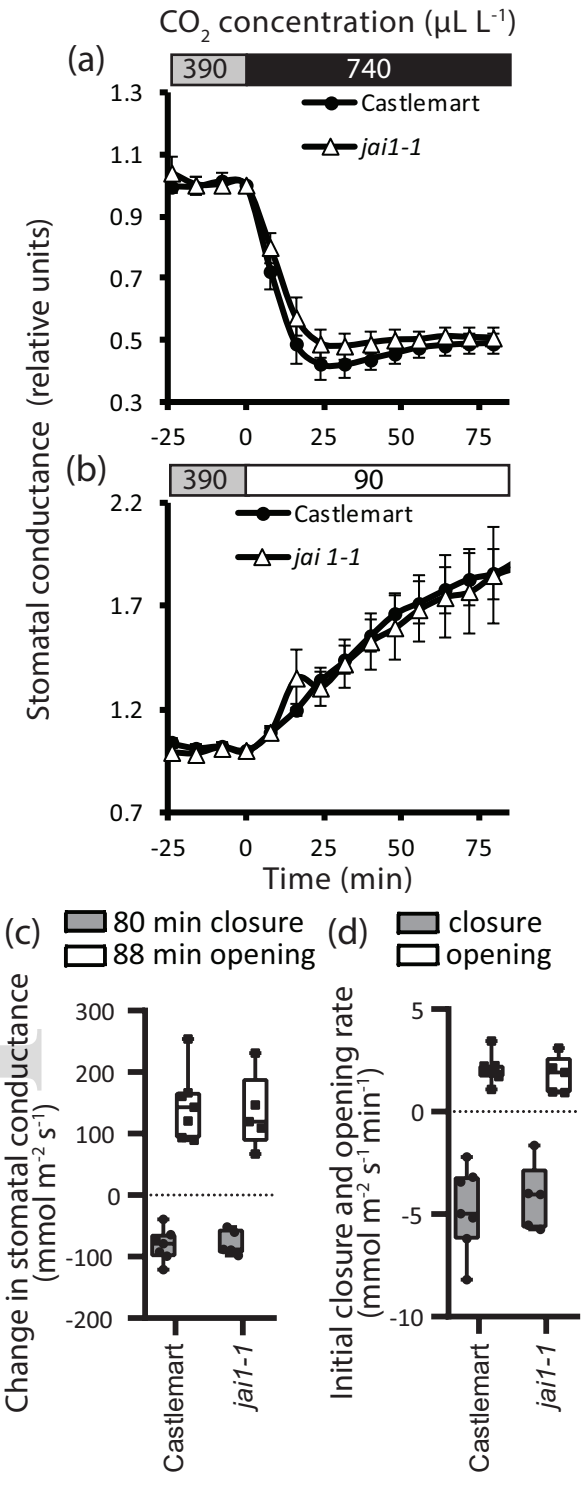

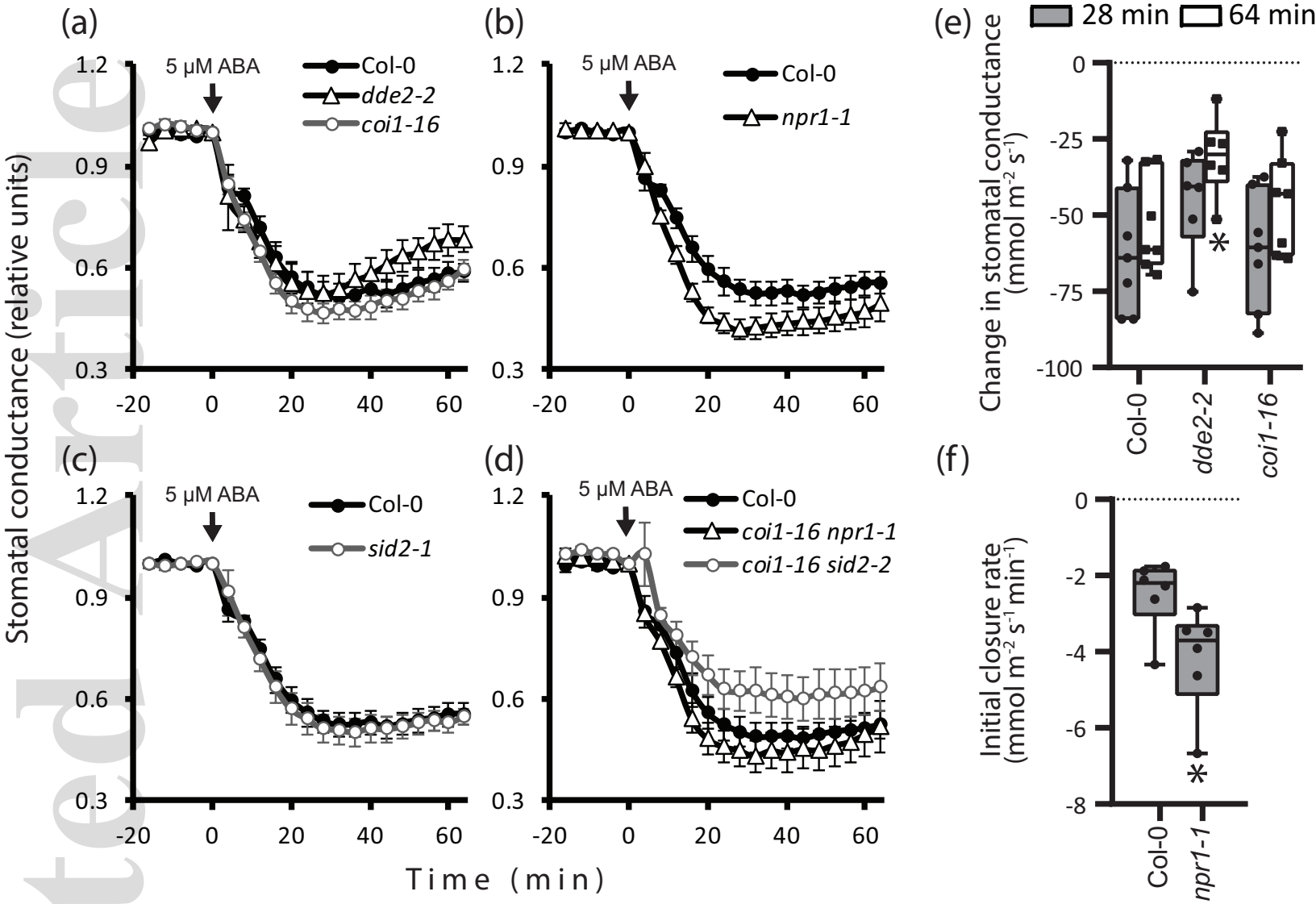

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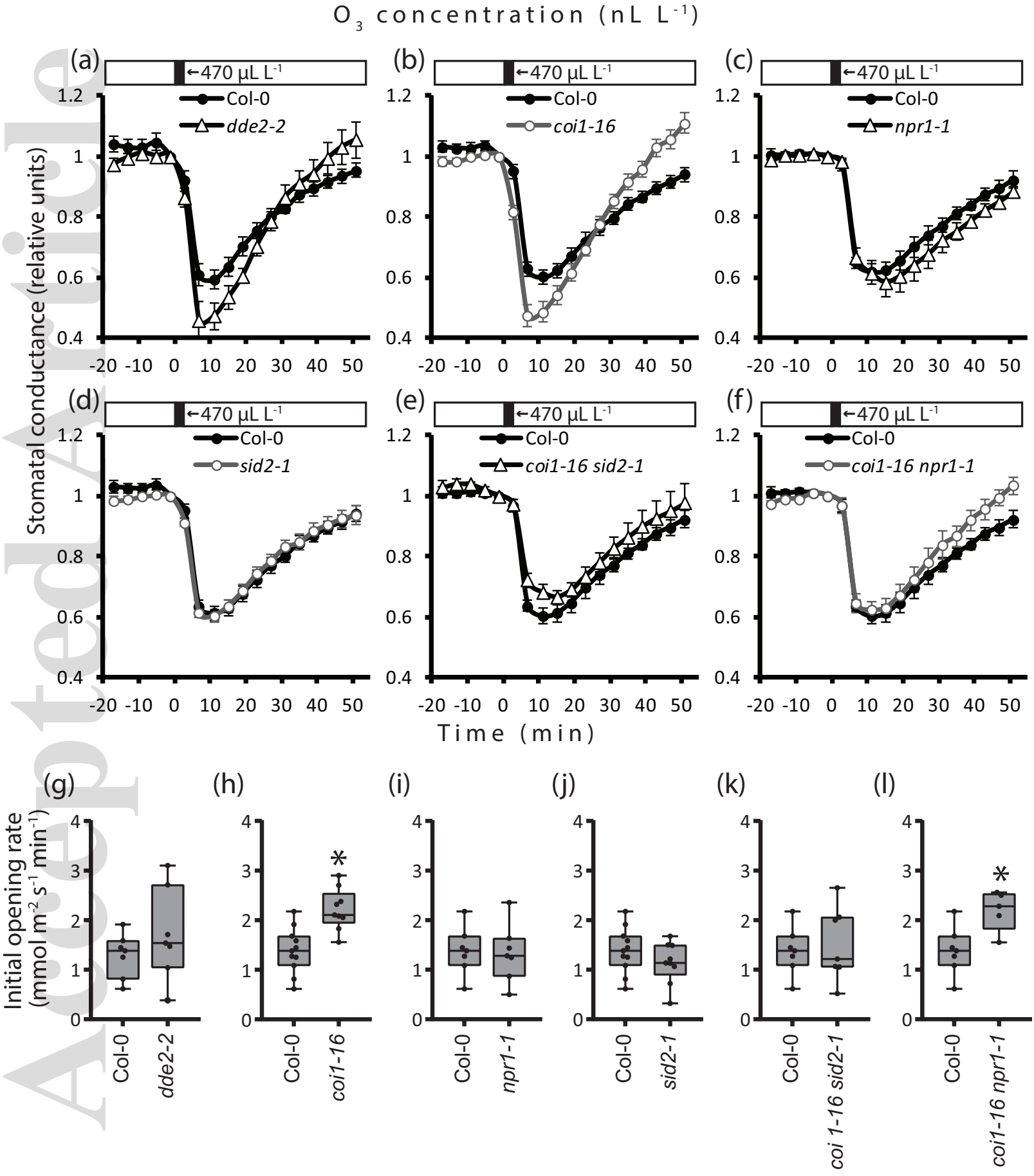

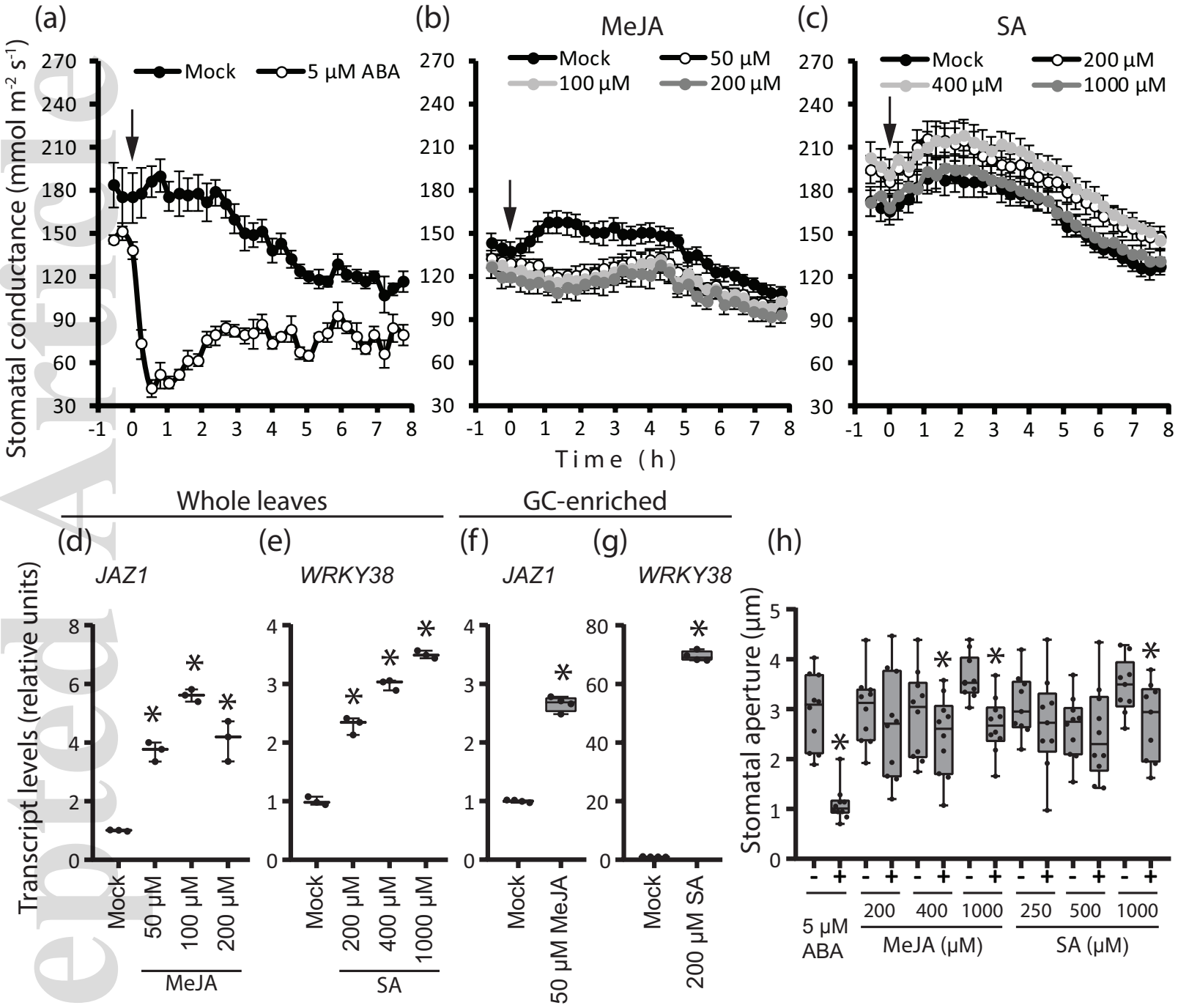

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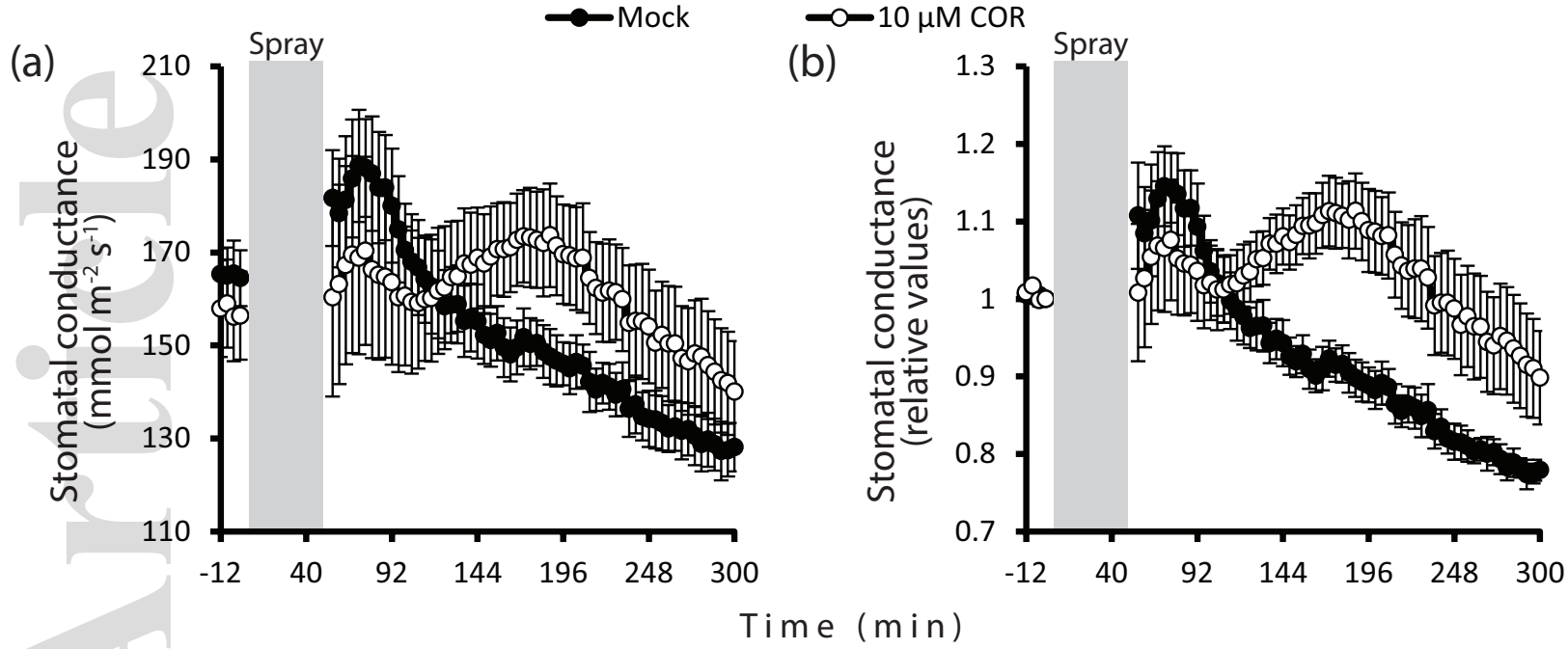

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