PROF. PHILIPPE REYMOND (Orcid ID : 0000-0002-3341-6200)

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Pieris brassicae eggs trigger inter-plant systemic acquired resistance against a foliar pathogen in Arabidopsis

Zigmunds Orlovskis and Philippe Reymond

Department of Plant Molecular Biology, University of Lausanne, 1015 Lausanne, Switzerland

Author for correspondence:

Philippe Reymond

Tel: +41 21 692 41 90

philippe.reymond@unil.ch

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ORCID Nr Zigmunds Orlovskis: 0000-0001-8583-9814

ORCID Nr Philippe Reymond: 0000-0002-3341-6200

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Summary

- Recognition of plant pathogens or herbivores activate a broad-spectrum plant defense priming in distal leaves against potential future attacks, leading to systemic acquired resistance (SAR). Additionally, attacked plants can release aerial or belowground signals that trigger defense responses, such as SAR, in neighboring plants lacking initial exposure to pathogen or pest elicitors. However, molecular mechanisms involved in inter-plant defense signal generation in sender plants and decoding in neighboring plants are not fully understood.
- We previously reported that *Pieris brassicae* eggs induce intra-plant SAR against the foliar pathogen *Pseudomonas syringae* in *Arabidopsis thaliana*. Here we extend this effect to neighboring plants by discovering an egg-induced inter-plant SAR via mobile root-derived signal(s).
- The generation of egg-induced inter-plant SAR signal requires pipecolic acid (Pip) pathway genes *ALD1* and *FMO1* but occurs independently of salicylic acid (SA) accumulation in sender plants. Furthermore, reception of the signal leads to accumulation of SA in the recipient plants.
- In response to insect eggs, plants may induce inter-plant SAR to prepare for potential pathogen invasion following feeding-induced wounding or to keep neighboring plants healthy for hatching larvae. Our results highlight a previously uncharacterized belowground plant-to-plant signaling mechanism and reveals genetic components required for its generation.

Keywords: systemic acquired resistance (SAR), insect eggs, plant–herbivore interactions, neighborhood effects, plant–plant interactions, belowground signals, plant pathogens

Introduction

Plants have evolved mechanisms to recognize molecular patterns from attacking pathogens and herbivores or their inflicted damage (Gust *et al.*, 2017; Ranf, 2017). This recognition of non-self or self molecules triggers defense responses not only in local but also in systemic organs, priming these tissues for future attack, a process called systemic acquired resistance (SAR) (Pieterse *et al.*, 2009; Fu & Dong, 2013). Furthermore, upon stress, plants release aboveground and belowground info-chemicals, including volatile organic compounds (VOCs) or root-exudates, which function in 1) direct defense against the attacking herbivores or pathogens, 2) indirect defense to recruit natural enemies or 3) serve as chemical cues to neighboring plants (Bais *et al.*, 2006; Dicke & Baldwin, 2010; Delory *et al.*, 2016; Ninkovic *et al.*, 2019). Plants that ''eavesdrop'' on the chemical status of the attacked neighbors may benefit from the emitted signals by priming or pre-inducing their own defenses against the oncoming attack, thereby reducing future damage (Heil & Karban, 2010; Karban *et al.*, 2014). Moreover, inter-plant signals may not only benefit the receiver but also increase the inclusive fitness of the emitter, and, thus, could be considered as mutually beneficial plant-to-plant chemical communication (Kalske *et al.*, 2019).

Insect eggs are recognized by plants and induce direct and indirect defenses (Reymond, 2013; Hilker & Fatouros, 2015). For insects, the site of oviposition is determinant for the hatching progeny and any mechanism enhancing larval survival may be favored. Studies have shown that previous oviposition affects performance of hatching larvae, although the effect is variable across plant species (Bruessow *et al.*, 2010; Pashalidou *et al.*, 2015; Austel *et al.*, 2016; Bandoly *et al.*, 2016; Bonnet *et al.*, 2017; Lortzing *et al.*, 2019). In Arabidopsis, insect eggs provoke cellular and molecular changes that are observed during infection with biotroph pathogens. Indeed, oviposition by the Large White butterfly *Pieris brassicae* triggers localized necrosis, accumulation of reactive oxygen species, and expression of hundreds of genes that are drastically distinct from those differentially regulated after larval feeding (Little *et al.*, 2007). Strikingly, egg-induced transcriptional profile is enriched with genes regulated by the salicylic acid (SA) signaling pathway (Little *et al.*, 2007). Accordingly, oviposition by *P. brassicae* leads to SA accumulation in local and systemic leaves and crude egg extract (EE) activate expression of SA- and innate immunity-dependent genes (Bruessow *et al.*, 2010; Gouhier-Darimont *et al.*, 2013). EE application enhances

further larval performance of the generalist *Spodoptera littoralis* by suppressing expression of jasmonic acid (JA)-dependent genes (Bruessow *et al.*, 2010). This effect is lost in the SA biosynthesis-deficient *sid2-1* mutant (Bruessow *et al.*, 2010), illustrating the known antagonistic interaction between SA- and JA-pathways (Pieterse *et al.*, 2012), and suggests that insect eggs may in some cases use the SA pathway to dampen defenses against generalist larvae.

Recently, we discovered that oviposition induces a SAR in Arabidopsis. When plants were pretreated with intact eggs or EE, growth of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) was significantly inhibited in local and distal leaves (Hilfiker *et al.*, 2014). By reducing bacterial infection on the plant, this egg-induced SAR may prove beneficial for hatching larvae. It was indeed shown that *P. brassicae* larval performance was reduced on Arabidopsis plants infected with *Pst* and that this effect was less pronounced when plants were pretreated with EE (Hilfiker *et al.*, 2014). SAR is commonly associated with a primary infection by a pathogen that results in a systemic protection upon a secondary challenge by a broad range of pathogens (Vlot *et al.*, 2008; Fu & Dong, 2013; Shah & Zeier, 2013). SAR depends on the SA pathway and primes systemic leaves for a stronger and prolonged expression of defenses genes (Návarová *et al.*, 2012; Fu & Dong, 2013; Shah & Zeier, 2013). The nature of the translocated signal(s) is however still debated but candidate SAR mobile molecules include methyl salicylate, azelaic acid, glycerol-3-phosphate, dihydroabietinal, and pipecolic acid (Pip) (Fu & Dong, 2013; Shah & Zeier, 2013). With regard to the lysine catabolite Pip, studies showed that Pip accumulates in local and systemic leaves after leaf inoculation with *Pseudomonas syringae* pv. *maculicola* (*Psm*), and that treatment by Pip enhances resistance to bacterial pathogens by stimulating SA accumulation and defense gene expression (Návarová *et al.*, 2012). The first step of Pip synthesis is carried-out by the aminotransferase AGD2-LIKE DEFENCE RESPONSE PROTEIN1 (ALD1) and *ald1* mutant is compromised in *Psm*-induced SAR (Návarová *et al.*, 2012; Ding et al., 2016; Hartmann et al., 2017). In addition, FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) is necessary for systemic accumulation of SA, SAR establishment and Pip-induced resistance, being therefore a SAR downstream component (Mishina & Zeier, 2006; Zeier, 2013). Recent data show that FMO1 is a N-hydroxylase that converts Pip to N-hydroxypipecolic acid (NHP), the critical regulator of SAR (Chen *et al.*, 2018; Hartmann *et al.*, 2018). Insect egg-induced SAR was abolished in *ald1* and

fmo1, implicating the Pip pathway in this response and suggesting a conserved mechanism between egg- and pathogen-induced SAR (Hilfiker *et al.*, 2014).

Recent evidence suggests that recognition of bacterial elicitors leads to generation of aboveground (Riedlmeier *et al.*, 2017; Wenig *et al.*, 2019) and belowground (Song *et al.*, 2016) inter-plant communication which is capable of inducing SAR in neighboring plants. However, the molecular mechanisms involved in the generation (encoding) of the inter-plant info-chemicals as well as receiving and decoding these signals in defense phenotypes are not yet elucidated. Looking at similarities and differences between systemic signal generation and decoding within and between plants may aid our understanding how such mechanisms evolve and enable researchers to genetically test ecological hypotheses about the potential benefits of plant-to-plant signals for their emitters and receivers. Moreover, this would further add to our understanding about the diversity of aboveground and belowground inter-plant signals and their interplay in response to different types of plant attacks.

Here, we found that that insect eggs induce inter-plant SAR against the foliar pathogen *Pseudomonas syringae* via mobile root-derived signal in Arabidopsis. Furthermore, the generation of the insect egg-induced inter-plant SAR ~~signal~~ was shown to require ALD1 and FMO1 but to occur independently of SA accumulation in the sender plants. We further discuss results in the context of ecological implications of such interplant-signals and current knowledge about SAR mechanisms in plants.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) was sown on potting compost (pasteurized at 100°C for 2 h) and vernalized for 2 days at 4°C. After vernalization, plants were incubated in a growth room (20°C; 70% RH; 10 h of light at 100 µE $s^{-1}m^{-2}$) for 4 weeks. Foliar fertilizer Wuxal (Aglukon, Germany) was applied to two week old seedlings by watering soil according to manufacturer's instructions. The following mutant or transgenic lines, described previously, were used in this study: *ald1* (Song *et al.*, 2004), *fmo1* (Mishina & Zeier, 2006), *sid2-1* (also known as *ics1*) (Nawrath & Métraux, 1999) and *nahG* (Delaney *et al.*, 1994). All following treatments were done with four week old plants.

Experimental setup

For intra- and inter-plant SAR experiments, 4 plants were grown equidistant from each other in conical plastic pots (r_{top}= 7cm, r_{base}= 5 cm, h= 5.5 cm; V_{soil}≈ 130 mL). For experiments with soil barriers, a custom-cut, impermeable plastic barrier or a nylon mesh (SEFAR NITEX® 03-11/6, pore size 10.68 (± 0.35 SE) µm) was placed inside each pot and secured with Micropore Tape (3M, 1530-1) on the sides and the bottom of the pot. For experiments with an aerial barrier, a transparent plastic film (h= 7 cm, l= 11 cm) was placed vertically on top of the conical pots. Small cut was made at the longer edge to allow the aerial barrier sit vertically 1 cm deep in the soil with 2 cm overhang from the edge of each pot.

Experiments with distance effects on inter-plant SAR were set up in rectangular plastic pots (h= 5 cm; l= 17.5 cm, w= 12.5 cm, V_{soil}≈ 1.09 L) by placing a row of 4 plants parallel the shortest edge. The distance between plants in a row approximates the distance between plants in the conical pots. The distance between two rows of 4 plants was set at 3 cm, 6 cm, 9 cm or 12 cm.

Oviposition and egg extract treatment

Cabbage white *(Pieris brassicae* Lynn.) was reared on Brussels sprouts (*Brassica oleracea var. gemmifera*) in 1 m^3 cages in a greenhouse (25 ± 5 °C, 60 ± 5% RH, 16/8 h light-dark cycle). For oviposition, a pot with four Arabidopsis plants was transferred into a perforated plastic bag and placed in a cage with adult butterflies. Narrow slits were cut in the bag to expose only two Arabidopsis leaves of selected plants to egg-laying by *P. brassicae* for 24 h. Each leaf received one to two egg batches consisting of 10-20 eggs each. Control plants were similarly placed in a cage without butterflies.

For egg extract treatment, eggs laid by *P. brassicae* colony on *B. oleracee* were manually removed and crushed with a pestle in 1.5 mL micro-centrifuge tubes. After centrifugation (15'000 g, 3 min), the supernatant ("egg extract", EE) was stored at −20 °C. For each plant, each of two leaves were treated with 2 µl of EE. This amount corresponds to one egg batch of ca. 20 eggs. No treatment was applied to control plants.

SAR assays

Cultivation of bacteria, plant infection and bacterial growth determination was done as described previously (Hilfiker *et al.*, 2014). Five days after the beginning of *P. brassicae* oviposition or five days after application of *P. brassicae* EE, two distal leaves of Arabidopsis were syringe-infiltrated with *Pseudomonas syringae pv. tomato* (*Pst*) DC3000 (OD_{600}=0.0005) suspension in 10 mM $MgCl_2$. After 48 h, two 0.77 cm^2 leaf discs per plant were collected in 2 mL micro-centrifuge tubes with glass beads, grinded using a TissueLyser II (Qiagen) and suspended in 500 mL of 10mM $MgCl_2$. Each sample was diluted in series of 1:10. 10 µl of each dilution were spotted on LB plates with rifampicin (50 µg/mL). Plates were incubated at 28°C for 48 h and CFUs were counted. Data were expressed as $\log_{10}$ (CFU counts per 1 cm^2 of leaf area).

For bacteria-induced SAR, two leaves of previously untreated Arabidopsis were syringe-infiltrated with *Pst* (OD_{600}= 0.0005) suspension in 10 mM $MgCl_2$. After 48 h, another two distal leaves were infiltrated with *Pst* identical to EE-induced SAR experiments described above.

Salicylic acid measurements

Salicylic acid was quantified in non-treated Arabidopsis leaves distal from EE-treated leaves using *Acinetobacter sp*. ADPWH_*lux*-based SA quantification method (Huang *et al.*, 2005; Zvereva *et al.*, 2016). Five days after EE application, a pooled sample from 6 plants (6 x 0.77 cm^2 leaf discs, total of 200 mg FW) was analyzed. Luminescence was integrated using a 485 ± 10 nm filter for 1 s. A SA standard curve diluted in untreated *sid2-1* extract amounts ranging from 0 to 60 ng was read in parallel to allow quantification.

Statistical analysis

Pooled datasets from at least 3 independent experiments were analyzed with linear mixed model fit by the restricted maximum likelihood (REML) algorithm (package 'lme4' in R) using repeated experiments as random factors. *t*-tests use Satterthwaite's method ['lmerModLmerTest'] as part of the linear mixed model. Differences among multiple treatments were determined with post-hoc Tukey's test. Bars represent standard errors calculated from pooled variance and pooled standard deviation of repeated experiments.

Results

Insect eggs induce inter-plant SAR

We had previously demonstrated that insect eggs and EE induce intra-plant SAR (Hilfiker *et al.*, 2014). This effect appears to be independent from plant-density per experimental pot, suggesting that inter-plant competition may not affect the magnitude of egg-induced SAR in distal leaves at the plant densities tested (Supporting Information Fig. S1). This finding allowed us to investigate further whether plant treatment with insect eggs induces SAR in the neighboring plants grown in the same pot. First, we exposed plants to natural oviposition by *P. brassicae* (see methods) and subsequently infected distal leaves with *Pst*. Surprisingly, insect eggs triggered up to 15-fold reduction in bacterial titer of neighboring plants, which was comparable to more than 10-fold reduction in *Pst* growth in the distal leaves of oviposited plants (Fig. 1a). This suggested that insect eggs induce inter-plant SAR in Arabidopsis. Next, we repeated the experiment with *P. brassicae* EE (Fig. 1b) and confirmed that both insect eggs and EE are capable of inducing intra- as well as inter-plant SAR against *Pst*. Together, these results indicate that eggs-exposed plants produce a signal that is received by egg-free neighboring plants to induce SAR against a foliar plant pathogen.

Inter-plant SAR is mediated by a distance-dependent mobile belowground signal.

We next investigated how the SAR-inducing signal from egg-exposed plants is reaching neighbor plants. Volatile plant-to-plant signals have been implicated in inter-plant SAR signaling in other studies (Riedlmeier *et al.*, 2017). To test whether physical contact between aerial parts of Arabidopsis rosettes or emitted volatiles may explain the observed inter-plant SAR phenotype, we placed an aerial barrier separating two plants treated with EE from two EE-free plants (Fig. 2). We positioned the pots in such a manner that the barrier was parallel to the airstream in the growth room, avoiding overflow of potential volatiles from one side to the other side of the barrier. As a control, we independently replicated the experiment described in Fig. 1b, making sure that the distance between EE-treated and EE-free plants in the separate pots is approximately the same as when grown together in the same pot. Plants on either side of the aerial barrier showed similar bacterial growth with differences less than 0.2 log-phase, as did EE-treated plants and their neighbors without a barrier. In contrast, EE-treated plants display more

than 10-fold (>1.0 log-phase) reduced *Pst* titer compared to EE-free plants growing separately (Fig. 2). This suggests that headspace volatiles from EE-treated plants or direct leaf-to-leaf contact with EE-free plants is not contributing to EE-induced inter-plant SAR.

To test whether EE-induced inter-plant SAR could be mediated by a root-derived signal, we placed a plastic barrier separating roots from EE-treated and EE-free plants. Strikingly, this abolished inter-plant SAR as EE-free plants displayed significantly greater bacterial titer (about 1.0 log-phase greater) compared to EE-treated plants on the other side of soil barrier (Fig. 2). However, when a permeable nylon mesh (11 µm pore size) was placed instead of the non-permeable plastic barrier, the differences in bacterial growth of EE-free plants on the one side and EE-treated plants on the other were not significant and did not exceed 0.2 log-phases (Fig. 2). Since the mesh prevents physical root-to-root contact, these findings strongly suggest that EE-induced inter-plant SAR is induced by a root-derived mobile signal.

Next, we asked whether the root-derived inter-plant SAR signal is distance-dependent. To this end, we planted EE-free plants with increasing distance away from EE-treated plants in the same growing tray. EE-free plants grown only 3 cm away from EE-treated plants displayed bacterial titer not more than 2-fold (0.2 log-phase) different from EE-treated plants but a significantly reduced titer by more than 14-fold (1.4 log-phase) compared to EE-free plants growing separately (Fig. 3). Furthermore, the inter-plant SAR effect in EE-free plants was progressively reduced as the distance from EE-treated plants increased (Fig. 3). To verify that the observed inter-plant SAR effect could not simply be explained by plant crowding, we measured bacterial titer in a set of EE-free plants growing at increasing distance from another set of uninfected EE-free plants. Differences in bacterial titer of EE-free plants grown at 3-12 cm distance from each other were not significant and did not exceed 0.2 log-phase or 0.5 log-phase from EE-free plants that were grown in separate trays (Fig. S2). This indicates that the effect size of EE-induced inter-plant SAR exceeds any random variation in bacterial titer of EE-free plants in absence of EE-treated neighbors.

Finally, to test whether the EE-induced inter-plant SAR signal can be relayed from signal-receiving plants to their nearest neighbors, we grew EE-free plants at regular intervals from a set of EE-treated plants within the same tray. Bacterial titer in plants grown at 3 cm away from EE-treated plants was only 0.4 log-phase different from EE-treated plants. However, bacterial titer in

plants grown at 6 cm and 9 cm away from EE-treated plants was significantly higher by about 1.0 or 1.5 log-phase respectively (Fig. S3), suggesting that the signal from EE-treated row of plants induced SAR in receiving plants at 3 cm but that these receiving plants did not relay the signal further to their nearest neighbors at an equal distance from them. Together, these data suggest that upon perception of insect eggs or EE, plants release a mobile, distance-dependent, belowground signal that induces SAR against *Pst* in the foliar tissue of receiver plants.

ALD1 and FMO1 are required for EE-induced inter-plant SAR

Given that plants receiving a signal from EE-treated sender plants do not propagate the inter-plant SAR signal (Fig. S3), production of the inter-plant signal appears to be independent from the establishment of SAR in the systemic aboveground tissues. Perception of insect eggs was shown to elevate SA and Pip levels in both local and distal leaves (Bruessow *et al.*, 20210; Hilfiker *et al.*, 2014), and both SA and Pip are required for pathogen-induced intra-plant SAR against *Pseudomonas syringae* (Bernsdorff *et al.*, 2016; Hartmann & Zeier, 2019). To investigate whether Pip accumulation in EE-treated plants is required for generation of inter-plant signal, we tested *ald1* and *fmo1*, which are mutants deficient in the Pip pathway. Mutants were treated with EE and SAR induction was measured in wild-type EE-free neighboring plants. The advantage of testing inter-plant SAR is that one can genetically separate the generation from the perception of a systemic signal, which is not possible in Arabidopsis where leaf grafting is not yet amenable.

Strikingly, when *ald1* or *fmo1* mutants were treated with EE, no SAR was induced in the wild-type neighbor plants, indicating that the Pip pathway is required for generating the inter-plant signal (Fig. 4a). ALD1 was shown to be also required for *Pst*-induced production of volatile monoterpenes and volatile-mediated inter-plant SAR triggered by *Pst* (Wenig *et al.*, 2019). Since we found that EE-triggered inter-plant SAR also depends on functional ALD1 in the sender plant (Fig. 4a) but does not rely on air-borne signals (Fig. 2), we wondered whether *Pst* infection can trigger inter-plant SAR in our experimental system, without the requirement of closed systems used in the studies on volatile-mediated inter-plant SAR (Riedlmeier *et al.*, 2017; Wenig *et al.*, 2019). We observed a *Pst*-induced intra-plant SAR but no inter-plant SAR (Fig. S4), suggesting that the ALD1-dependent root-derived inter-plant signal described in our study is specific to the perception of insect eggs but not *Pst*. Thus, there may be different inter-plant SAR signal

generation and perception mechanisms activated by different biotic stresses. However, since we measured secondary infection rate 48 h after the initial infection, which is the time commonly used for bacterial SAR (Návarová *et al.*, 2012), there is the possibility that a root-derived signal takes longer to trigger inter-plant SAR. Further studies should address this hypothesis.

Next, we tested the requirement of SA in the generation of the EE-induced inter-plant SAR signal. To this end, we treated the SA biosynthesis mutant *sid2-1* and the SA-degrading transgenic line *nahG* with insect EE and measured bacterial growth in wild-type EE-free neighbors. Surprisingly, EE treatment of *sid2-1* and *nahG* still triggered inter-plant SAR, similar to wild-type sender plants (Fig. 4b). This strongly suggests that SA accumulation is not required for the generation of the inter-plant SAR signal.

Altogether, we thus demonstrate that the generation of EE-induced inter-plant SAR signal in the sender plant requires functional ALD1 and FMO1 but occurs independently from SA accumulation.

EE triggers elevated SA levels in neighboring plants

While SA does not seem to be required for the generation of EE-induced inter-plant signal in the sender plants, we investigated whether SA could be involved for SAR activation in receiver plants, like it is the case for distal leaves in intra-plant SAR (Bernsdorff *et al.,* 2016). We showed previously that EE induces a strong accumulation of SA in EE-treated leaves, whereas levels stay close to control levels in distal leaves (Bruessow *et al.*, 2010). We thus measured changes in SA levels in receiver EE-free plants growing next to EE-treated plants. First, SA levels in distal leaves from EE-treated plants were not significantly different from levels in untreated control plants. However, plants growing 3 cm to EE-treated plants displayed more than 10-fold higher SA levels than EE-free plants growing alone and this effect diminished to only 2.5 fold as the distance to EE-treated plants increased (Fig. 5). The observed distance-dependent accumulation of SA in receiver plants is thus correlated with the establishment of distance-dependent inter-plant SAR against *Pst* (Fig. 3), implying that elevated SA levels in the receiver plants may contribute to the SAR phenotype. The observation that SA levels are enhanced in neighboring plants but only weakly in distal leaves from EE-treated plants suggests that the root-derived signal triggers a

strong SA biosynthesis in receiving plants. Further work should address the connection between root-derived signal perception and activation of SA biosynthesis.

Discussion

In this study, we have discovered an intriguing natural phenomenon where plants oviposited by *P. brassicae* or exposed to the elicitors in EE generate a mobile belowground signal that is able to induce SAR against the foliar bacterial pathogen *Pst* in receiving neighbor plants which have not prior been exposed to insect eggs themselves. Strikingly, ALD1 and FMO1 are both required for the generation of egg-induced inter-plant SAR signal, like the establishment of egg-induced intra-plant SAR (Hilfiker *et al.*, 2014). Similarly, *Pst AvrRpm1*-triggered generation of inter-plant SAR-inducing volatile signal depends also on ALD1 in the sender plants (Wenig *et al.*, 2019). It appears thus that a common mechanism is involved in the generation of intra- and inter-plant signals to trigger systemic immunity. However, since belowground signals do not appear to mediate *Pst*-induced inter-plant SAR in our experimental system, different above- and belowground signals may be generated by different biotic stresses to trigger SAR in the recipient plants. It also remains to be tested whether the generation of inter-plant signal depends on the establishment of SAR in the distal tissues of the sender plant or requires ALD1 and FMO1 independently from their roles in SAR establishment. SA and NHP, the biologically active form of the Pip pathway, act synergistically in SAR (Hartmann & Zeier, 2019), including in the promotion of each other's synthesis in distal tissues (Bernsdorff *et al.*, 2016). However, although we show that SA accumulates in the receiver plants in response to the inter-plant signal, SA is not important for generating the egg-dependent inter-plant signal and thus sending the signal might not require establishment of full intra-plant SAR. Future research should aim at deciphering the molecular mechanisms that link the Pip pathway to the production of signals involved in inter-plant SAR.

We show here that the inter-plant signal produced by EE-treated plants is mediated by plant roots. Root exudates (soluble phytochemicals) or root VOCs have been investigated in context of diverse biotic interactions such as allelopathic plant-plant interactions, root-soil microbiome interactions as well as direct defense against soil pathogens or recruitment of natural enemies against soil herbivores, and are extensively reviewed in (Bais *et al.*, 2006; De-la-Peña *et al.*, 2012; Baetz & Martinoia, 2014; Haichar *et al.*, 2014; Delory *et al.*, 2016). However,

only few studies have described root exudates as inter-plant signals regulating defense in the neighboring plants. Evidence for inter-plant role of aggressive weed couch-grass (*Elytrigia repens)* root exudates was provided (Glinwood *et al.*, 2003). When barley plants were treated with *E. repens* root exudates or synthetic compounds therein, barley plant acceptance to bird cherry-oat aphid (*Rhopalosiphum padi*) was significantly reduced. Nevertheless, it remains to be investigated whether this is due to sequestration of *E. repens* exudates with direct toxic or herbivore repellent effects or due to exudate-induced systemic defense (or priming) responses. Pea aphid-colonized bean plants produce root exudates that induce the release of parasitoid-attractive volatiles in un-colonized neighboring plants (Guerrieri *et al.*, 2002). Similarly, root exudates from spider mite-infested lima beans rendered un-infested recipient plants more attractive to predatory mites (Dicke & Dijkman, 2001). Tobacco leaves infiltrated with either BHT (SAR-inducing SA agonist) or avirulent *P. syringae pv. syringae (Psy)* triggered root-mediated inter-plant SAR against soil-borne pathogen *Ralstonia solanacearum* as well as foliar pathogen *P. syringae pv. tabaci (Pta)* (Song *et al.*, 2016). Moreover, the root exudates of BHT-treated plants contained elevated SA levels, and drench application of exogenous SA reduced disease severity of *Ralstonia solanacearum* (Song *et al.*, 2016). However, since we show that the generation of egg-induced inter-plant signal is independent from SA accumulation in the sender plant, it is unlikely that the observed increased accumulation of SA in neighboring plants is due to the absorption of SA from root exudates. In contrast, Pip application in the soil enhances resistance against *Psm* (Návarová *et al.*, 2012). Future research should test for the presence of Pip in roots and root exudates of egg-treated plants.

Few other belowground compounds could be considered as potential mobile signals in inter-plant SAR. Root-applied azelaic acid (AZA) can induce systemic resistance of Arabidopsis aerial tissues against *P. syringae* (Cecchini *et al.*, 2019). However, the study showed that AZA does not move from root to shoot and would require additional internal mobile signals to induce SAR in the leaves. Furthermore, while pathogen infection increases AZA and other oxylipin accumulation in leaves, no significant changes in oxylipin profiles were observed in the roots (Mukhtarova *et al.*, 2011). It remains an open question whether root-derived inter-plant mobile signals could originate in the aerial tissues and are transported to roots or are synthesized and excreted by the roots themselves.

Stress-induced root volatiles may have a potential function in plant-to-plant defense signaling. However, to date, induced or constitutive root volatiles have been implicated in allelopathic inhibitory or growth-stimulating plant-plant interactions among hetero- or conspecifics, as reviewed by (De-la-Peña *et al.*, 2012; Delory *et al.*, 2016) and demonstrated in a recent study by (Gfeller *et al.*, 2019). Additionally, root volatiles contribute to indirect plant defense against herbivores by attracting natural enemies (Rasmann *et al.*, 2005; Delory *et al.*, 2016) as well as participate in direct defense against soil pathogens or pests (Delory *et al.*, 2016; Lackus *et al.*, 2018). To our knowledge, studies showing stress-induced belowground VOCs that trigger defense responses against attackers in neighboring plants are lacking, except for demonstration of increased susceptibility of dandelion *Taraxacum oficinale* to herbivorous cockchafer *Melolontha melolontha* after treatment with the constitutively produced root VOCs from sympatric spotted knapweed (*Centaurea stoebe*) (Huang *et al.*, 2019). Therefore, future studies should focus on untargeted metabolomic analysis of compounds produced in the rhizosphere of in egg-treated plants, with the goal to identify mobile signals that can trigger defenses in neighboring plants.

Potential ecological role of egg-induced inter-plant SAR

Insect eggs pose an herbivore threat to plants as eggs hatch into chewing larvae. It has been demonstrated that herbivore damage in bittercress (*Cardamine cordifolia*, Brassicaceae) under field conditions produces shifts in phyllosphere microbial diversity and increases overall leaf microbial load which is mainly driven by infection intensity of *P. syringae* and another potential pathogenic *Pseudomonas spp*. (Humphrey *et al.*, 2014; Humphrey & Whiteman, 2020). Furthermore, herbivory-induced (JA-related) plant defenses could contribute to such shifts in leaf microbiome and favor the infection of pathogenic microbes (Humphrey & Whiteman, 2020). In addition, subsequent plant-to-plant movement and distribution of herbivores is predicted to largely depend on induced leaf defenses (Rubin *et al.*, 2015). Hence, chewing herbivores may not only increase infection load in the plants they hatch on but are likely to disperse to the neighbors in search of supplementary feed and avoidance of induced host defenses. Perception of egg-induced inter-plant signal and induction of SAR may thus benefit the receiver plants to

counteract the negative effects of microbial infection during herbivory. In contrast, in an insect-centric view of the inter-plant SAR, protecting neighboring plants from infection may ensure healthy food for moving larvae. We showed indeed that *P. brassicae* larvae perform significantly less when they feed on *Pst*-infected Arabidopsis (Hilfiker et al., 2014). Intra- and inter-plant SAR would have thus evolved as a mechanism to favor insect development on oviposited and neighboring plants.

It remains to be elucidated whether egg-induced intra- and inter-plant SAR would have any fitness benefits to the host plant and/or feeding larvae in natural environments under pathogen pressure. The composition of the leaf microbiome may play an important role in the unexpected increase of plant performance under simultaneous herbivore and pathogen attack (Saleem *et al.*, 2017). Moreover, a recent study demonstrated that herbivory can act as selective force in the evolution of VOC-mediated plant-to-plant communication in *Solidago altissima* (Kalske *et al.*, 2019), and rising temperature and insect herbivory in an Arctic tundra ecosystem interacted synergistically to alter plant VOC emissions (Li *et al.*, 2019). Collectively, these studies suggest that 1) multiple biotic and abiotic factors can affect plant info-chemical production, 2) biotic interactions can select for inter-plant communication that results in resistance phenotypes in signal receiving plants, 3) the fitness benefits of inter-plant signaling can be an integral measure of plant performance under multiple biotic and abiotic stresses.

In addition to the adaptive hypotheses about a potential fitness benefits to the host plant when minimizing infection during herbivory or a benefit to the herbivore from less infected host, we also consider the neutral hypothesis that egg-induced inter-plant SAR may be a physiological by-product or an epiphenomenon of activating defenses within the plant. However, given that other biotic stresses such as leaf damage or leaf chewing do not trigger the SA pathway and a SA-inducing biotic stress factor such as *Pst* infection does not trigger root-mediated inter-plant SAR (Figure S4), it is probable that insect egg-triggered inter-plant signal is a more fine-tuned response, rather than a mere by-product of co-opting plant defenses or SA pathway. Moreover, our finding that EE-free plants accumulate SA in response to root-derived signals from EE-treated plants (Fig. 5) but do not further relay the signal to their EE-free neighbors (Figure S3) suggests that SA accumulation alone may not be sufficient to produce SAR-inducing inter-plant signal by

roots. Whether eggs from other insect species induce the same response in Arabidopsis or other plants is another important question that remains to be investigated in future studies.

In summary, we have discovered an intriguing phenomenon where *P. brassicae* eggs trigger inter-plant SAR in Arabidopsis against *Pst* infection. Future work is necessary to further characterize the nature of the root signal(s), the genetic components and mechanisms involved in the generation of egg-induced inter-plant signal(s), as well as the decoding of such information in neighboring plants. Whether other insects induce the same response in Arabidopsis and other plants is another important question.

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Author Contribution

ZO and PR conceived and designed the research. ZO performed experiments. ZO and PR analyzed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Intra-plant SAR is independent of plant density

Fig. S2 *Pseudomonas syringae* titer is not affected by distance between *Pieris brassicae* egg extract-free plants

Fig. S3 Egg extract-induced inter-plant systemic acquired resistance signal is not relayed by receiver plants

Fig. S4 *Pseudomonas syringae* (*Pst*) induces intra-plant systemic acquired resistance (SAR) but no inter-plant SAR against *Pst.*

Figure legends

Figure 1. *Pieris brassicae* eggs and egg extract induce intra-plant and inter-plant SAR against *Pseudomonas syringae pv. tomato* DC3000 in Arabidopsis. (**a**) Insect eggs significantly reduce bacterial titer by more than a log-phase (10-fold) in distal leaves when compared to egg-free plants grown separately (intra-plant SAR; F=97.01, *P*=1.065e-12) but no difference in bacterial titer is observed when oviposited and egg-free plants are in the same pot (inter-plant systemic acquired resistance (SAR); F=0.1071, *P*=0.745). Each bar represents mean of bacterial titer (± SE) in ≥24 individual plants pooled from 3 independent experiments. Dots indicate individual results. (**b**) *P. brassicae* egg extract (EE) significantly reduces bacterial titer by about a log-phase (10-fold) in distal leaves when compared to EE-free plants grown separately (intra-plant SAR; F=81.56, *P*=2.108e-14) but no difference in bacterial titer is observed when EE-treated and EE-free plants are in the same pot (inter-plant SAR; F=1.6646, *P*=0.1998). Each bar represents mean of bacterial titer (± SE) in ≥ 48 individual plants pooled from 6 independent experiments. Dots indicate individual results. Black triangles indicate infected sampled leaves, yellow dots indicate site of egg oviposition or EE treatment. Grey bars represent egg- or EE-free plants, yellow bars represent oviposited or EE-treated plants. Significant difference between control and EE-treated plants is indicated (linear mixed model, ***, *P*<0.001; ns, not significant).

Figure 2. Inter-plant SAR against *Pseudomonas syringae* is mediated by a mobile belowground signal. Bacterial growth is reduced by more than 10-fold (1.0 log-phase) in *Pieris brassicae* egg

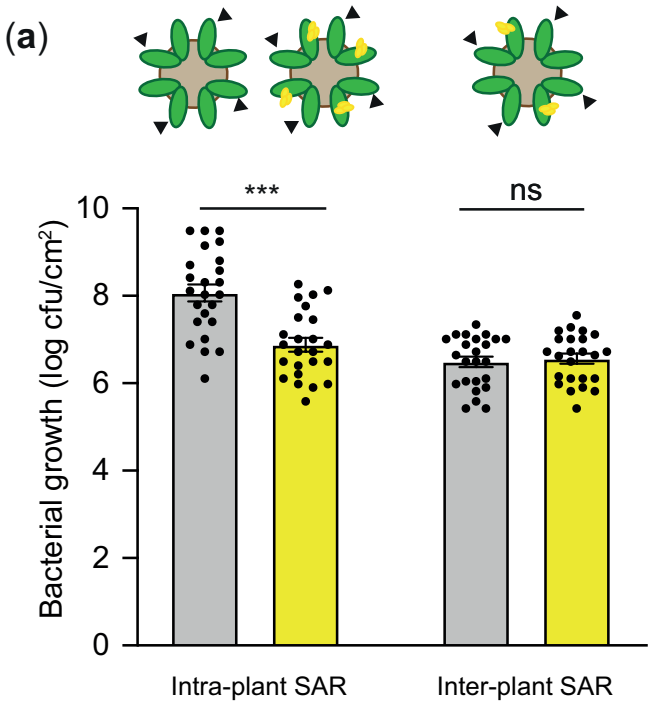
extract (EE)-treated Arabidopsis plants compared to EE-free plants growing in separate pots (F=65.359, *P*=3.051e-10). EE-treated and EE-free plants show similar bacterial titer with less than 2-fold (0.2 log-phase) difference when grown in the same pot without (F=1.8057, *P*=0.1874) or with a barrier that separates plant aerial parts (F=1.5299, *P*=0.2218). Inter-plant systemic acquired resistance (SAR) is abolished when a non-permeable soil barrier is placed in between the two plants (F=37.739, *P*=2.261e-07) but maintained when plants are separated by a 11 µm nylon mesh in the soil (F=2.5494, *P*=0.1177). Each bar represents mean of bacterial titer (± SE) in ≥24 individual plants pooled from 3 independent experiments. Dots indicate individual results. Black triangles indicate infected sampled leaves, yellow dots indicate site of EE treatment. Grey bars represent EE-free plants, yellow bars represent EE-treated plants. Blue arrows indicate airflow direction. Significant difference between control and EE-treated plants is indicated (linear mixed model, *** *P*<0.001; ns, not significant).

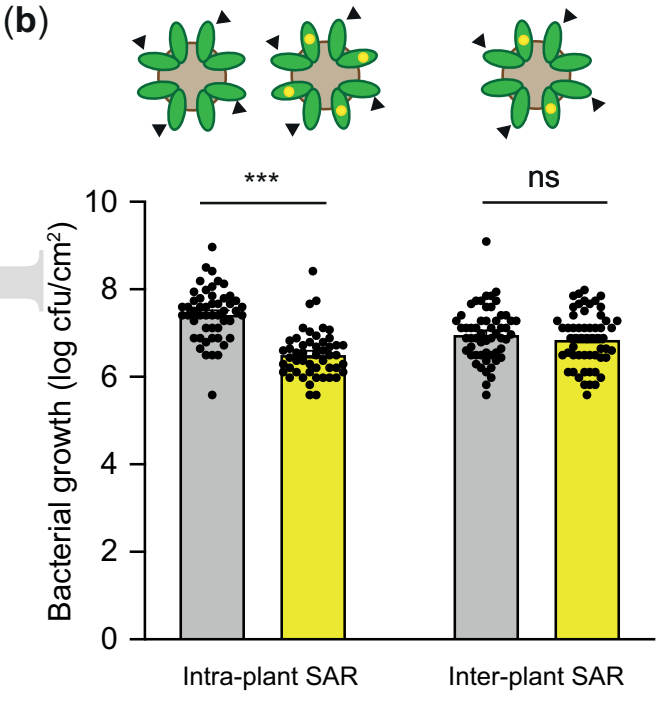
Figure 3. Egg extract-induced inter-plant systemic aquired resistance signal is distance-dependent. Reduction in *Pseudomonas syringae* titer in *Pieris brassicae* egg extract (EE)-free Arabidopsis plants is dependent on the proximity to EE treated neighbor plants (F=52.56, *P*<2.2e-16). Each bar represents mean of bacterial titer (± SE) in ≥24 individual plants pooled from 3 independent experiments. Dots indicate individual results. Significant differences are displayed as letters (Tukey's HSD post-hoc, *P*<0.5). Black triangles indicate infected sampled leaves, yellow dots indicate site of EE treatment. Grey bars represent EE-free plants, the yellow bar represents EE-treated plants. CTL, control plants grown alone.

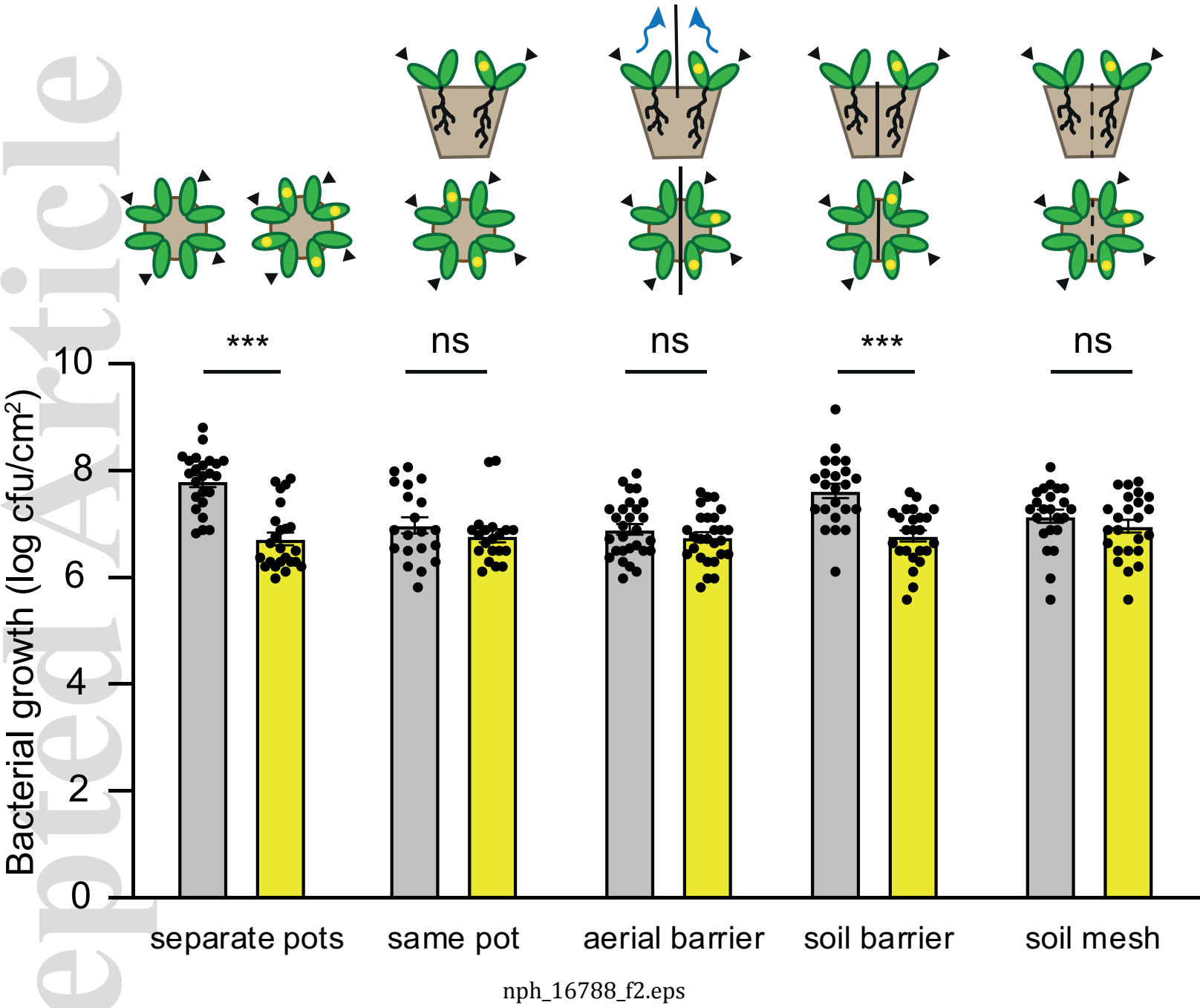
Figure 4. ALD1 and FMO1 are required for generation of egg extract-induced inter-plant SAR signal. (**a**) Induction of systemic acquired resistance (SAR) against *Pseudomonas syringae* (*Pst*) in the receiver Arabidopsis plants of *Pieris brassicae* egg extract (EE)-induced inter-plant signal is dependent on functional ALD1 and FMO1 module in the sender plants (F=12.063, *P*=3.547e-08). (**b**) Induction of SAR against *Pst* in the receiver plants of EE-induced inter-plant signal is not dependent on functional salicylic acid (SA) accumulation in the sender plants (F=7.359, *P*=2.67e-05). Each bar represents mean of bacterial titer (± SE) in ≥24 individual plants pooled from 3 independent experiments. Dots indicate individual results. Significant differences are displayed

as letters (Tukey's HSD post hoc). Black triangles indicate infected sampled leaves, yellow dots indicate site of EE treatment. Grey bars represent EE-free plants, yellow bars represent EE-treated plants. Plant genotypes are color-coded.

Figure 5. *Pieris brassicae* egg extract triggers elevated salicylic acid levels in the neighboring plants. Egg extract (EE)-free plants growing at 3 cm to EE-treated Arabidopsis plants display 9.2-fold (4.8-fold at 6 cm, 3.3-fold at 9 cm, 2.3-fold at 12 cm) elevated salicylic acid (SA) levels compared to the systemic leaves of EE-treated plants 5 days after EE treatment ($F=126.1$, $P=6.07e-10$). Each bar represents mean levels of SA (± SE) in 18 individual plants pooled from 3 independent experiments. Dots indicate individual results. Significant differences are displayed as letters (Tukey's HSD post hoc, $P<0.5$). Black triangles indicate sampled leaves, yellow dots indicate site of EE treatment. Grey bars represent EE-free plants, the yellow bar represents EE-treated plants.







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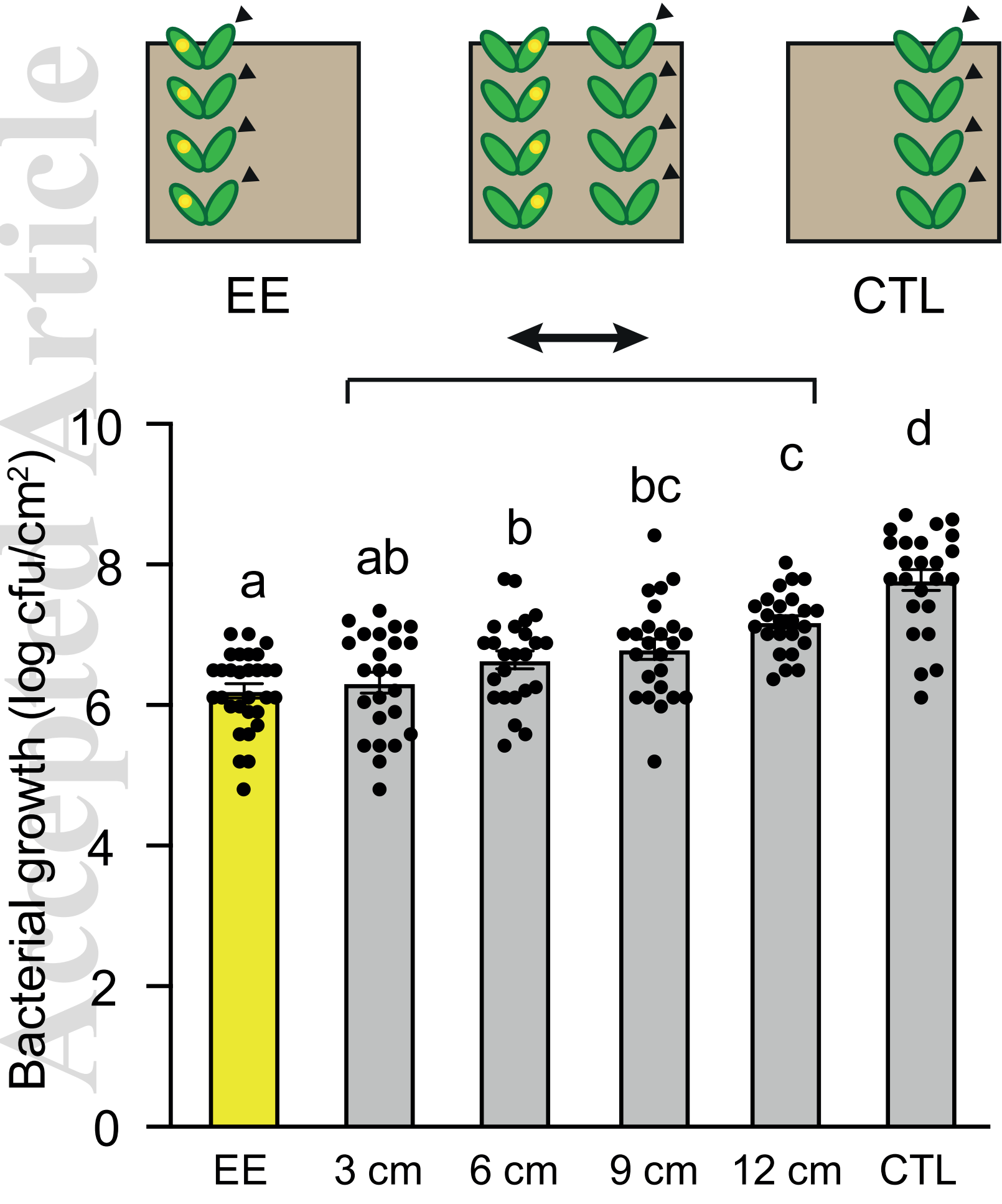

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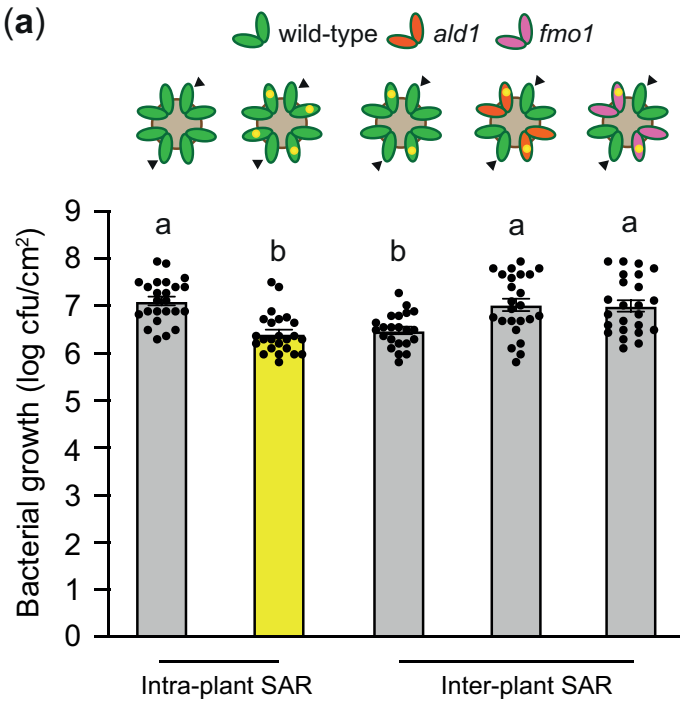

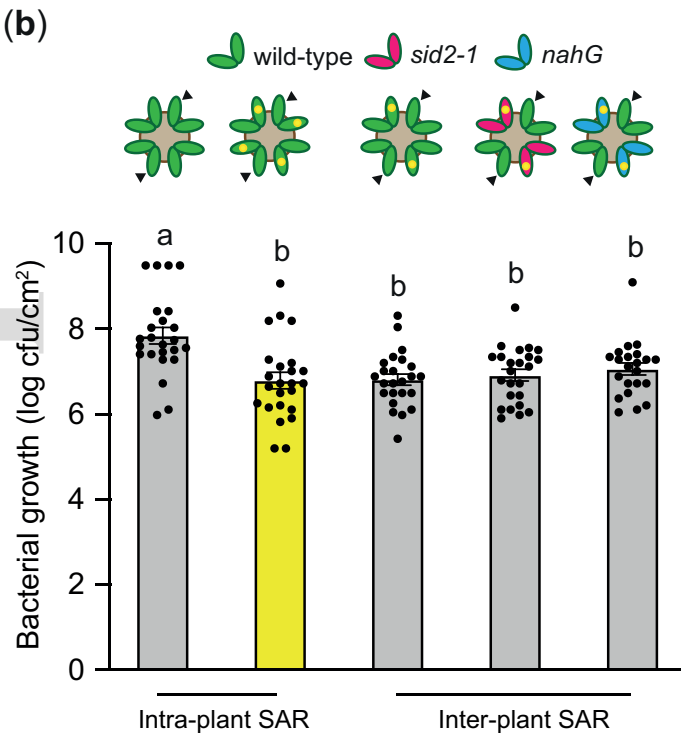

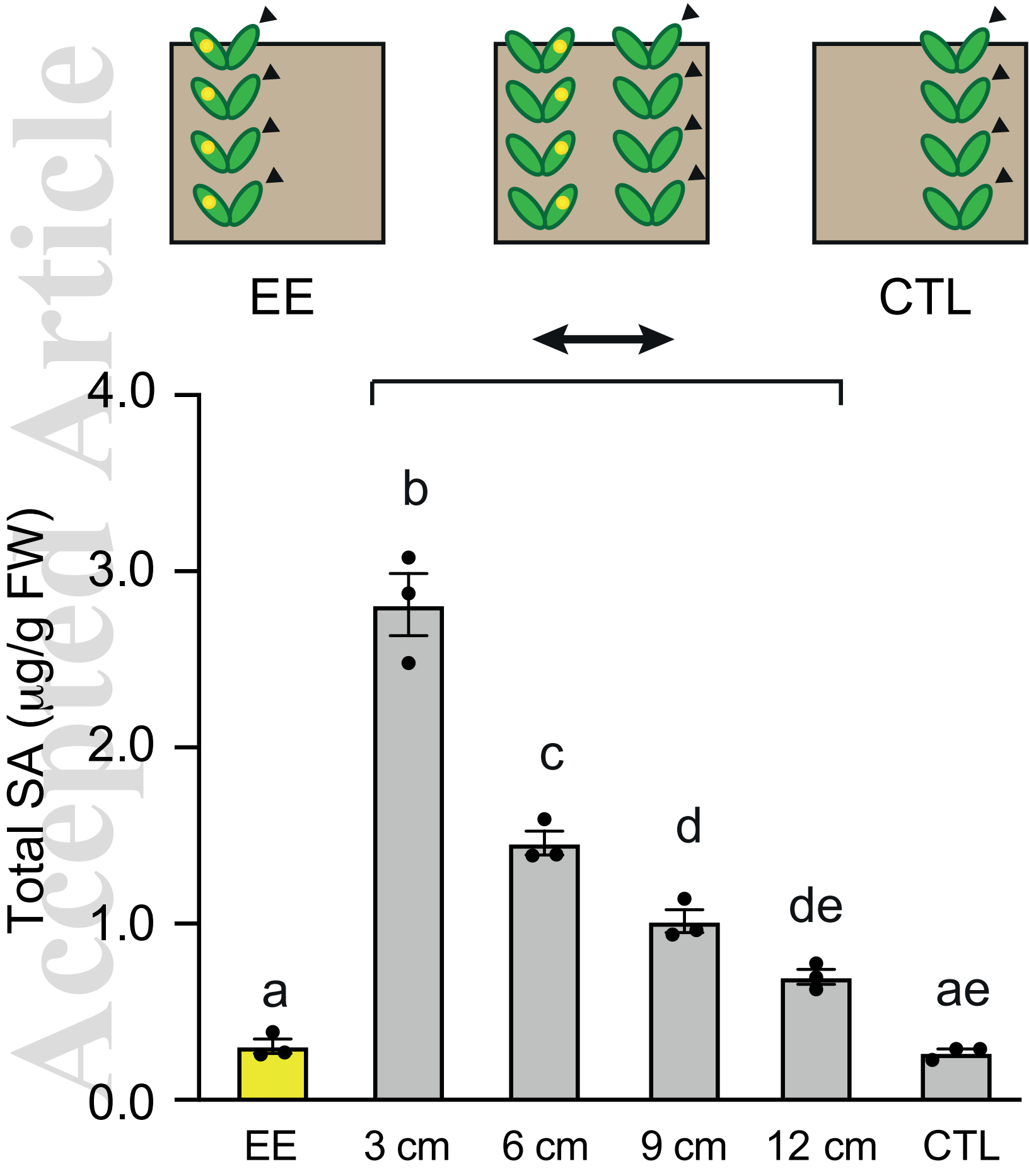

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