DR EDGAR PEITER (Orcid ID : 0000-0002-9104-3238)

DR AXEL MITHÖFER (Orcid ID : 0000-0001-5229-6913)

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ANNEXIN1 mediates calcium-dependent systemic defense in Arabidopsis plants upon herbivory and wounding

Running title: ANNEXIN1 in herbivore defense

Jaiana Malabarba[1,2], Anja K. Meents[1,3], Michael Reichelt[4], Sandra S. Scholz[3], Edgar Peiter[5], Julia Rachowka[6], Dorota Konopka-Postupolska[6], Katie A. Wilkins[7], Julia M. Davies[7], Ralf Oelmüller[3], Axel Mithöfer[1,8]

[1]Research Group Plant Defense Physiology, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

[2]Postgraduate Program in Cell and Molecular Biology, Biotechnology Center, Federal University of Rio Grande do Sul, Porto Alegre – RS, 90040-060, Brazil

[3]Plant Physiology, Matthias-Schleiden-Institute for Genetics, Bioinformatics and Molecular Botany, Faculty of Biological Sciences, Friedrich-Schiller-University Jena, 07743 Jena, Germany

[4]Department of Biochemistry, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

[5]Institute of Agricultural and Nutritional Sciences, Faculty of Natural Sciences III, Martin Luther University of Halle-Wittenberg, 06120 Halle (Saale), Germany

[6]Plant Protein Phosphorylation Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland
[7]Department of Plant Sciences, University of Cambridge, CB24 6DG Cambridge, UK

Orchid addresses:
Jaiana Malabarba: 0000-0002-1263-9026
Anja K. Meents: 0000-0003-2267-6952
Michael Reichelt: 0000-0002-6691-6500
Sandra S. Scholz: 0000-0002-5389-5296
Edgar Peiter: 0000-0002-9104-3238
Julia Rachowka: 0000-0002-4148-9928
Dorota Konopka-Postupolska: 0000-0002-3181-5478
Katie A. Wilkins: 0000-0001-6513-856X
Julia M. Davies: 0000-0003-2630-4339
Ralf Oelmüller: 0000-0002-3878-0044
Axel Mithöfer: 0000-0001-5229-6913

[8]**Correspondence:** Priv. Doz. Dr. Axel Mithöfer
amithoefer@ice.mpg.de
phone:+49 3641 571263
fax:+49 (0)3641 57 1256

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SUMMARY

- Cellular calcium transients are endogenous signals involved in local and systemic signaling and defense activation upon environmental stress, including wounding and herbivory. Still, not all Ca^{2+} channels contributing to the signaling have been identified, nor are their modes of action fully known. Plant annexins are proteins capable of binding to anionic phospholipids and can exhibit calcium channel-like activity. Arabidopsis ANNEXIN1 is suggested to contribute to calcium transport.
- Here, we report that wounding- and simulated herbivory-induced cytosolic free calcium elevation was impaired in systemic leaves in *ann1* loss of function plants. We provide evidence for a role of ANNEXIN1 in local and systemic defense of plants attacked by herbivorous *Spodoptera littoralis* larvae.
- Bioassays identified ANNEXIN1 as a positive defense regulator. *S. littoralis* feeding on *ann1* gained significantly more weight compared with larvae feeding on wild-type, while those feeding on ANNEXIN1 overexpressing lines gained less weight. Herbivory and wounding both induced defense-related responses on treated leaves such as jasmonate accumulation and defense gene expression. These responses remained local and were strongly reduced in systemic leaves in *ann1* plants.
- Our results indicate that ANNEXIN1 plays an important role in activation of systemic rather than local defense in plants attacked by herbivorous insects.

Keywords

Calcium signaling, herbivory, jasmonates, plant defense, *Spodoptera*, systemic signaling.

INTRODUCTION

Plants are challenged throughout their life by various abiotic and biotic stress factors. These changes in the environment require fast adaptation. Consequently, plants evolved a multilayered metabolic barrier, composed of mechanical and chemical defenses (Maffei *et al.*, 2012; Mithöfer & Boland, 2012). An attack by herbivorous insects represents a major threat to the plant's survival. In particular an attack by chewing insects is a combination of plant tissue wounding and application of insect-specific herbivore-associated molecular patterns (HAMPs), mainly present in their oral secretions (OS) (Mithöfer & Boland, 2008; Vadassery *et al.*, 2012b; Kiep *et al.*, 2015). The establishment of chemical defenses to such an insect herbivory is mediated by a network of signaling pathways (including Ca^{2+} ions, protein phosphorylation, phytohormones, and reactive oxygen and nitrogen species) that finally initializes synthesis and accumulation of a plethora of defensive metabolites (Zebelo *et al.*, 2014; Seybold *et al.*, 2014). The elevation in cytosolic free calcium, $[Ca^{2+}]_{cyt}$, is one of the earliest signaling events initiated upon the plant's interaction with feeding insects (Maffei *et al.*, 2004; Kiep *et al.*, 2015; Toyota *et al.*, 2018). Jasmonates represent the most important class of wound-induced phytohormones to be activated, with the main components being jasmonic acid (JA) and its biologically active isoleucine conjugate (+)-7-*iso*-Jasmonoyl-*L*-isoleucine (JA-Ile) (Wasternack, 2007; Mithöfer & Boland, 2008; Mithöfer & Boland, 2012). The connection between jasmonates and $[Ca^{2+}]_{cyt}$ is likely mediated by Ca^{2+}-sensing proteins. In plants, canonical calmodulins (CAMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and Ca^{2+}-dependent protein kinases (CDPKs) are good candidates (Swarbreck *et al.*, 2013; Yan *et al.*, 2018; Mohanta *et al.*, 2019; Tai *et al.*, 2019). In particular, for CML42 and CML37 a connection between $[Ca^{2+}]_{cyt}$ and jasmonate signaling has been shown (DeFalco *et al.*, 2010; Vadassery *et al.*, 2012a,b; Scholz *et al.*, 2014, 2016; Heyer *et al.*, 2018b).

However, upstream of jasmonates, Ca^{2+}-sensing proteins, and $[Ca^{2+}]_{cyt}$ changes, an influx of Ca^{2+} is necessary to cause $[Ca^{2+}]_{cyt}$ elevations. Here, the opening of ion channels localized at the plasma membrane or intracellular compartments is involved. In Arabidopsis, ligand-gated channels such as glutamate receptor-like channels (GLR) and cyclic nucleotide-gated channels (CNGC) plus potentially stretch-activated Ca^{2+} channels OSCA (reduced hyperosmolality-induced Ca^{2+} increase) and MCA (MID1-complementing activity) are the four main families of plasma membrane Ca^{2+}-

permeable channels (Dodd *et al.*, 2010; Yuan *et al.*, 2014). In addition, the vacuolar two-pore channel 1 (TPC1) is localized at the tonoplast (Peiter *et al.*, 2005; Peiter, 2011).

Strikingly, Arabidopsis senses local herbivore attack and transmits this information to unwounded vascular-connected systemic leaves through a long-distance signaling system (Mousavi *et al.*, 2013; Kiep *et al.*, 2015). Systemic signaling has also been identified leading to activation of jasmonate accumulation and signaling in distal leaves (Mousavi *et al.*, 2013; Heyer *et al.*, 2018b). Recently it has been shown that wound-induced electrical signals precede vascular Ca^{2+} fluxes as xylem contact cells and phloem sieve elements function together for leaf-to-leaf electrical signaling (Nguyen *et al.*, 2018). Probably, the systemic electrical signaling is mediated by glutamate receptor GLR-type cation channels, because in *glr3.3 glr3.6* double mutants the wound activated electrical signal propagation, as well as propagation of $[Ca^{2+}]_{cyt}$ signals between leaves, is attenuated (Toyota *et al.*, 2018). In addition, TPC1 was shown to be involved in systemic $[Ca^{2+}]_{cyt}$ elevations (Kiep *et al.*, 2015). This channel trio of GLR3.3, GLR3.6 and TPC1 also operates in local $[Ca^{2+}]_{cyt}$ elevations induced by aphid feeding (Vincent *et al.*, 2017). Very recently, it was demonstrated that a rapidly activated cyclic nucleotide-gated Ca^{2+} channel (CNGC19) also plays a partial role in wounding-induced Ca^{2+} influx (Meena *et al.*, 2019). Thus, it becomes more and more evident that herbivory–induced $[Ca^{2+}]_{cyt}$ elevation involves multiple channels and pathways regulating local and long-distance $[Ca^{2+}]_{cyt}$ signals.

Nevertheless, some studies showed that conventional channels might not always be responsible for Ca^{2+} influx pathways. Thus, the involvement of other passive Ca^{2+} transport-mediating proteins such as annexins becomes an interesting possibility (Laohavisit & Davies, 2009, 2011; Davies, 2014; Ma *et al.*, 2019).

Annexins are found in eukaryotic organisms and form a diverse multigene superfamily of Ca^{2+}-dependent membrane-binding proteins that serve as targets for Ca^{2+} in most eukaryotic cells. In angiosperms, annexins are found in vegetative and generative organs (Laohavisit & Davies, 2011; Clark *et al.*, 2012). They are composed of 60 to 70 amino acid-long motifs, repeated four times. The ability of annexins to conduct Ca^{2+} has become evident from *in vivo* and *in vitro* assays (Demidchik & Maathuis, 2007; Laohavisit *et al.*, 2009, 2012; Richards *et al.*, 2014; Ma *et al.*, 2019). Unlike conventional Ca^{2+} channels, which are routed from the Golgi complex to reside in a specific membrane, annexin proteins are able to occupy multiple cellular locations simultaneously. This characteristic makes annexins capable of a fast-recruitment response that can be driven by localized stimulation of membrane regions and might be independent of vesicular delivery (reviewed by Laohavisit & Davies, 2009, 2011; Clark *et al.*, 2012).

Among the eight annexins described to date in *Arabidopsis thaliana*, ANNEXIN1 (ANN1) is the best-studied one. It was initially detected in the cytosol of cells and later on in the plasma membrane, endoplasmic reticulum, vacuole, mitochondria, chloroplast, and in the cell wall (Laohavisit & Davies, 2011). *ANN1* overexpression has a protective effect on plant survival under drought conditions, while lack of expression increases stress sensitivity (Konopka-Postupolska *et al.*, 2009). Studies on Arabidopsis roots have correlated its localization in the plasma membrane with the presence of a Ca^{2+} conductance, which is activated by voltage hyperpolarization and extracellular hydroxyl radicals and is involved in the elongation of root hair cells (Foreman *et al.*, 2003; Laohavisit *et al.*, 2012). The Arabidopsis *ann1* knock-out mutant, (*ann1*), lacks this Ca^{2+} channel-like activity in the plasma membranes of root epidermal cells and root hairs. Furthermore, *ann1* mutants also have shorter roots compared to wild-type (Col-0) plants (Laohavisit *et al.*, 2012). More recent studies have shown that ANN1 is involved in root and seedling $[Ca^{2+}]_{cyt}$ elevation in response to hydrogen peroxide (Richards *et al.*, 2014; Zhao *et al.*, 2019).

As ANNEXIN1 is firmly implicated in $[Ca^{2+}]_{cyt}$ elevation and this occurs during insect feeding, our aim was to elucidate a putative role for this annexin in plant responses to herbivory-related cues. Therefore, we performed a set of assays to characterize *ann1* mutants. The effect of the lack of ANN1 was analyzed by observing larval growth of the crop-pest moth *Spodoptera littoralis* on two different *ann1* knock-out and two ANN1 overexpressing lines. Further, the plant's response to mechanical injuries (i.e. mechanical wounding with and without the addition of larval oral secretion) and after lesions caused by *S. littoralis'* feeding on leaves was investigated. We found a role for ANNEXIN1 in Arabidopsis for both local and systemic defense responses against *S. littoralis* attack by mediating $[Ca^{2+}]_{cyt}$ elevations, jasmonate level, and defense-related gene expression. This study contributes to our understanding of the molecular identity of Ca^{2+} channels involved in the plant response to wounding and herbivory.

MATERIALS AND METHODS

Plant growth and treatment

Arabidopsis thaliana Columbia-0 (Col-0) wild-type, *ANNEXIN1* knock-out mutant lines *ann1-1* (SALK_015426 originally characterized by Lee *et al.* (2004), *ann1-2* (WiscDsLox477–480P11) and *ANNEXIN1* overexpressing lines *ANN1-OE10, ANN1-OE12* (Konopka-Postupolska *et al.*, 2009) were used. For $[Ca^{2+}]_{cyt}$ measurements Col-0 and *ann1-1* were transformed by floral dip to express cytosolic (apo)aequorin driven by the 35S promoter. Floral dip employed *Agrobacterium tumefaciens* GV3101 pNEW 35S::AEQ pGreen vector containing the 35S::AEQ insert cut from pMAQ (Knight *et al.*, 1991). All plants used in aequorin assays were at least three generations post-transformation

Plants were kept in short-day conditions after stratification for two days at 4 °C. Four to five-week-old plants grown in 10 cm round pots were used for all experiments. The growth chamber was adjusted to 50-60 % humidity and 21 °C with a 10-h-light/14-h-dark photoperiod and a light intensity of 100 µmol m^{-2} s^{-1}. For experiments investigating the systemic response and translocation of metabolites, the leaves of each plant were counted according to their age (Dengler, 2006; Farmer *et al.*, 2013; Kiep *et al.*, 2015).

MecWorm (Mithöfer *et al.*, 2005) treatment was used for mechanical wounding of the plant with punches every 5 sec (12 punches per min) on treated leaf 8. To investigate the systemic response upon treatment of leaf 8, the local and systemic leaves 5, 8, 9, and 13 were analyzed. Untreated plants were used as control and had the same growth and handling conditions as the treated ones.

To study the mechanical wounding-induced systemic response, wounds were generated on leaf 8 with a pattern wheel (6 vertical movements on each side of the midrib), and 20 µL of water (MW+W; mechanical wounding + water) or of *S. littoralis* oral secretion (OS), diluted 1:1 in water (MW+OS), was applied to the wounds (Vadassery *et al.*, 2012a). Treated plants were kept in the growth chamber with a cover to prevent evaporation. Samples of leaf 8 and selected systemic leaves were harvested in liquid nitrogen and kept at -80 °C till further analysis.

Analysis of $[Ca^{2+}]_{cyt}$ elevations

For the analysis of $[Ca^{2+}]_{cyt}$ in whole plants, leaves of 4-week-old Arabidopsis rosettes were numbered according to their phyllotactic sequence (Dengler, 2006). The day before the experiment, plants were sprayed with 10 µM coelenterazine in 0.01% (v/v) Tween 20 and incubated in the dark for 16 h for Aequorin reconstitution. Aequorin imaging was performed according to Kiep *et al.*, (2015) using a high-resolution photon-counting camera system (HRPCS218; Photek, St. Leonards-on-Sea, UK) comprising an intensified CCD camera (ICCD218; Photek) and a camera controller (HRPCS4; Photek). The camera was mounted on a darkbox (DB-2; Photek). Signal acquisition and processing were performed with the IFS32software (Photek). Photons were captured in photon-counting mode with a 200 ms frame rate, and cumulative images were integrated offline after the experiments as indicated in the figure legends. At the end of each treatment, the rosettes were flooded with 40 mL discharge solution (1 M $CaCl_2$, 10% (v/v) ethanol) to achieve a complete discharge of Aequorin to enable calibration of the obtained data and determine the cytosolic Ca^{2+} concentration according to Knight *et al.,* (1996). The identical regions of interest (ROIs) found in treatment and discharge images were identified, and the average signal intensity in the ROIs at a given time point, as well as the cumulative counts in the ROIs were determined by using the IFS32 software. The wounding treatment consisted of mechanically wounding the midrib of leaf 8 with a pattern wheel and adding 20 µL of water (MW+W) or 20 µL of OS (MW+OS) across all holes of the mechanically wounded leaf.

Insect material and feeding assays

Larvae of the generalist herbivore *Spodoptera littoralis* were hatched and reared on artificial diet at 23–25 °C with 10-h-light/14-h-dark cycles (Bergomaz & Boppré, 1986). The oral secretion (OS) was collected from *S. littoralis* larvae fed on *Arabidopsis* Col-0 plants and stored on ice. The OS was centrifuged at 10,000 x *g* and 4 °C to remove residual plant tissue pieces and diluted with water (1:1) as previously described (Vadassery *et al.*, 2012a).

For short-term feeding assays, 3rd instar *S. littoralis* larvae were used after being kept separately without food overnight. This treatment ensures an immediate start of feeding after placement on the plant. The locally fed leaves were collected in liquid nitrogen after the indicated time points and kept at -80 °C until further analysis.

For local larval feeding assays, overnight-starved 3rd instar larvae were placed on leaf 8 for direct feeding. Each plant received one larva. After feeding on ~40% of the leaf, which took between 5-10 min, the larva was removed. After 90 min, the local and systemic leaves were harvested for phytohormone extraction and gene expression analysis.

For one-week feeding assays, 30 1st instar larvae were placed on 10 Col-0, *ann1,* and *ANN1 OE* plants, respectively (3 larvae per plant). To achieve similar starting conditions, all larvae determined for one plant genotype were pooled and weighed prior to the experiment. The minimal starting weight of 30 larvae was set to 60 mg. After 1 week, the weight of all larvae found again was recorded separately. Due to a limited number of 1st instar larvae available, the experiment was carried out several times and the weight data of each genotype were combined.

Gene expression

Total RNA was extracted from frozen material (approximately 50-100 mg) using the Trizol method according to the manufacturer's protocol (Thermo Fisher). Genomic DNA in total RNA samples was removed using the TURBO DNAse-free kit (Ambion, Thermo Fisher) according to the manufacturer's protocol. The integrity and amount of RNA were monitored by agarose gel electrophoresis and spectrophotometric quantification, respectively. Complementary DNAs were synthesized using the GeneAmp Core PCR RNA Kit (Applied Biosystems) according to the manufacturer's instructions. The pair of primers specific for *ANN1* (AT1G35720; Forward 5'-ATGGCGACTCTTAAGGTTTCTGAT-3' according to Clark *et al.* (2001) and Reverse 5'-GCCTGATGACTTTCCTCTGTTCAG-3') was used, producing a product size of 151 bp. For *VSP2* (AT5G24770) we used (Forward 5'-ACGACTCCAAAACCGTGTGCAA-3' and Reverse 5'-CGGGTCGGTCTTCTCTGTTCCGT-3', Vadassery *et al.*, 2012b), and for *JAZ10* (AT5G13220; Forward 5'-TCGAGAAGCGCAAGGAGAGATTAGT-3' and Reverse 5'-AGCAACGACGAAGAAGGCTTCAA-3', Scholz *et al.*, 2014). qRT-PCR was performed on a CFX96 Real Time System (BIO-RAD). Brilliant II QPCR SYBR green Mix (Agilent) was used to monitor the synthesis of dsDNA. Each biological sample was analyzed in technical triplicates. The cycle protocol consisted of: 95 °C for 10 min followed by 40 cycles of (95 °C for 15 sec, 60 °C for 1 min) and ended by a dissociation curve determined between 60 °C and 95 °C. The specificity of PCR amplifications was evaluated by the presence of a single peak in denaturation curves and by visualization of simple amplification products of expected size in ethidium bromide gel electrophoresis. The primer efficiencies were calculated using LinRegPCR (version 11.0, (Ruijter *et al.*, 2009). The mean relative expression of the gene was calculated according to Pfaffl (2001), using $^{\Delta\Delta}$Ct with the *ribosomal protein S18* gene (AT1G34030) as reference (Scholz *et al.*, 2014) (*RPS18,* Forward 5'-GTCTCCAATGCCCTTGACAT-3'; Reverse 5'-TCTTTCCTCTGCGACCAGTT-3').

Extraction and quantification of phytohormones

A total of 250 mg of leaf material was used for phytohormone analyses. The extraction procedure and the determination of JA and JA-Ile were performed as previously described (Vadassery et al., 2012a) with some modifications. An API5000 triple quadrupole mass spectrometer (AB Sciex, Darmstadt Germany) was used for detection (Heyer *et al.*, 2018a). Moreover, in this study, a different mixture of labeled jasmonates was used as internal standard. Instead of 15 ng of JA- [13 C6]-conjugate used in the previous study, 12 ng of D6-JA-Ile (HPC Standards GmbH, Cunnersdorf, Germany) were used. In addition, the 60 ng of 9,10-D2-9,10-dihydrojasmonic acid was replaced by 60 ng of D6-JA (HPC Standards GmbH, Cunnersdorf, Germany) as previously reported by Scholz *et al.*,(2017).

Statistics

To ensure reproducibility, all experiments have been repeated with independent biological replicates. The exact number of replicates is indicated in the particular Figure legends. For statistical analyses one- or two-way analysis of variance (ANOVA) followed by post hoc (Student–Newman–Keuls SNK; Šidák; Tukey) test or Student's *t*-test were used as indicated in the Figure legends. Different letters indicate significant differences between treatments or leaves. GraphPad Prism 6 and OriginPro 9.3 software were used for data analysis and graph composition.

RESULTS

ANNEXIN1 is induced by mechanical wounding and herbivory

Initially, in order to learn if ANN1 were involved in the plant's defense response against wounding and herbivory, we implemented different wounding-related treatments on Col-0 and *ann1-1* knock-out plants then analyzed *ANN1* expression levels. Different treatments were performed on leaf number 8:

i) mechanical wounding (MW) with a pattern wheel and applying water (MW+W) or oral secretion of *S. littoralis* (MW+OS) simulating herbivory and ii) direct feeding of *S. littoralis* larvae on one leaf. Col-0 plants showed a high accumulation of *ANN1* transcripts after all treatments, while in the *ann1-1* mutant no induction of *ANN1* was detected (Fig. **1a, b**). No significant difference between the different treatments was detected (Fig. **1a**), suggesting a universal response of *ANN1* expression in response to mechanical wounding, with or without the presence of larval oral secretion, and to herbivore attack.

Local and systemic calcium signaling is affected in *ann1* plants

Since on the one hand a $[Ca^{2+}]_{cyt}$ signal precedes jasmonate accumulation and subsequent defense-related responses upon wounding and herbivory (Fisahn *et al.*, 2004; Maffei *et al.*, 2007; Bricchi *et al.*, 2010; Toyota *et al.*, 2018; Meena *et al.*, 2019) and on the other hand annexins are components of $[Ca^{2+}]_{cyt}$ signal generation, we aimed to investigate how the elevation of $[Ca^{2+}]_{cyt}$ upon stress is influenced by the presence or absence of the ANN1 protein. Therefore, we used Col-0 and *ann1-1* plants, both containing the $[Ca^{2+}]_{cyt}$ reporter (apo)aequorin. The $[Ca^{2+}]_{cyt}$ elevation was analyzed in whole plant rosettes according to Kiep et al. (2015). Leaf number 8 was wounded with a pattern wheel before adding 20 µL water or OS (1:1 diluted) to the wounds. We observed an immediate and monophasic local elevation of $[Ca^{2+}]_{cyt}$, with a peak after around 30-45 sec, and a return to background levels after about 4 min (Fig. **2, S1**; Supporting video **1-4**). The local response was similar to those described in previous studies, but presenting a slightly faster $[Ca^{2+}]_{cyt}$ peak response (c.f., 1 min (Verrillo *et al.*, 2014; Kiep *et al.*, 2015). In case of MW+OS treatment, the Col-0 $[Ca^{2+}]_{cyt}$ elevation lasted longer when compared with the MW+W (water) treatment. Although slightly weaker than in the Col-0 plants, the local $[Ca^{2+}]_{cyt}$ response was clearly detectable in the wounded *ann1-1* leaf and moved into the petiole. Strikingly, neither water nor OS induced a systemic $[Ca^{2+}]_{cyt}$ response in the *ann1-1* genotype (Fig. **2, S1**). In contrast, upon MW+W treatment, a systemic $[Ca^{2+}]_{cyt}$ response (also monophasic) was observed in Col-0 plants (systemic leaf 5), which started after 4 min and reached a maximum at 4.5 minutes before decreasing to background levels (Fig. **S1**). Upon MW+OS treatment, the systemic response was more pronounced. Here, leaves 5, 6, 7, 10 and 11 responded in Col-0 (Fig. **2c**). This response was highly significant different when compared with *ann1*. It was possible to observe a $[Ca^{2+}]_{cyt}$ wave running from the treated leaf to the connected leaves in Col-0 (Supporting video **1, 3**) while only the local response was detected in treated *ann1* plants (Supporting video **2, 4**). Therefore, ANN1 seems to be an important player in the systemic $[Ca^{2+}]_{cyt}$ wave upon wounding and herbivory challenge.

ann1 plants are more susceptible to herbivore feeding while *ANN1* overexpressing plants are more resistant

To further evaluate the impact of ANN1 on the defense against chewing herbivores, we carried out feeding assays using first instar *S. littoralis* larvae on Col-0 wild-type plants and two different knock-out lines (*ann1-1; ann1-2*), and two different *ANN1* overexpressor lines (*ANN1-OE12; ANN-OE10)*. We performed two independent sets of experiments, each with Col-0 as wild type control, one knock-out and one overexpressor line. Our results show that larvae feeding on *ann1* plants gained significantly more weight than wild-type-fed larvae (Fig. **3**). This effect was also observed when we evaluated the larval growth on the APOAEQUORIN-containing *ann1* mutant (*ann1-1/AEQ)* (Fig. **S2**). The opposite happened when the larvae were feeding on the *ANN1-OE* plant lines, where they gained significantly less weight than those feeding on Col-0 plants (Fig. **3**).

Spodoptera littoralis feeding-induced jasmonate accumulation is affected in *ANN1* mutant plants

Jasmonates are rapidly induced upon wounding and herbivory. To test if these were differentially induced in *ann1* or *ANN1-OE10* lines compared to the Col-0 wild type, we analyzed jasmonate concentrations after herbivore feeding. In independent short-term (30 min, 90 min) feeding assays, the levels of both JA and JA-Ile increased significantly in the treated leaves of all genotypes at both time points (Fig. **4**). Even fed leaves of *ann1-1* and *ann1-2* plants still accumulated both JA and JA-Ile; however, their levels were significantly lower compared to Col-0 plants (Fig. **4**). In contrast, in the overexpressing line *ANN1-OE10* the level of JA-Ile was significantly higher than in Col-0 although the JA level was not (Fig. **4c, d**).

Systemic transcriptional responses to *S. littoralis* attack are impaired in *ann1-1* plants

In order to elucidate whether ANN1 is also involved in systemic defense, the effect of herbivory on local and systemic defense responses was analyzed in parallel, focusing on the full knock-out line *ann1-1* (Fig.1b). We performed an experiment in which *S. littoralis* was allowed to feed on one defined local leaf (leaf 8) followed by leaf sampling after 90 min (leaf numbering according to Dengler, 2006; Farmer *et al*., 2013; Kiep *et al*., 2015). In addition to the treated leaf 8, unwounded systemic leaves 5 and 13 (vascular-connected to leaf 8) and leaf 9 (unconnected to leaf 8) were sampled and analyzed for the expression of two jasmonate-responsive genes, *VSP2* and *JAZ10*. Compared with the non-treated Col-0 plants, *VSP2* was induced 10-fold in the local leaf 8 and even more strongly in the directly vascular-connected leaf 13, while no change in expression was detectable in the *ann1-1* mutant (Fig. **5a**). Also, *JAZ10* was significantly induced in the treated leaf 8 in Col-0 as well as in all systemic leaves, again with the highest expression in leaf 13. In addition, in *ann1-1* plants, *JAZ10* showed a significant induction in systemic leaves 5 and 13 (Fig. **5b**).

Herbivory-like feeding-induced systemic jasmonate accumulation is abolished in *ann1-1* plants

Considering that ANN1 is involved in jasmonate-related defense induction in systemic leaves, we wanted to study whether mechanical wounding (MW) alone employs ANN1 or if a chemical signal of the oral secretion (OS) of the larvae is necessary. Thus, we tested the systemic jasmonate response after local leaf wounding with a pattern wheel followed by either water or OS application to the small wounds. The results show a clear local jasmonate response in Col-0 as well as in *ann1-1* plants upon wounding and water (Fig. **6**). This local response was significantly higher when wounded sites were treated with OS. Interestingly, only OS treatment induced a systemic response in the vascular-connected leaves 5 and 13 in Col-0, represented by a strong increase of JA and JA-Ile. This was not the case in *ann1-1* plants where the systemic effect was completely abolished (Fig. **6**). These results were supported by other measured jasmonates. The biosynthetic precursor *cis*-OPDA as well as the catabolite OH-JA both showed very similar results as found for JA and JA-Ile (Fig. **S3**).

Previous work has shown that pattern wheel wounding does not fully represent the insect feeding-like wounding as, for example, the use of MecWorm does (Mithöfer *et al*., 2005). Therefore, MecWorm-mediated wounding was applied in an additional experiment. Based on the studies of Heyer *et al.* (2018b) we wounded leaf 8, including the midrib. As shown in Fig. **7**, the continuous mechanical wounding of leaf 8 inflicted by MecWorm significantly elevated the local levels of JA-Ile, which we focused on as the bioactive form of the jasmonates, in both Col-0 and *ann1-1* plants. Strikingly, in contrast to Col-0, in *ann1-1* plants local accumulation of jasmonates in leaf 8 was not accompanied by a comparable systemic increase of jasmonates. Moreover, a highly significant difference in JA-Ile accumulation in directly connected leaf 13 was observed when Col-0 and *ann1-1* plants were compared (Fig. **7**).

DISCUSSION

Various studies have demonstrated that a rapid, early and transient increase of $[Ca^{2+}]_{cyt}$ is involved and essential for the successful induction and regulation of jasmonate accumulation and further downstream plant defense strategies upon wounding and insect herbivory (Maffei *et al.*, 2004, 2007; Fisahn *et al.*, 2004; Arimura *et al.*, 2008, 2011; Scholz *et al.*, 2014; Yan *et al.*, 2018; Toyota *et al.*, 2018; Meena *et al.*, 2019; Kumari *et al.*, 2019). Such stress-induced $[Ca^{2+}]_{cyt}$ elevation occurs both locally and systemically (Kiep *et al.*, 2015). There are various channels that have been shown to be involved in wounding or herbivory-related Ca^{2+} influx into the cytosol such as the Two Pore Channel 1 (TPC1; Kiep *et al.,* 2015; Vincent *et al.,* 2017), glutamate receptor-like channels (GLRs; Mousavi *et al.,* 2013; Toyota *et al.,* 2018), and cyclic nucleotide-gated channel 19 (CNGC19; Meena *et al.,* 2019). However, Ca^{2+} influx may not be mediated only by conventional channels - additional, unconventional ones such as annexins might also contribute (Laohavisit & Davies, 2011; Laohavisit *et al.*, 2012; Davies, 2014; Ma *et al.*, 2019). Diverse studies in plants have gathered evidence of annexins' ability to influence Ca^{2+} transport. A growing body of data suggests that annexins play a role in plant response to nematode parasitism. Some cyst-secreted effectors are annexin-like and able to affect plant defense possibly by mimicking the endogenous annexin functions and impairing H_2O_2-induced $[Ca^{2+}]_{cyt}$ transients (Patel *et al.*, 2010; Zhao *et al.*, 2019). Moreover, it was shown that compared with wild-type, overexpression of *ANN1* and *ANN4* decreases susceptibility against *Meloidogyne incognita* nematode infection of roots while the *ann1* and *ann4* lines were more susceptible (Zhao *et al.*, 2019). Our study demonstrates a role for *ANN1* in systemic leaf defense responses against herbivore attack and mechanical wounding.

ANNEXIN1 is induced upon insect herbivory

Biotic interaction has been shown to influence annexin transcription in crops such as alfalfa (*Medicago sativa*), Indian mustard (*B. juncea)*, tomato (*Solanum lycopersicum*), and wheat (*Triticum aestivum*) (Kovács *et al.*, 1998; Jami *et al.*, 2009; Lu *et al.*, 2012; Xu *et al.*, 2016). In multiple studies it was shown that Arabidopsis *ANNEXIN1* gene expression is influenced by diverse environmental signals (Konopka-Postupolska *et al.*, 2009; Clark *et al.*, 2010; Guelette *et al.*, 2012). Here, we demonstrate the expression response of Arabidopsis *ANN1* to herbivory. The assays were designed to understand the attack in a mechanistic and holistic way, which we achieved by dissecting the insect attack into different modules of stress: mechanical wounding alone (MW+W), mechanical wounding plus OS (MW+OS) or the complex stress of larval feeding (Fig. **1a, b**). The high expression of *ANN1* upon the different but complementary stresses showed that *ANN1* transcription activation is triggered quickly (here after 90 min) by insect feeding-related damage but also by wounding alone. The latter confirms earlier results showing *ANN1* induction 24 and 48 h after wounding (Konopka-Postupolska *et al.*, 2009).

ANNEXIN1 is involved in the defense against herbivores

As with other cellular components that are involved in $[Ca^{2+}]_{cyt}$ signaling (Vadassery *et al.*, 2012b; Scholz *et al.*, 2014; Meena *et al.*, 2019), here we show that ANN1 is also an important player in the regulation of insect feeding-induced defense. Using two different knock-out and two different overexpression lines, we showed that *S. littoralis* larvae feeding on *ann1* plants gained substantially more weight (in total +27.6 %) while those feeding on ANN1-OE plants were much smaller (-26.9 %) compared to larvae feeding on Col-0 plants (Fig. **3, S2**). Thus, ANN1 is a positive regulator in herbivory-induced defense in Arabidopsis and contributes to resistance against *S. littoralis*, comparable with CNGC19 and GLRs.

To gain further insight into the putative role of ANN1 in systemic defense-related signaling, we performed a series of experiments in which a defined local leaf was treated and systemic leaves were analyzed (Mousavi *et al.*, 2013; Kiep *et al.*, 2015). Using (apo)aequorin-expressing *ann1-1* and wild-type plants we demonstrated that ANN1 is indispensable for the systemic $[Ca^{2+}]_{cyt}$ response; this is comparable to what has been found for TPC1 and GLRs before (Kiep *et al.*, 2015; Toyota *et al.*, 2018). In contrast, the local response was slightly but not significantly reduced in the *ann1-1* mutant (Fig. **2, S1**). However, compared with other studies (Nguyen *et al.,* 2018; Toyota *et al.*, 2018) the systemic $[Ca^{2+}]_{cyt}$ response was found to be weaker. This can be explained by different experimental conditions; first, in the other studies the GCaMP3 fluorescent protein–based $[Ca^{2+}]$ sensor was used, a highly sensitive calcium fluorescent reporter, while we used bioluminescent aequorin. Second, their mode of wounding was much harsher. Nguyen and colleagues (2018) even destroyed half of the leaf tissue while we used a pattern wheel causing only few small holes in the tissue. This supports the view that the intensity of wounding correlates with the intensity of the response (Nguyen *et al.,* 2018). As read-out for the defense response, the expression of jasmonate-responsive genes *VSP2* and *JAZ10* was examined. Upon larval feeding, a local increase was detected in Col-0 for both genes as well as a strong increase in the vascular-connected leaf 13 that was even higher than the local response (Fig. **5**). Such a strong systemic increase was found neither for *VSP2* nor *JAZ10* in *ann1-1* plants, suggesting that the feeding-related signals necessary to induce the systemic gene activation do not reach the distal leaves. The fact that in this and other experiments leaf 13 is stronger responding and to a lesser extent leaf 5, can be explained by the direct (leaf 13) and indirect (leaf 5) vascular connection to leaf 8 (Dengler, 2006).

As $[Ca^{2+}]_{cyt}$ elevations initiate Ca^{2+} signaling (Kudla *et al.*, 2010; Mithöfer & Boland, 2012; Scholz *et al.*, 2014; Vadassery *et al.*, 2014) and precede downstream signals such as phytohormone accumulation, we further investigated the concentrations of the jasmonates JA and JA-Ile at two time points (30 and 90 min). These jasmonates were found to be strongly induced locally in both Col-0 and *ann1* upon herbivore attack, but accumulation was significantly lower in *ann1* plants while in the *ANN1-OE10* at least the JA-Ile level was significantly higher (Fig. **4**). This is in accordance with the finding that $[Ca^{2+}]_{cyt}$ signals in *ann1* plants are affected in local leaves to a certain extent and suggests that a full induction of jasmonates in response to insect herbivores is not possible in those plants. Strikingly, the induction of *ANN1* upon herbivory or wounding (Fig. **1**) is high if compared with downstream responses such as jasmonate or gene induction. Very likely the reason behind this finding is that gene expression not always reflects the corresponding protein expression. Lee et al. (2004) already noted that *ann1-1* transcript level does not necessarily correspond to the ANN1 protein. Besides using larvae, we further evaluated the phytohormonal responses in local and systemic leaves of plants treated with mechanical wounding. We chose this approach because such experiments can be better standardized than exposure to larvae that might feed or not, in particular when only a short time period is investigated. Pattern wheel-mediated mechanical wounding, as well as wounding by MecWorm, supported the finding of local jasmonate induction in wild type and mutant (Fig. **6, 7**). Strikingly, after MW+OS treatment the response of JA-Ile in *ann1-1* plants was significantly reduced when compared to wild type (Fig. **6**). In addition, an elevation of jasmonates was observed also in systemic leaves of OS-treated plants in wild type but not in *ann1-1* mutant plants. This further indicates that ANN1 is involved in systemic $[Ca^{2+}]_{cyt}$-dependent jasmonate elevation. This notion was supported by results obtained by mechanical wounding alone, using MecWorm treatment. In *ann1-1* plants, leaf 13 showed significantly lower accumulation of JA-Ile than in Col-0 plants (Fig. **7**) suggesting that ANN1 might not only be involved in systemic OS-specific signaling but also in systemic wound-induced jasmonate accumulation.

Conflating the data obtained we propose that ANN1 is a positive factor of the $[Ca^{2+}]_{cyt}$-dependent systemic defense response against wounding and larval feeding. Thus, the absence of this unconventional Ca^{2+} channel causes an impaired systemic response. ANN1 can exist as an integral plasma membrane protein (Alexandersson *et al.*, 2004; Marmagne *et al.*, 2007). Nevertheless, as small amphipathic proteins, annexins are distributed throughout cells and can be transported within the plant *via* the phloem (Guelette *et al.*, 2012). It is possible that annexins may be recruited directly to membranes, independently of vesicle delivery, to operate in stimulus-specific signaling (Laohavisit & Davies, 2011; Laohavisit *et al.*, 2012; Davies, 2014; Espinoza *et al.*, 2017). Therefore, a plant that contains the conventional and unconventional Ca^{2+} channels should be able to recruit annexins to the tissues under stress when needed, while the *ann1* genotype can only launch reduced $[Ca^{2+}]_{cyt}$-mediated defense responses. It should be kept in mind that annexins might also act *via* the regulation of channel activities, for example by selective channel delivery to or retraction from membranes, in a similar way as the KAT1 plasma membrane K^+ channel is cycled during ABA-induced stomatal closure (Sutter *et al.*, 2007). However, the observed effect of abolishing systemic $[Ca^{2+}]_{cyt}$-induced responses in *ann1* plants may be specific for certain stresses such as herbivory and is not necessarily involved in all types of biotic and abiotic stress responses. An example is a study showing that ANN1 was not necessary for systemic signaling and development of acquired resistance in uninfected leaves during challenge with avirulent bacteria *Pseudomonas syringae* pv tomato (Carella *et al.*, 2016).

The success of the plant's defense response against stresses does not depend on one single signaling component alone. Instead, it is a coordinated local and systemic communication between cells and distant organs. For that, various signals such as reactive oxygen species (ROS), hydraulic pressure, as well as electropotential waves, $[Ca^{2+}]_{cyt}$ and others are employed in a tightly linked manner (Foreman *et al.*, 2003; Zimmermann *et al.*, 2009; Maischak *et al.*, 2010; Mousavi *et al.*, 2013; Farmer *et al.*, 2013; Davies, 2014; Seybold *et al.*, 2014; Ranjan *et al.*, 2015; Peiter, 2016; Alonso *et al.*, 2019; Saijo & Loo, 2019; Gully *et al.*, 2019). In previous studies using epidermal root tissue, hydroxyl radical-activated plasma membrane conductance of Ca^{2+} and K^+ were absent in the *ann1-1* mutant (Laohavisit *et al.*, 2012). Expression and protein levels of Arabidopsis *ANN1* also correlated with the occurrence of the radical-activated plasma membrane Ca^{2+} conductance in the root epidermis and at the apex of root hairs (Clark *et al.*, 2001; Dinneny *et al.*, 2008). These results strongly suggest that ANN1 is very likely a Ca^{2+}-permeable protein in Arabidopsis and might provide a molecular link between reactive oxygen species and $[Ca^{2+}]_{cyt}$ in the systemic defense-related signaling in plants. Further studies will address this hypothesis.

In conclusion, we investigated the role of ANNEXIN1 in local and systemic plant defense against wounding and herbivorous insects in Arabidopsis. Plant tissue wounding and cell disruption caused by feeding insects strongly induced *ANN1* expression demonstrating that it is part of the rapid defense response against invertebrate pests; neither jasmonates nor defense-related genes were upregulated systemically in *ann1* mutants. ANN1 mediates plant defense affecting larval growth and is crucial for the induction of signaling upon herbivory within the whole plant. ANN1 is an important part of systemic $[Ca^{2+}]_{cyt}$ signaling, thereby connecting $[Ca^{2+}]_{cyt}$ to subsequent downstream signals and defense responses against herbivores.

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AUTHOR CONTRIBUTIONS

J.M. and A.M. conceived the project and research plans; J.M., A.K.M., M.R. performed the experiments; J.M., A.K.M., S.S.S., R.O. and A.M. designed the experiments and analyzed the data; E.P. provided and assisted with the equipment for calcium analyses; D.K.-P., J.R., J.M.D, K.A.W. generated and provided seed material; J.M., A.K.M, S.S.S. and A.M wrote the article with contributions from all authors.

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SUPPORTING INFORMATION (SI)

Figure S1. $[Ca^{2+}]_{cyt}$ response upon mechanical wounding (MW) and water (H_2O) in Col-0 and annexin1 mutants.

Figure S2. Weight of *Spodoptera littoralis* larvae fed on Arabidopsis Col-0 and *ann1-1* plants expressing *Aequorin.*

Figure S3. Accumulation of related jasmonates after wounding and oral secretion (OS) treatment.

Video S1. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina.

Video S2. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + oral secretion (OS) of the leaf lamina.

Video S3. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana* Col-0 rosette induced by mechanical wounding + water of the leaf lamina.

Video S4. Time-lapse of $[Ca^{2+}]_{cyt}$ response in *Arabidopsis thaliana ann1-1* rosette induced by mechanical wounding + water of the leaf lamina.

FIGURE LEGENDS

Figure 1. Response of *Annexin1* in *Arabidopsis thaliana* after treatment with different stresses. Levels of *ANN1* transcripts (± SE) were determined on the wild-type and the annexin1 mutant genotype (a, Col-0; b, *ann1-1*) after injuring leaf 8 with a pattern wheel, applying either water (MW+W) or oral secretion (MW+OS) to the wounds, or promoting *Spodoptera littoralis* larvae feeding (Larvae, 3rd instar) feeding on leaf 8 until ~40% of the leaf was eaten). Per genotype and treatment 9 replicates (n=9) were done. All plants were incubated for 90 min before sampling leaves. Leaves of untreated plants were used as controls. Differences between treatments within the same

genotype were analyzed by 2-way ANOVA (SNK post hoc test); significant differences are indicated by different letters ($P < 0.05$); n.s = not significant.

Figure 2. $[Ca^{2+}]_{cyt}$ response upon mechanical wounding (MW) and oral secretion (OS) in Col-0 and annexin1 mutant. (**a**) Cumulative images over 10 min of local and systemic $[Ca^{2+}]_{cyt}$ signals in 4-week-old whole *Arabidopsis thaliana* Col-0 (top) and *ann1-1* (bottom) rosettes in response to MW+OS applied to leaf 8 (left) and after discharge (right). Discharge was used to determine L_{total} to calculate the $[Ca^{2+}]_{cyt}$ in individual leaves. (**b**) Kinetics of a representative $[Ca^{2+}]_{cyt}$ response in individual leaves in Col-0 and *ann1-1* after treatment. (**c**) $[Ca^{2+}]_{cyt}$ response (mean ± SE) in individual leaves in Col-0 and *ann1-1* after treatment. Per genotype and treatment 9 replicates (n=9) were done. cul= cumulative; dis= discharge. 2-way ANOVA (Sidak post hoc test) (*** $P < 0.001$).

Figure 3. Weight of Spodoptera littoralis larvae after feeding on *Arabidopsis thaliana* Col-0 plants, two *ann1* mutants, and two *ANN1* overexpression lines. 30 first instar larvae of *S. littoralis* were pre-weighed, and 3 larvae were placed on each plant. In each independent experiment, 10 plants per genotype were used. Larva weight was measured individually after 7 days of feeding. Each set of experiment were independently repeated (**a**, n=3; **b**, n=4). The combined total number of larvae recovered after 1 week (N) is indicated. The box indicates the middle 50% of the data points, the black line within the box the median. Whiskers are defined as 1.5 fold interquartile range, dots represent outliers. Statistical differences between the genotypes after feeding were analyzed by 1-way ANOVA (Tukeys' post hoc test) and indicated by different letters ($P < 0.001$).

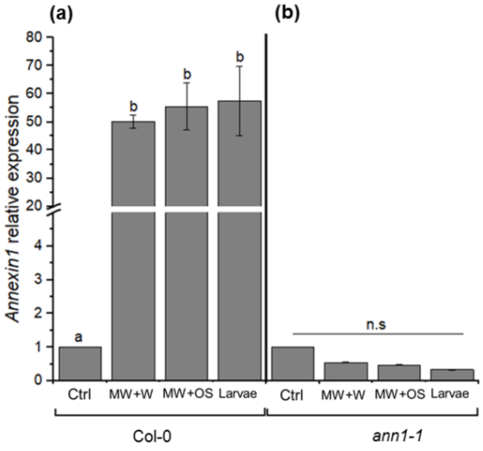
Figure 4. Local accumulation of jasmonates in *Arabidopsis thaliana* leaves after short-time feeding assay with *Spodoptera littoralis*. (**a, c**) JA and (**b, d**) JA-Ile levels (mean ± SE) from *A. thaliana* leaves were analyzed after 30 min and 90 min of feeding from a single 3rd instar *S. littoralis* larva. Phytohormones were determined only from fed leaves. 5-8 replicates, (n=5-8) were done per time point. Leaves of untreated plants were used as controls. The differences of JA and JA-Ile levels

between time points and genotypes were analyzed by 2-way ANOVA (Sidak post hoc test); significant differences are indicated by different letters (P <0.005).

Figure 5. Local and systemic transcriptional responses to *Spodoptera littoralis* feeding. (**a**) Levels of *VSP2* and (**b**) *JAZ10* transcripts were determined in *Arabidopsis thaliana* Col-0 and *ann1-1* genotypes in different leaves after local *S. littoralis* (Larvae) feeding for 90 min on leaf 8 (t). Treated leaf 8 and untreated leaves 5, 9, and 13 were analyzed (mean ± SE). Per genotype and treatment 9 replicates (n=9) were done. Leaves of untreated plants were used as controls. *RPS18* was used as a reference gene to normalize the data. Statistical differences between treatments were analyzed by 2-way ANOVA (SNK post hoc test); significant differences are indicated by different letters (P <0.05).

Figure 6. Local and systemic accumulation of jasmonates after wounding and oral secretion (OS) treatment. Levels of (**a**) JA and (**b**) JA-Ile were determined in *Arabidopsis thaliana* Col-0 and *ann1-1* genotypes in different leaves 90 min after wounding of leaf 8 with a pattern wheel (mechanical wounding = MW) and applying water (MW+W) or oral secretion (MW+OS) on the wounds (mean ± SE). Per genotype and treatment 7 replicates (n=7) were done. Untreated leaves 5, 9 and 13 and treated leaf 8 (t) were analyzed. Leaves of untreated plants were used as controls. Statistical differences found in leaves between treatments, or between genotypes were analyzed by 2-way ANOVA (* P <0.05; ** P <0.005; *** P <0.001; SNK test). No indication, no significant difference.

Figure 7. Accumulation of JA-Ile in leaves of *Arabidopsis thaliana* after mechanical wounding with MecWorm. JA-Ile levels were analyzed in Col-0 and *ann1-1* genotypes in different leaves after mechanical wounding for 90 min including the midrib. In treated plants, leaf 8 (t) was subjected to mechanical damage, untreated leaves 5, 9 and 13 and treated leaf 8 were analyzed (mean ± SE). Per genotype and treatment 6-8 replicates (n=6-8) were done. Leaves of untreated plants were used as controls. Statistical differences between leaves of control and treated plants on the same genotype, and between Col-0 and *ann1-1* leaves upon MecWorm were analyzed by an unpaired Student's *t*-test (* P <0.05; ** P <0.005). No indication, no significant difference.



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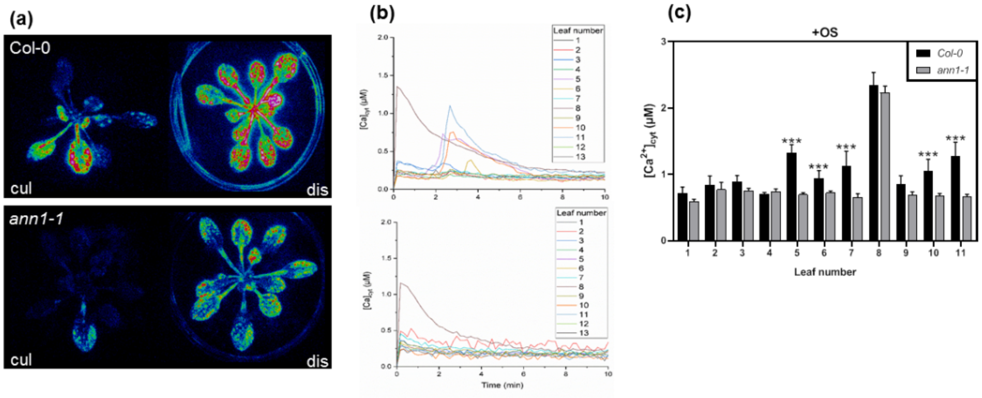

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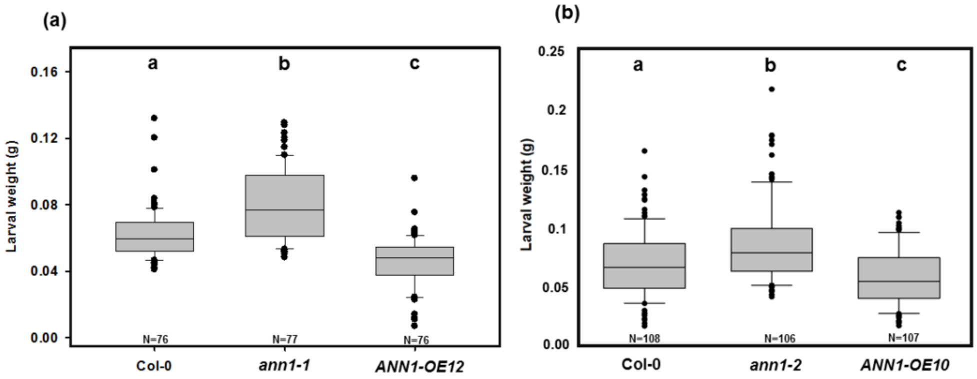

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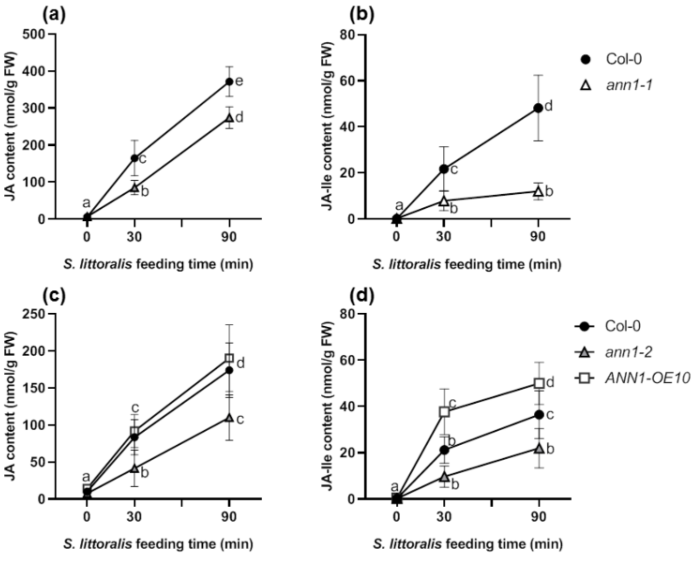

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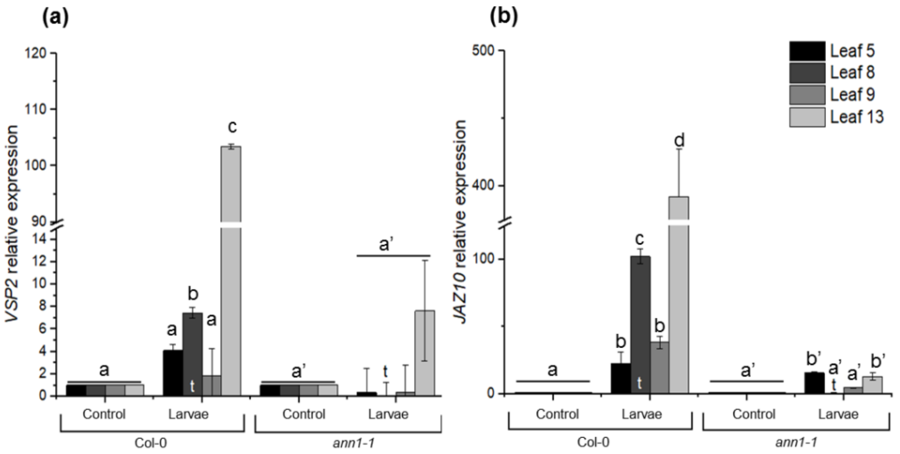

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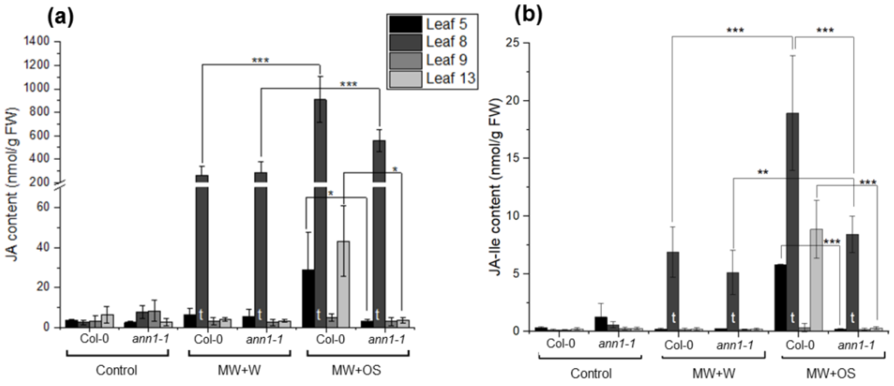

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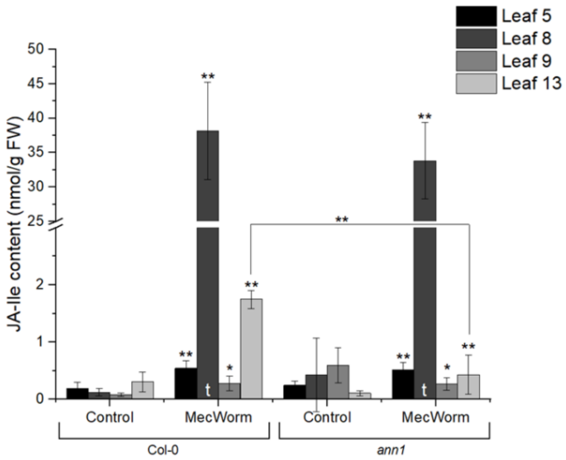

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