SHORT COMMUNICATION

Specialist Aphid Feeding Causes Local Activation of Salicylic and Jasmonic Acid Signaling in *Arabidopsis* Veins

Nikoleta Rubil,^{1,2,3} Tetiana Kalachova,^{1,†} Thure Pavlo Hauser,³ and Lenka Burketová¹

¹ Institute of Experimental Botany, The Czech Academy of Sciences, Rozvojová 263, 165 02, Prague 6, Czech Republic

² Czech University of Life Sciences, Kamýcká 129, 165 00 Prague 6–Suchbát, Czech Republic

³ Department of Plant and Environmental Sciences, Copenhagen University, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

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Aphids, the phloem sap feeders, probe into leaf tissues and activate a complex network of plant defense responses. Phytohormonal signaling plays a major role in this network; however, the dynamics of the signal spreading is yet to be clarified. Despite the growing knowledge about transcriptomic changes upon infestation, results often differ due to sampling, varying strongly between the tissues collected at the single feeding site, individual leaves, pooled infested leaves, or whole plant rosettes. This study focuses on activation of salicylic acid (SA) and jasmonic acid (JA) signals in *Arabidopsis* leaves during infestation by cabbage aphid (*Brevicoryne brassicae*) in high spatio-temporal resolution. We used genetically encoded fluorescent biosensors, histochemistry, and quantitative reverse transcription-PCR to precisely map activation of distinct branches of phytohormonal signaling. We found a rapid induction of SA and JA signaling markers in cells surrounding stylet puncture, colocalizing with callose deposition. For both *PRI* and *JAZ10*, we detected activation at 24 h postinfestation (hpi), increasing and spreading along the veins until 72 hpi and, to a lesser extent, within the epidermal pavement cells. The SA signaling wave appeared in parallel with JA-associated signaling and continued to increase in time. Our results first show a local activation of SA- and JA-related responses after stylet penetration of *Arabidopsis* leaves and bring a detailed insight into the spatio-temporal complexity of plant defense activation during specialist aphid attack.

Keywords: *Arabidopsis thaliana*, *Brevicoryne brassicae*, callose, infestation, jasmonic acid, salicylic acid, phytohormonal signaling

Plants are frequently attacked by herbivores and pathogens and have evolved complex defense mechanisms to counter this,

[†]Corresponding author: T. Kalachova; kalachova@ueb.cas.cz

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including localized as well as remote reactions. The immediate local responses are often accompanied by spreading signals to induce more remote reactions; however, the dynamics of these signaling responses are still not understood in detail. As phloem sap feeding insects, aphids evolved a characteristic elongated mouthpart, called a stylet, by which they probe into plant tissue, secrete saliva, and, finally, reach for the nutrients inside the sieve elements. For successful colonization, specialized molecules in aphid saliva, termed effectors, manipulate plant defense responses during probing and feeding periods. During probing, the stylet follows an intercellular pathway in the mesophyll while excreting gelling saliva. Many cells are punctured intracellularly along the stylet track and are filled with watery saliva (Miles 1959). Stylets are immediately retracted in the moment of cell puncture and continue to move between the cells in the direction of sieve elements, aiming to avoid the activation of plant defense mechanisms (Miles 2007; Tjallingii 2006). During the stylet penetration into sieve elements, aphids start a massive secretion of watery salivary molecules, with the goal to prevent sieve-tube occlusion by interacting with calcium ions (Miles 2007; Tjallingii 2006; Will et al. 2009). Hence, when a stylet finally punctures phloem cells, Ca^{2+} increases in these and triggers protein and callose synthesis, which occludes sieve tubes to prevent loss of phloem sap (Knoblauch and van Bel 1998; Silva-Sanzana et al. 2020). In response to this plant reaction, the attacker tries to manipulate plant defense responses by injecting Ca^{2+} -binding proteins, with the goal to suppress the blockage of nutrients (Tjallingii 2006; Will et al. 2007, 2009). However, the host plant may be able to recognize aphid-derived molecules or elicitors, resulting in an activation of a complex series of molecular defense responses, such as triggering of intracellular signaling cascades in which phytohormones, e.g., salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), play major roles (Jaouannet et al. 2014; Kuśnierczyk et al. 2008).

Up to now, the spatial dynamics of defense activation in leaf tissues in response to aphid feeding remains rather enigmatic. Despite an increasing amount of data, the sensitivity and accuracy of transcriptome studies is largely limited by various factors: i) sample preparation, as a certain amount of leaf tissues needs to be homogenized, ii) highly heterogeneous infestation due to the distinct number of individuals feeding on the leaf, and iii) different methods of sampling between studies. Several studies reported the induction of SA and JA signaling responses during the aphid infestation (De Vos et al. 2005; Moran and Thompson 2001), while attackers differing in a range of host plants, i.e., specialists or generalists, trigger distinct changes in plant transcriptome (Kuśnierczyk et al. 2007; Mewis et al. 2005;

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Moran et al. 2002). On the other hand, some of the studies reported changes in gene expression when sampling the whole rosettes of *Arabidopsis* (Kuśnierczyk et al. 2008; Mewis et al. 2005; Onkokesung et al. 2019) or the individual aphid-infested leaves (Kuśnierczyk et al. 2007; Moran and Thompson 2001; Moran et al. 2002). However, feeding aphids are not homogeneously dispersed on the leaf but mostly localize along the veins, which might introduce a significant bias into sampling.

Our goal was to investigate the dynamics of *Arabidopsis thaliana* defense responses during the infestation with a specialist aphid, *Brevicoryne brassicae*, in higher spatial resolution, with a special focus on the cells in close contact with the stylet. For this, we used a combination of biochemical and molecular biology methods with novel visualization tools to dissect the role of individual cell reactions and cell-to-cell communication in response to herbivorous attacks. To study the spatial progression of the signal induced by feeding aphids, we combined the following approaches: i) quantitative reverse transcription PCR (qRT-PCR) of marker genes for different signaling pathways, sampling 6-mm leaf discs around the aphid feeding site; ii) monitoring activation of marker genes in vivo in the area surrounding the feeding site, using a set of visualization tools, including plant lines expressing promoter-fluorescent reporter constructs for genes associated with SA, JA, and ET signaling pathways (Marhavý et al. 2019); and iii) monitoring the localization of *PR1* activation using plants expressing the *PR1::GUS* construct.

Four-week-old *A. thaliana* plants were cultivated in a short-day photoperiod before infestation with *B. brassicae*, and samples were collected at 6, 24, 48, and 72 h postinfestation (hpi). Based on previously published results about transcriptome remodeling upon infestation, several genes marking activation of distinct hormone signaling pathways were chosen for qRT-PCR: SA (*PATHOGENESIS-RELATED 1, 2, and 5* [*PR1, PR2, PR5*], *ISOCHORISMATE SYNTHASES 1 and 2* [*ICS1, ICS2*]), JA (*LIPOXYGENASE 2* [*LOX2*], *Allene Oxide Synthase* [*AOS*], *Jasmonate ZIM-Domain 8 and 10* [*JAZ8, JAZ10*]), ET (*ACC SYNTHASES 2 and 6* [*ACS2, ACS6*]), and ET/JA (*PLANT DEFENSIN 1.2* [*PDF1.2*], *HEVEIN-LIKE/PATHOGENESIS-RELATED 4* [*HEL/PR4*]). Cycle threshold values of defense genes were normalized to the geometric mean of two housekeeping genes, namely *ACT8* and *SAND*, and the transcription of both genes is shown in Supplementary Figure S1. A list of analyzed genes and primers is available in Supplementary Table S1. Furthermore, to visualize the activation of marker genes on a cellular level in different leaf tissues surrounding the stylet insertions, we used a set of promoter-reporter transgenic lines of *A. thaliana* (Marhavý et al. 2019), expressing fusions of defense gene promoters with mVenus reporter and a nuclear localization signal (NLS). The lines were originally designed to study gene activation in roots during infestation by the nematode *Heterodera schachtii*. Hence, the authors monitored nematode performance from the first moment of root invasion until the feeding establishment and analyzed SA, JA, and ET-associated responses. During this cellular damage interval, the induction of SA and JA-associated markers was not detected, while the expression of ET marker was significantly induced. We found this method applicable for experiments with aphids, as with this approach the single aphid “effect zone” can be studied. We tested several lines to dissect activation of separate hormone-associated signaling pathways: ET (*ACS6*), JA (*AOS* and *JAZ10*), ET/JA (*PR4/HEL*), and SA (*PR1*). Unfortunately, high background noise was observed in noninfested leaves with reporter constructs for *ACS6*, *AOS*, and *PR4/HEL*, which brought difficulties for data interpretation (Supplementary Fig. S2). Reporter lines for SA (*PR1*) and JA (*JAZ10*) were found suitable for our system.

Aphid feeding caused upregulation of several genes involved in defense signaling and biosynthesis of defense-related hormones,

as found by our qRT-PCR analysis of leaf discs at feeding sites (Fig. 1). Changes in the expression of one gene involved in SA synthesis (*ICS1*) and stress-responsive SA-related genes (*PR1, PR2, PR5*) were detected at 24 h after *B. brassicae* infestation compared with noninfested control. *ICS1* showed fivefold induction at 24 hpi, while *ICS2* was significantly induced only from 48 hpi, suggesting this gene is involved in maintaining rather than in the establishment of the response. Transcription of *PR1* was over 100-fold higher in aphid-infested leaf discs at 24 hpi compared with noninfested control and about 1,000-fold higher at 48 and 72 hpi (Fig. 1). As for *PR2* and *PR5*, the pattern of induction was similar to that of *PR1* (gradual increase in transcription starting from 24 hpi). Previous studies also reported strong induction of *PR1* gene 72 hpi by a specialist aphid (Kuśnierczyk et al. 2007, 2008; Moran et al. 2002). However, *PR1* fold change was considerably higher in our study, likely because we analyzed leaf discs closely surrounding the feeding sites while other researchers analyzed whole leaves or even rosettes. Kuśnierczyk et al. (2007) infested three different ecotypes of *A. thaliana* (Ws, Cvi, and Ler) with *B. brassicae* and, after harvesting the whole leaves, the induction of *PR1* as a consequence of aphid infestation was five-, two-, and 21-fold in Ws, Cvi, and Ler ecotype, respectively. Moreover, Moran et al. (2002) reported 23-fold induction of *PR1* in infested leaves of *A. thaliana* ecotype Col-0 at 72 hpi. Surprisingly, Onkokesung et al. (2019) observed the induction of *PR1* while sampling whole rosettes of *A. thaliana* ecotype Col-0 (up to 50-fold) already at 6 h after infestation with *B. brassicae*; however, no induction was observed at 24, 48, and 72 hpi. The significant and strong induction at later timepoints found in our study could be caused by our spatial sampling technique, as, for the whole leaf and rosette sampling, the signal may be masked by the background level of many noninduced cells.

In addition to the SA-related marker genes, our qRT-PCR analysis found an early induction of several ET and JA marker genes in leaf tissues surrounding feeding sites. Infestation triggered the expression of the gene coding for the ZIM-domain protein, *JAZ8*, already at 6 hpi and strongly increased the expression of *JAZ8* and *JAZ10* genes at 24 and 72 hpi (Fig. 1). On the contrary, genes involved in the octadecanoid signaling pathway or wound-inducible JA pathway (*LOX2, AOS*) were significantly upregulated in aphid-infested samples at 24 and 48 hpi. On the other hand, Kuśnierczyk et al. (2007) observed rather low induction of JA biosynthetic genes in the whole leaves of different ecotypes of *Arabidopsis* only at 72 hpi; the change in *LOX2* expression ranged from three- to sixfold and, in *AOS* expression, from two- to threefold. Later on, Kuśnierczyk et al. (2008) also reported lower but still significant induction of *JAZ10* gene in the whole rosettes of *A. thaliana* ecotype Ler at 6, 24, and 48 hpi (up to 1.4-fold).

As for the genes important for ET synthesis, *ACS2* and *ACS6*, our qRT-PCR analysis showed the induction already at 6 hpi, indicating an early response to *B. brassicae* attack; then, low but significant induction was observed at 24 hpi, followed by the strong upregulation at 48 and 72 hpi (Fig. 1). Our results are in line with other studies reporting the involvement of ET responses during aphid infestations in whole leaves and rosettes (Kuśnierczyk et al. 2007, 2008; Smith and Boyko 2007). Kuśnierczyk et al. (2007) also observed an increase in *ACS6* transcription in *B. brassicae*-infested leaves of *A. thaliana* ecotype Ws (up to twofold), Cvi (up to 13-fold), and Ler (up to twofold) at 72 hpi. In our setup, genes involved in both JA and ET signaling pathways, *PR4/HEL* and *PDF1.2*, were also found to be induced in infested leaf discs at 24, 48, and 72 hpi (Fig. 1). Interestingly, Moran et al. (2002) reported higher induction of *PDF1.2* in *B. brassicae*-infested leaves of *A. thaliana* ecotype Col-0 (up to 48-fold) after 72 h of aphid feeding, while Kuśnierczyk et al. (2008)

Fig. 1. Gene expression changes in leaf discs around feeding sites of the specialist aphid *Brevicoryne brassicae* in wild-type plants of *Arabidopsis thaliana* (Col-0) after 6, 24, 48, and 72 h of infestation. Graphs represent genes involved in the salicylic acid signaling pathway (*PR1*, *PR2*, *PR5*, *ICS1*, *ICS2*), jasmonic acid (JA) signaling pathway (*LOX2*, *AOS*, *JAZ8*, *JAZ10*), ethylene (ET) biosynthetic pathway (*ACS2*, *ACS6*), and ET/JA signaling pathway (*PDF1.2*, *HEL/PR4*). Cycle threshold values of target genes were normalized to the geometric mean of two housekeeping genes, namely *ACT8* and *SAND*. Control and infested samples were compared within each timepoint with the Student's *t* test; *P* value is indicated for comparisons that passed the significance threshold; normality of data distribution was checked using the Shapiro-Wilk test; *n* = 4. Error bars represent standard error of the mean. Dashed line on *y* = 1 is set to a normalized mean of noninfested control for each timepoint.

reported lower induction of *PDF1.2* in infested rosettes of Ler ecotype 48 hpi (up to twofold). Similarly to our results, Kuśnierczyk et al. (2007, 2008) reported gradual upregulation of the *HEL* gene in the leaves and whole rosettes of *A. thaliana* ecotype Ler at 6, 24, 48, and 72 hpi; however, the induction was relatively low (up to 2.7-fold).

Using fluorescent reporter lines, we were also able to detect an induced transcription of *PR1* and *JAZ10* in the cells surrounding the stylet puncture at 24 hpi in comparison with noninfested

plants (Fig. 2A and B). Notably, the induction started from the sites of stylet punctures (visible as an autofluorescent line [Fig. 2B]) at 24 hpi, and, until 48 hpi, was spreading along the veins, while, at 72 hpi, the reporter fluorescence was detected also in the leaf epidermis pavement cells outside the vein (Fig. 2B). The induction of *PR1* and *JAZ10* was evaluated by manual counting of the number of cells with nuclear fluorescence per leaf disc. Statistically significant induction of *PR1* and *JAZ10* was detected in the cells of aphid-infested plants at each timepoint (Fig. 2C).

Fig. 2. Local activation of *PR1* and *JAZ10* transcription in *Arabidopsis thaliana* veins upon infestation around the feeding sites of *Brevicoryne brassicae*. **A**, **B**. *brassicae* feeding on the leaves of *pPR1::NLS3xVenus* and *pJAZ10::NLS3xVenus* 48 h postinfestation (hpi). Scale bar = 500 μ m. **B**, Representative images of *PR1* and *JAZ10* induction. Yellow arrows point to the nuclei with induced reporter construct expression, blue arrows point to the stylet puncture autofluorescence. Scale bar = 10 μ m. **C**, *PR1* and *JAZ10* quantification of induced cells per 6-mm leaf disc. Control and infested samples were compared within each timepoint with the Student's *t* test; normality of data distribution was checked using the Shapiro-Wilk test; *P* value is indicated for comparisons that passed the significance threshold; *n* = 10. Error bars represent standard error of the mean.

To complement previous results regarding visualization of spatio-temporal dispersion of *PR1* induction from the sites of aphid feeding, *Arabidopsis* plants expressing *PR1::GUS* were infested and analyzed by X-GLUC assay sampling whole

infested leaves (Supplementary Methods S1). As expected, β -glucuronidase (GUS) activity was detected, as the *PR1* gene was slightly induced in infested plants at 24 hpi and more strongly in later timepoints (Fig. 3A and B) and was detectable around the

Fig. 3. *Brevicoryne brassicae* triggers *PR1* expression and callose deposition. **A**, Histochemical staining of β -glucuronidase (GUS) activity in leaves of *PR1::GUS* at 24, 48, and 72 h postinfestation (hpi) by *B. brassicae*. **B**, Dynamics of *PR1::GUS* expression and callose deposition in leaves at 24, 48, and 72 hpi by *B. brassicae*. Yellow arrows indicate the sites of stylet insertion, blue arrows point to deposited callose. Scale bar = 100 μ m.

sites of stylet puncturing (Fig. 3B, yellow arrows). Indeed, the strong induction of *PR1* seems to occur locally around the aphid feeding zones, as we found both while sampling leaf discs for qRT-PCR analysis (Fig. 1) and when using promoter-reporter line *pPR1::NLS3xVenus* (Fig. 2A and B).

Callose, a β -glucan polymer synthesized at the plasma membrane of plant cells, plays an important role in plant defense responses to aphid infestation (Silva-Sanzana et al. 2020). To study whether callose deposition is spatially connected with defense gene activation, we subsequently stained *PR1::GUS* leaves with aniline blue for callose deposition analysis after X-GLUC assay (Supplementary Methods S1). Callose deposition was detected at all studied timepoints at the sites of *B. brassicae* stylet insertions; hence, it colocalized with *PR1* gene activation (Fig. 3B). Our findings confirmed previously observed callose formations (Kuśnierczyk et al. 2008) around the stylet insertion sites during *B. brassicae* wounding of *Arabidopsis* cells. Interestingly, generalist aphids seem to trigger similar responses in *Arabidopsis*. De Vos et al. (2005) observed *PR1* gene induction around aphid feeding sites in *PR1::GUS*-expressing leaves but only after 72 h of *Myzus persicae* feeding. This could be explained by variation in protein concentration and composition of saliva between specialist and generalist aphid species that causes different response intensity from plant defense systems (Will et al. 2009).

With the development of novel visualization tools, the research on plant-pest interactions is getting more precise and detailed. We show that the stylet penetration during attack by the *B. brassicae* aphid causes a rapid activation of plant defense signals in the cells surrounding the stylet puncture in *A. thaliana*. In time, the induction spreads along the veins and, to a lesser extent, radially to the leaf tissues. In this work, we particularly show that subtle, though biologically important, changes in transcriptomic response can be detected by performing precise sampling procedures (i.e., collecting leaf discs around feeding sites instead of pooling material from the whole infested rosettes or leaves). Local transcriptomic remodeling seems to occur faster in tissues close to feeding sites than in the rest of the leaf when compared with previously published studies. Our study therefore also suggests that important dynamics in space and time soon after herbivore attack may be missed if not using spatially explicit sampling, as also found for the reaction of leaf photosynthesis to herbivory (Moustaka et al. 2021). We wish to draw attention to the need for a detailed description of sampling protocols that might influence data output and drive conclusions, especially in high-throughput methods like transcriptomics or metabolomics.

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