Tissue-specific systemic responses of the wild tobacco *Nicotiana attenuata* against stem-boring herbivore attack

Gisuk Lee[1†], Youngsung Joo[2†], Ian T. Baldwin[3] and Sang-Gyu Kim[1*]

Abstract

Background: Plants are able to optimize defense responses induced by various herbivores, which have different feeding strategies. Local and systemic responses within a plant after herbivory are essential to modulate herbivore-specific plant responses. For instance, leaf-chewing herbivores elicit jasmonic acid signaling, which result in the inductions of toxic chemicals in the attacked leaf (tissue-specific responses) and also in the other unattacked parts of the plant (systemic responses). Root herbivory induces toxic metabolites in the attacked root and alters the levels of transcripts and metabolites in the unattacked shoot. However, we have little knowledge of the local and systemic responses against stem-boring herbivores. In this study, we examined the systemic changes in metabolites in the wild tobacco *Nicotiana attenuata*, when the stem-boring herbivore *Trichobaris mucorea* attacks.

Results: To investigate the systemic responses of *T. mucorea* attacks, we measured the levels of jasmonic acid (JA), JA-dependent secondary metabolites, soluble sugars, and free amino acids in 7 distinct tissues of *N. attenuata*: leaf lamina with epidermis (LLE), leaf midrib (LM), stem epidermis (SE), stem pith (SP), stem vascular bundle (SV), root cortex with epidermis (RCE), and root vascular bundle (RV). The levels of JA were increased in all root tissues and in LM by *T. mucorea* attacks. The levels of chlorogenic acids (CGAs) and nicotine were increased in all stem tissues by *T. mucorea*. However, CGA was systematically induced in LM, and nicotine was systematically induced in LM and RCE. We further tested the resource allocation by measuring soluble sugars and free amino acids in plant tissues. *T. mucorea* attacks increased the level of free amino acids in all tissues except in LLE. The levels of soluble sugars were significantly decreased in SE and SP, but increased in RV.

Conclusions: The results reveal that plants have local- and systemic-specific responses in response to attack from a stem-boring herbivore. Interestingly, the level of induced secondary metabolites was not consistent with the systemic inductions of JA. Spatiotemporal resolution of plant defense responses against stem herbivory will be required to understand how a plant copes with attack from herbivores from different feeding guilds.

Keywords: Tissue-specific defense, Stem herbivore, Jasmonic acid, Systemic response, *Trichobaris mucorea*, *Nicotiana attenuata*

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Background

In nature, plants face attack from multiple herbivores from a broad range of feeding guilds, e.g., leaf chewing, sap-sucking, stem-borer, and root-feeder. For instance, aphids penetrate the phloem in vascular bundles using their stylets to extract plant nutrients (Will et al., 2013). Leaf-mining larvae live inside of leaves, and most only feed on leaf mesophyll tissues (Sinclair and Hughes, 2010). In addition, many stem-boring beetles (Coleoptera) specifically feed on parenchymal tissues in stems (Lee et al., 2016; Michaud & Grant, 2005), while lepidopteran stem-borers feed on various tissue types in stems (Potter and Held, 2002). Therefore, responses that are fine-tuned to the feeding guild of the attacker could increase the efficiency of plant defense.

Jasmonic acid (JA) induces specific defense responses in attacked tissue (Erb and Reymond, 2019). Exogenous treatment with methyl jasmonate elicits different responses in the shoots and roots of *Arabidopsis thaliana* (Tytgat et al., 2013). JA signaling also activates systemic defense responses in unattacked tissues that result in shoot-shoot and shoot-root signaling in response to herbivore attacks. JA-mediated systemic responses allow plants to anticipate and prepare for attack in unattacked leaves (Li et al., 2002). In addition, systemic JA inducibility is tissue-specific. When *Manduca sexta* attacks *Nicotiana attenuata* leaves, diurnal rhythms of primary and secondary metabolites in the attacked leaf are differentially altered compared with the rhythms in systemic leaves and roots (Kim et al., 2011). Some systemic responses are asymmetrically activated. For example, stem-boring herbivores increases JA levels in attacked *N. attenuata* stems but not in systemic leaves, whereas leaf-chewing herbivores increase JA levels in both attacked leaves and unattacked stems (Lee et al., 2017).

Toxic "secondary" metabolites elicited by JA are commonly derived y from primary metabolism, with amino acids being a major class precursor. For instance, phenolic compounds are synthesized from the shikimate and phenylpropanoid pathways, which are derived from the aromatic amino acids (tryptophan, phenylalanine, and tyrosine) (Tohge et al., 2013). Amino acids are also used in phytohormone biosynthesis, commonly by forming conjugates, e.g., JA-isoleucine and tryptophan-conjugated indol-3-acetic acid (Fonseca et al., 2009). Leaf herbivory reshapes resource allocation in plants: source strength in damaged leaves is decreased and carbon source allocation to systemic roots is increased (Schwachtje and Baldwin, 2008). Hence, to understand JA-elicited tissue-specific defense responses, it is essential to examine both local and systemic alterations in both primary and secondary metabolites.

In this study, we examine systemic defense responses of *N. attenuata* when the stem-borer *T. mucorea* attacks.

N. attenuata has been used as a model system to study plant-insect interactions. JA signaling in *N. attenuata* induces many defense-related metabolites, e.g., nicotine (Steppuhn et al., 2004), chlorogenic acid (Lee et al., 2017), diterpene glycosides (Heiling et al., 2010), phenolamides (Kaur et al., 2010; Onkokesung et al., 2012), and volatile compounds (Schuman et al., 2012). We hypothesized that this stem-boring herbivore alters the levels of both primary and secondary metabolites in the systemic tissues of *N. attenuata* to fine-tune plant defense.
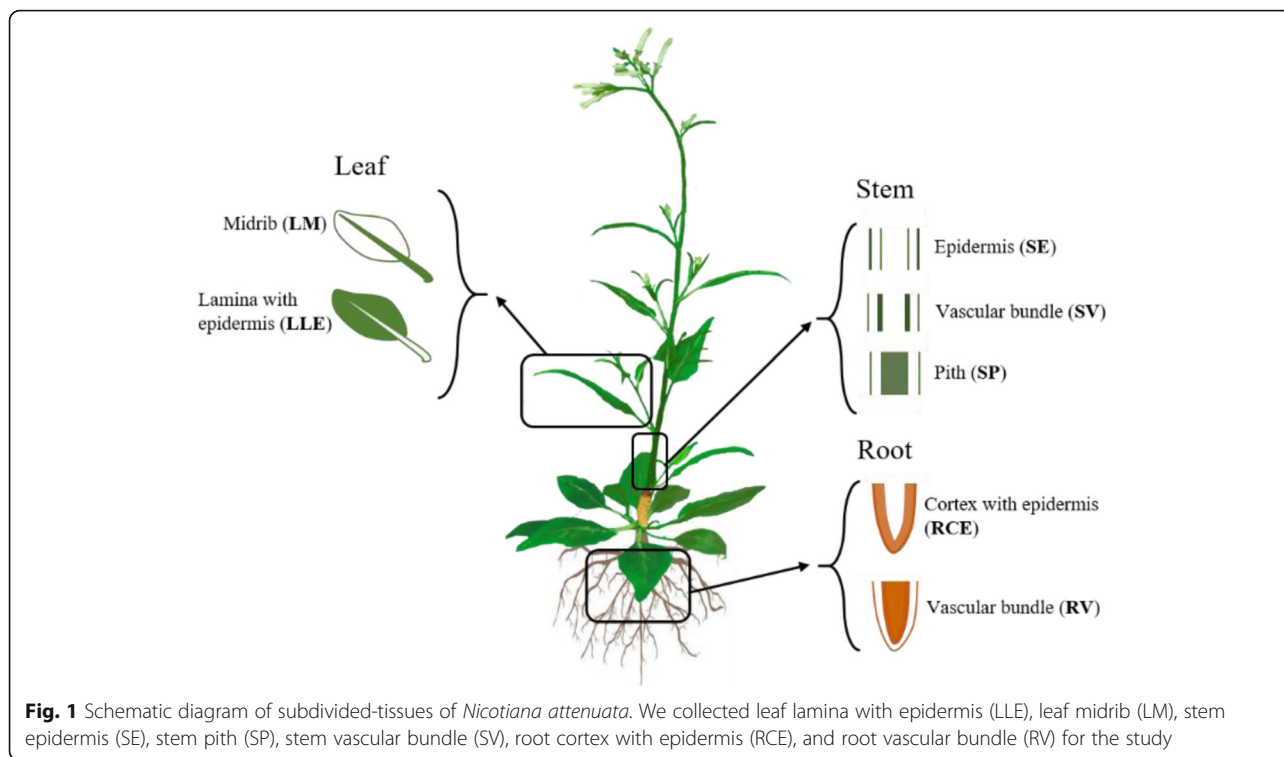
Materials and methods

Plant and insect growth conditions

We used the 31st inbred generation of *N. attenuata* seeds which were originally collected from a native population at a field site located in Utah, USA. Germination procedures and glasshouse conditions have been described previously (Krügel et al., 2002). Young seedlings were planted individually in Teku plastic pots after 10 days' germination; another 10 days later, early rosette plants were transferred to soil in 1-l pots and grown in the glasshouse with 16-h day (26–28°C)/8 h (22–24°C) night, under supplemental light from Master Sun-T PIA Agro 400 or Plus 600 high-pressure sodium lamps with an automatic glasshouse watering system.

For stem herbivore treatments, we used laboratory colonies of *T. mucorea* and the egg inoculation method, reported in our previous study (Lee et al., 2016). Briefly, we inoculated a single egg per plant that was in the early flowering stage, and 3 weeks later, we sampled various plant tissues in *T. mucorea*-attacked plants and control plants. Stem samples were collected 5 cm above from the *T. mucorea*-attacked site, and the 2nd or 3rd stem leaf was collected from the attacked and control plants. Lastly, root samples were collected from the taproot. To investigate tissue-specific systemic responses against stem-boring herbivore attack, we divided plants into 7 tissues for further analyses (Fig. 1): leaf midrib (*LM*), leaf lamina with epidermis (*LLE*), stem epidermis (*SE*), stem vascular bundle (*SV*), stem pith (*SP*), root cortex with epidermis (*RCE*), and root vascular bundle (*RV*). *T. mucorea* adults and larvae were grown in a growth chamber with a 16-h light (26°C)/8-h dark (24°C) cycle and 65% humidity.

Plant metabolites and phytohormones analysis

Aliquoted 100 mg of homogenized plant samples were placed in liquid nitrogen-precooled 96-well bio-tubes. All homogenized samples were ground in the mortal with the pestle. Samples were extracted with acidified methanol (methanol, distilled water, and formic acid in a ratio of 15:4:1) spiked with isotope-labeled phytohormone standards and 4-methylumbelliferone. The samples with the extraction solvent were shaken in a

Fig. 1 Schematic diagram of subdivided-tissues of *Nicotiana attenuata*. We collected leaf lamina with epidermis (LLE), leaf midrib (LM), stem epidermis (SE), stem pith (SP), stem vascular bundle (SV), root cortex with epidermis (RCE), and root vascular bundle (RV) for the study

GenoGrinder 2000 for 60 s at 1150 strokes. After centrifugations at 13,000g, a part of the supernatant was used for the primary metabolites (amino acids and sugars). The remaining supernatant was purified on two solid-phase extraction (SPE) columns for hormones, following the protocol described by Schäfer and colleagues (Schäfer et al., 2016). For all liquid chromatograph-mass spectrometry (LC-MS) analysis, an ultra-high-performance liquid chromatography (UHPLC, Dionex UltriMate 3000) was used with a chromatography column (Agilent Zorbax Eclipes XDB-C18, 50 x 3.0mm, 1.8μm) as the stationary phase, connected a mass spectrometer, Bruker Elite EvoQ triple-quadropole MS equipped with a heated electrospray ionization (HESI) ion source. The samples analyzed in multiple reaction monitoring and post-run analysis was performed with the Bruker MS Workstation version: 8.1.2. Analyses were carried out with six biological replicates in each subdivided tissue from each damaged and control plants.

Unbiased metabolites analysis

For non-targeted metabolite analysis, approximately frozen 100 mg homogenized samples were extracted by the extraction buffer (60% solution of acetic acid and ammonium acetate adjusted to pH 4.8 with 1M ammonium hydroxide, and 40% (v/v) methanol). The samples were homogenized by a GenoGrinder 2000 for 60 s at 1200 strokes. Supernatants were collected after 20 min of centrifugation at 16,100g at 4°C as described in a previous

study (Heiling et al., 2010). The extracted supernatants were performed on a microTOF-Q II (Time-of-Flight, Bruker Daltonics) equipped with an electrospray ionization (ESI) source operated in positive ionization mode. Extractions used six biological replicates for analysis. The output results were preprocessed with *R* packages (version 3.4.1) XCMS and CAMERA which used a previously designed precursor-to-product assignment pipeline (Broeckling et al., 2013). Peak identification, peak matching, and retention time correction from the XCMS package were performed using the centWave algorithm with the following parameter settings: ppm=20, snthresh=10, peakwidth=c (5,18). Missing peak values were filled using the fillPeaks function. The CAMERA package was used for annotating isotope and adduct ions, and after 75 percentile normalization and $\log_2$ transformation, the final output results from each subdivided tissue and treatment (damaged and control) were analyzed in the MetaboAnalyst platform (www.metaboanalyst.ca) for using a cluster analysis.

Statistical analysis

Data analysis was mainly conducted with Origin 2019 (OriginLab Cop. Northampton, USA). We used *R* packages (*qqnorm*) to test whether data is normally distributed. The metabolites result of control and attacked tissue was analyzed by a Student's *t*-test. A cluster analysis by heat map method was performed on each group following the annotated dataset from XCMS and

CAMERA *R* packages in subdivided tissue and treatments, using the following parameters: Pearson distance measure, ward clustering algorithm, and auto-scale features standardization.

Results

We experimentally controlled stem-boring herbivore damage by inoculating an egg into a stem (Lee et al., 2016). Since herbivores induce tissue-specific and systemic responses, we divided herbivore attacked plants into 7 different tissues. First, we measured the level of JA in the 7 tissue types of *T. mucorea*-infested plants

and control plants (Fig. 2a). JA levels were highly induced in all stem tissues (SE, SV, and SP), and JA levels were systematically induced in other tissues (LM, RCE, RV), except LLE. Systemic induction of JA is much higher in the root tissues than in the leaf tissues (Fig. 2a). We also measured salicylic acid (SA) and abscisic acid (ABA) to test the systemic induction of other phytohormones (Fig. 2b, c). In *T. mucorea*-attacked plants, levels of ABA were significantly induced in SE and SV, but slightly decreased in RCE (Fig. 2b). SA was mostly unchanged in all subdivided tissues but significantly decreased in SV (Fig. 2c).
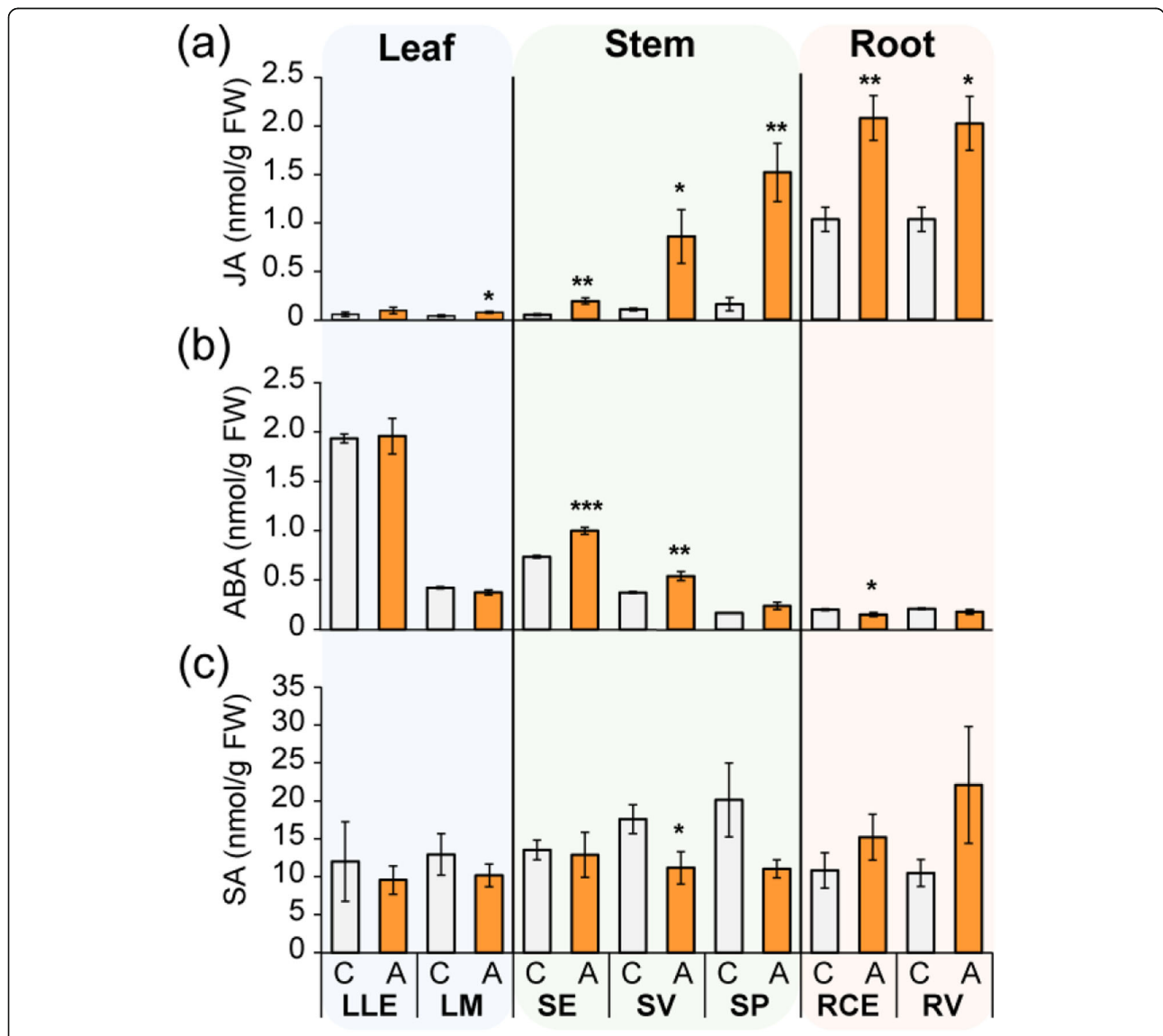

Fig. 2 The levels of phytohormones in seven different tissues from *Trichobaris mucorea*-attacked and control plants. **a** Mean (± SE) levels of JA were significantly increased in the attacked LM, all stem and root tissues comparing with control tissues. **b** Mean (± SE) levels ABA were only increased in SE, SV, and RCE. **c** Mean (± SE) levels SA were not significantly different between subdivided tissues from control and attacked plants. C represents non-attacked (control) plants, and A represents *T. mucorea*-attacked plants. Asterisks indicate significant differences among treatments (Student *t*-test; *$p <$ 0.05; **$p <$ 0.01; ***$p <$ 0.001; $n = 6$)

Fig. 3 Tissue-specific induction of secondary metabolites in *T. mucorea*-attacked and control plants. **a** Mean (± SE) levels of chlorogenic acid (CGA) were highly induced in all stem tissues (SE, SP, and SV) and LM. **b** Mean (± SE) levels of nicotine were significantly increased LM, all stem tissues, and RCE. C represents non-attacked (control) plants, and A represents *T. mucorea*-attacked plants. Asterisks indicate significant differences among treatments (Student *t*-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 6$). FW, fresh weight
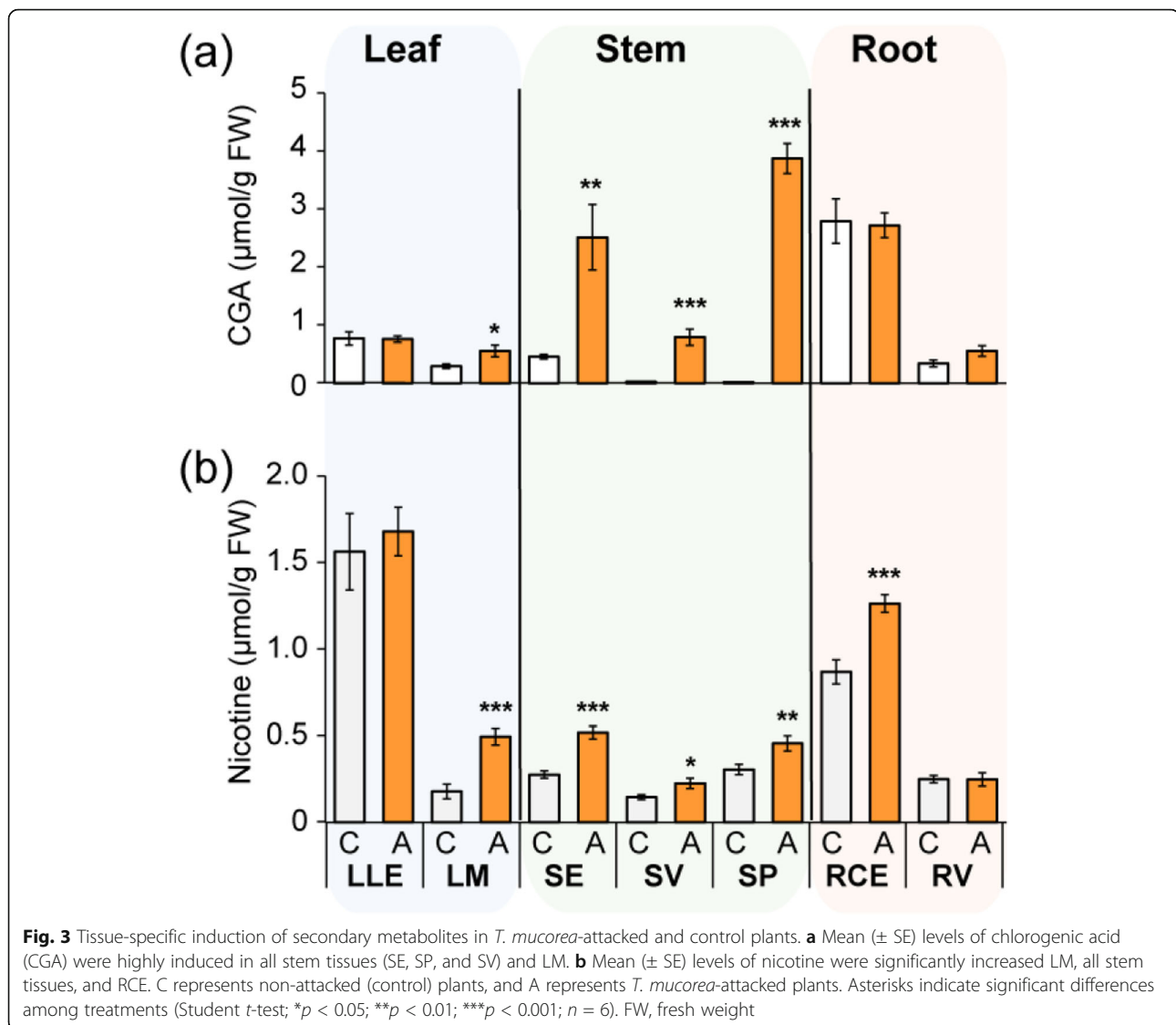
We have further compared plant secondary metabolites between *T. mucorea*-attacked plants and control unattacked plants to test systemic effects of stem-boring herbivore attack in the different tissues. Among various toxic secondary metabolites in *N. attenuata*, we measured nicotine as a non-tissue-specific defense compound (Li et al., 2020; Steppuhn et al., 2004) and CGA as a stem-specific defense compound (Lee et al., 2017) (Fig. 3). In response to stem-boring herbivore attack, CGA was highly induced in all stem tissues (SE, SV, and SP) and systematically only induced in LM against the stem-boring herbivore attack (Fig. 3a). The constitutive level of CGA was highest in RCE, but CGA levels were not changed in the root attacked plants (Fig. 3a). Constitutive levels of nicotine were highest in LLE, but nicotine also was induced in all stem tissues (SE, SV, and SP), LM, and RCE (Fig. 3b).

To investigate the systemic effects of stem-boring herbivore attack in plant primary metabolism, we measured eighteen free amino acids and three soluble sugars in the different tissues from *T. mucorea*-attacked and control plants (Fig. 4). Interestingly, free amino acids were increased in all tissues except LLE attacked plants, which was consistent with the patterns of JA induction (Fig. 4a). In contrast, soluble sugars (mostly fructose and glucose) significantly decreased only in two stem tissues (SE and SP). Interestingly, the level of soluble sugars was significantly increased in RV (Fig. 4b).

Untargeted metabolomic profiling by LC-qTOF-MS allowed for a more holistic analysis of metabolic changes in the different tissues of *T. mucorea*-attacked plants. The analysis resulted in an integrated data matrix consisting of 1263 mass features, being *m/z* signals detected at particular retention times. We used hierarchical

Fig. 4 Mean (± SE) levels of free amino acids and soluble sugars in seven subdivided tissues when stem herbivores attack. **a** Total amounts of free amino acids in LM and all parts of root tissue systemically increased in response to stem herbivore attack. In contrast, **b** total amounts of soluble sugars in the different tissues of attacked tended to decrease in comparison to those of control plants. Soluble sugars in SP and SV were significantly decreased compared to the those of control plants, but soluble sugars in RV were significantly increased compared with those of control plants (Student t-test; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n=6). FW, fresh weight. C represents non-attacked (control) plants, and A represents *T. mucorea*-attacked plants

clustering and Venn diagram analysis of the normalized data set of spectral intensities (Fig. 5). We found four major clusters in the hierarchical clustering (Fig. 5a): (1) LLE from the attacked plants and SP, RCE, and RV from control plants; (2) LLE and SE from control plants; (3) LM and SE from the attacked plants and LM and SV from control plants, and (4) SP, SV, RCE, and RV from the attacked plants. Venn diagrams revealed that stem and root tissues shared only three substances among 691 upregulated mass features in attacked plants. There were no features which were identically elicited between the stem and leaf tissues (Fig. 5b).

Discussion

Different plant tissues are not homogenously defended against herbivorous insects. Plants appear to prioritize the allocation of defenses to different tissues in accordance with the tissue's probability of being attacked and their fitness value for the plant, as predicted by the Optimal Defense Hypothesis (ODH) (McKey, 1974). As not all parts of a plant are equally valuable or equally likely to be attacked, defense concentrations vary considerably amongst plant parts (Schuman and Baldwin, 2016). Consistent with ODH predictions, the inducibility of both JAs and the metabolites they elicit are also highly variable among plant parts (Meldau et al., 2012; Pichersky and Lewinsohn, 2011; Wolinska and Berens, 2019). In nature, plants encounter more than one biotic and/or abiotic stress condition. Zandalinas et al. show that combination of heat and light stresses induce different systemic responses in plants compared with systemic responses induced by single heat or light stress (Zandalinas et al., 2020). Although we found that leaf herbivore attacks did not alter the level of metabolites induced by stem-boring herbivore in our previous study (Lee et al., 2017), it will be interesting to examine how biotic (root herbivore, pathogen) and abiotic (drought, nutrient) stresses modulate tissue-specific responses in plants for optimal defense.

Here, we showed that the levels of JA and CGA were systemically induced in LM, but not in LLE (Figs. 2a and 3a). *T. mucorea* larvae do not feed on the leaf lamina, but neonate *T. mucorea* larvae frequently feed on leaf petioles as they move into stems (unpublished data). Thus, the systemic induction patterns of JA and secondary metabolites are consistent with the chance of being attacked by this stem-boring herbivore, again consistent with ODT predictions. Interestingly, the induced JA levels in root tissues (RCE and RV) were similar to those in SP, which is a tissue damaged by the herbivore (Fig.
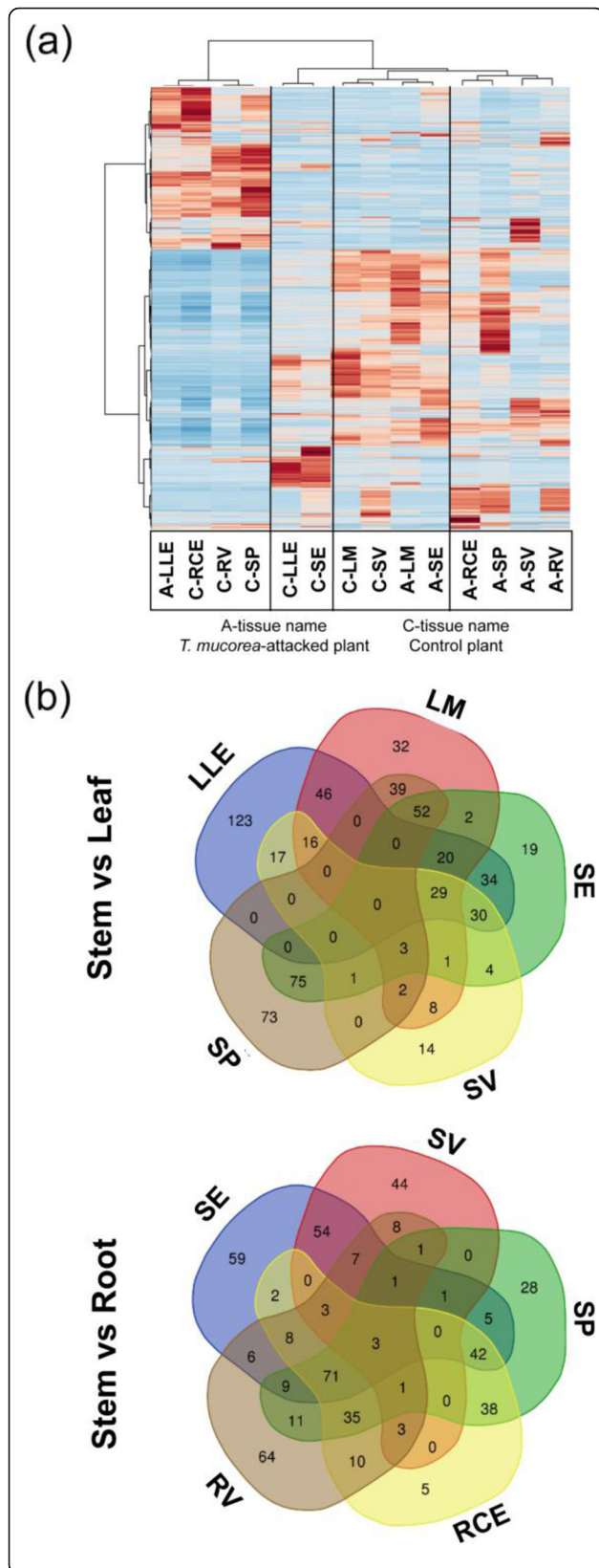
Fig. 5 Hierarchical clustering and Venn diagram analysis using an integrated dataset of annotated metabolites from different tissues of *T. mucorea*-attacked and control plants. **a** Four main clusters appeared from the hierarchical clustering analysis based on Pearson distance calculations. The heat-map coloring reflects the scaled intensities. **b** Venn diagram of different stem versus root tissues and stem versus leaf tissues. The numbers indicate upregulated mass features detected in different tissues when plants are attacked by *T. mucorea* larvae

2a). Of the two major secondary metabolites quantified in the roots, only nicotine concentrations increased in RCE; however, soluble sugar level significantly increased in RV and free amino acid significantly increased in all root tissues (Figs. 3 and 4). In response to folivore attack, carbon is allocated to roots in many plant species (Schwachtje and Baldwin, 2008); our results suggest that attack of stems results in a similar allocation of resources to roots. Systemic induction of JA levels in the roots may represent the phytohormonal mechanism by which the levels of soluble sugars in the roots increase (Erb et al., 2012; Huang et al., 2014; Soler et al., 2013). Therefore, tissue-specific plant systemic responses can affect to the pattern of resource and secondary metabolites allocation between the stem and the root.

In a previous study, we found that CGA induction is JA-dependent in the stem, but not in the leaf (Lee et al., 2017). Here we also showed that CGA was not induced in the root though JA levels in the root were systemically induced by stem-boring herbivore attack (Figs. 2a and 3a). JA-dependent CGA regulation is clearly highly organ-specific. It remains unclear why *N. attenuata* dramatically increases quinic acid-conjugates (e.g., CGA) in response to attack to stems. Nicotine, the most abundant alkaloid in *N. attenuata*, is known as an efficient defense metabolite in leaf tissue against leaf-chewing herbivores (Baldwin, 2001; Steppuhn et al., 2004). A recent study which independently manipulated another class of induced phenolic conjugates, the phenolamines, and nicotine levels in response to *T. mucorea* attack found that larvae fed on nicotine-free plants performed much better and that phenolamide levels had little influence on larval performance (Li et al., 2020). It is possible that the CGA response plays a role in the stem-lignification response, which has recently been shown to be activated by *T. mucorea* attack and functions as a defense response (Joo et al., 2021). Nicotine accumulation is JA- and IAA-dependent both in leaves and roots (Li et al. 2020; Fragoso et al., 2014; Baldwin 1989), and the systemic induction patterns of nicotine reported here (Figs. 2a and 3b) are consistent with those of JA. These results highlight the complexity of plant responses to herbivore attack and the importance of disentangling the different components of this complex response at level of the tissues that are attacked to understand their function.

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Unbiased metabolites analysis of subdivided tissues under biotic stress and abiotic stress can contribute important information about the tissue-specific distributions of induced metabolites and their fate upon growth or reproduction. In fruits, some metabolites, such as glycosylated flavonoids including rutin, kaempferol rutinoside, and a quercetin trisaccharide, was not equally distributed during the fruit ripening (Moco et al., 2007). In *N. attenuata*, tissue-specific metabolic specialization has been studied by combining tissue-specific non-targeted mass spectral data acquisition, information theory analysis, and tandem MS molecular networks (Li et al., 2016). Particularly, there are compelling patterns of metabolic specialization in floral limb and anthers (Li et al., 2016). In the results presented here, root and stem tissues produce more metabolites than did leaf tissues when plants responded to stem-boring herbivore attack (Fig. 5), again highlighting the importance of tissue-specific responses.

Conclusions
In this study, we demonstrate that plant systemic responses are not identical across tissues and organs and that the responses to stem-boring herbivore attack are highly tissue-specific. JA levels were systemically induced more in roots than in leaves, but inductions of secondary metabolites were not always consistent with those of JA. Changes in primary metabolites in roots in response to stem-borer attack were also correlated with changes in JA.

Abbreviations
ABA: Abscisic acid; CGA: Chlorogenic acid; HESI: Heated electrospray ionization; JA: Jasmonic acid; LC-MS: Liquid chromatography-mass spectrometry; LC-qTOF-MS: Liquid chromatography-quadrupole time-of-flight-mass spectrometry; ODH: Optimal defense hypothesis; SA: Salicylic acid; SPE: Solid-phase extraction; UHPLC: Ultra-high-performance liquid chromatography

Authors' contributions
GS and YS planned and designed the study. GS and YS collected and analyzed the data. GS and YS wrote the manuscript. SK and ITB reviewed/edited the manuscript. SK and YS made equal contributions. All authors read and approved the final manuscript.

Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author (Professor Sang-Gyu Kim, sgkim1@kaist.ac.kr) on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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