

Plants recognize identity and intensity of herbivores to adaptively reconfigure metabolism

Corresponding Author: Professor Youngsung Joo

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Choi et al. investigate how *Arabidopsis thaliana* differentially adjusts its leaf metabolome in response to four herbivore species (*Lipaphis pseudobrassicae*, *Myzus persicae*, *Plutella xylostella*, *Spodoptera litura*) applied at three distinct attack intensities. Using untargeted metabolomics combined with herbivore performance assays, the authors show that plants mount species-specific and intensity-dependent metabolic responses that go beyond simple feeding-guild or host-range classifications. These responses are quantitatively characterized through chemical distance metrics, chemical diversity, and MS/MS-based annotations. The study further dissects the regulatory underpinnings using hormone (JA, SA) treatments and mutants impaired in phytohormone signaling (*myc2/3/4*, *npr1*) as well as membrane co-receptors (*bak1-5*, *sobir1*). Finally, the authors perform correlation-based screening to identify putatively resistance-associated metabolites, validate allantoin as a novel resistance compound against *L. pseudobrassicae*, and discuss the adaptive nature of the observed metabolic reconfiguration.

This work provides a comprehensive metabolomics analysis. The mechanistic dissection is particularly interesting. By comparing wild-type and mutant lines impaired in JA/SA signaling and membrane receptor complexes, the study goes beyond descriptive metabolomics and provides new evidence linking both upstream receptor-mediated recognition (via BAK1 and SOBIR1) and downstream phytohormonal pathways to herbivore-specific metabolic fingerprints. This adds a new layer to the plant defense signaling paradigm, traditionally dominated by phytohormones. Overall, this is an impressive study that could potentially advance our understanding of plant defense specificity. I have only a few minor points that need to be addressed to improve the paper.

1. The authors used different initial numbers of herbivores (1, 2, 3 for caterpillars; 2, 4, 6 for aphids) to create three intensity levels. However, the actual damage or feeding pressure may not scale linearly with initial insect number, especially for aphids that can reproduce during the 6-day feeding assays. Did the authors measure final insect numbers or feeding damage (e.g., leaf area consumed, honeydew production) to verify that the intended intensity levels were achieved?

2. The functional Hill number increased for Lp and Mp, whereas chewers (Px and SI) decreased diversity (Fig. 3h). The decrease in metabolic diversity is consistent with previous results in *Nicotiana attenuata* consumed by chewing insects and with optimal defense theory. How do the authors interpret the increased diversity observed upon phloem-feeding insect attack?

3. SA levels were induced by Lp, Mp, and Px, but not by SI (Fig. 2c). However, MeSA treatment significantly reduced diversity (Fig. 3i), a pattern contrasting with the increased diversity induced by Lp and Mp. This discrepancy should be further discussed.

4. The authors acknowledge that *bak1-5* and *sobir1* are pleiotropic co-receptors affecting many membrane receptors, including those for MAMPs/DAMPs. This limits the interpretability of their specificity loss, as the effect could stem from impaired general stress perception rather than herbivore-specific recognition. Do the authors have evidence (e.g., from early marker gene expression) that BAK1/SOBIR1 are specifically engaged by herbivore elicitors/treatment in their system? Such evidence would strengthen the conclusion that membrane receptors “fine-tune herbivore-specific metabolic responses”.

5. The authors show that allantoin is highly induced by Lp, Px, and SI and acts as a resistance compound against Lp. Is the

accumulation of allantoin affected in the phytohormone or co-receptor mutants? If so, this would further connect functional interpretation with mechanistic regulation and complement the correlation-based screening of resistance compounds.

6. In line 932, “heling” should be corrected to “helping”.

7. In Figure 4, “Foldenrichment” should have a space (“Fold enrichment”).

Reviewer #2

(Remarks to the Author)

This manuscript details a study on the metabolomic effects of four different herbivores on *Arabidopsis* plants. The research aims to analyze the specificity of the plant's response based on the herbivore's feeding mode (sap-sucking vs. chewing) and diet breadth (specialist vs. generalist). To further understand the underlying mechanisms, the researchers also subjected mutant plants, defective in jasmonic or salicylic acid signaling and membrane receptors, to the same treatments. The authors correlated changes in the plant's metabolic profiles, and hormone levels (jasmonic, salicylic, and abscisic acid) after six days of herbivore feeding to herbivore and plant biomass, thereby identifying patterns related to the number of herbivores and their feeding style. By correlating changes in the metabolome to herbivore feeding levels, they identified allonin as a most responsive compound and experimentally tested its effect on aphid performance. Based on their data, they concluded that plants respond specifically to particular herbivores, and also that some of the signaling pathways involved may not have to be intact for the plant to show a response. They critically discuss the issues related to their analysis, which is highly appreciated.

Overall, this paper presents a highly valuable dataset on the effect and specificity of herbivore-induced responses. The hypotheses are clearly introduced and visualized in figure 1. Several different statistical (multivariate) analyses have been performed and joined to interpret the data set and the figures elaborately show the results obtained. Overall, the text is well written and clear but it may need editing by a native English speaker.

Having said that, the authors overstate the lack of studies comparing induced responses to multiple types of herbivores. One of the most comprehensive – even though on herbivore-induced volatile responses – may be Danner H, et al (2018) *New Phytologist* 220:726-738. doi: 10.1111/nph.14428. This one, and others (e.g. from the Poelman group at Wageningen University, Netherlands) are missing in the Supplementary excel table (minor comment; please give full bibliographic information, not only titles).

Similarly, the MS would benefit by looking at studies providing a strong conceptual framework as well as experimental evidence for the functional role of metabolic diversity, including: Petrén H, et al (2023) Quantifying chemodiversity considering biochemical and structural properties of compounds with the R package chemodiv. *New Phytologist* 237:2478-2492. doi: <https://doi.org/10.1111/nph.18685>; Whitehead SR, et al (2021) Interaction diversity explains the maintenance of phytochemical diversity. *Ecology Letters* 24:1205-1214. doi: 10.1111/ele.13736; Walker et al (2023) Leaf metabolic traits reveal hidden dimensions of plant form and function. *Science Advances* 9:eadi4029. doi: 10.1126/sciadv.adi4029; Walker et al (2022) Functional Traits 2.0: The power of the metabolome for ecology. *Journal of Ecology* 110:4-20. doi: <https://doi.org/10.1111/1365-2745.13826>). These previous studies would be a great support to interpret the data in the proper conceptual framework to close the gap between ecology and metabolomics (lines 418-419).

Several aspects of the experimental setup require clarification in the materials and methods section. First, the number of plants used for each herbivore treatment is not specified. This information is essential for understanding the statistical power and robustness of the experiment. Second, it is unclear whether individual plants were harvested and analyzed separately or if plants were pooled into biological replicates. If pooling was performed, the number of plants constituting each pool must be detailed. Third, the manuscript does not state whether control plants were established for the methyl jasmonate and methyl salicylate treatments. The plants were sprayed with solutions of these compounds in 0.2% Tween and 10% ethanol. The solvent solution alone, containing only Tween and ethanol, may have elicited physiological or metabolic responses in the plants. Therefore, a control group treated only with the solvent solution is necessary to isolate the specific effects of methyl jasmonate and methyl salicylate.

It is also questionable how much of the primary metabolome was sufficiently captured with the metabolic approach used (LC-MS). The excel list with the m/z data suggests that amino acids were detected but the masses suggest that most are not amino acids proper.

Regarding the herbivory treatments, particularly with the chewing herbivores, it is important to know if the amount of leaf mass removed was measured. These data would be valuable for several reasons. It could reveal whether the plant's response correlated with the amount of removed biomass or the total area of leaf damage. Furthermore, this measurement is highly relevant to the subsequent analysis presented in Figure 7D, where plant biomass is correlated with metabolomic profiles. While a higher degree of herbivory is expected to induce a stronger metabolic response, it also directly reduces plant biomass, especially in the case of chewing herbivores. This reduction in biomass, which increases with herbivore pressure, could act as a confounding factor in the analyses presented in figure 7D, which has not been accounted for in the analysis.

There were two figures that I struggled with to understand or interpret properly. One is figure 6, presenting several network analyses converted in distribution plots. Without knowing the exact background of these analyses or which biological

process they inform us on, their meaning is really difficult to understand. In figure 7, I was struggling with understanding panels c and d. Apart from the fact that I think that d may have suffered from a confounding factor (see above), it was not clear why there were no dots for m/z features in the middle. I assume it has something to do with the enrichment. In the figure legend, it said that only NC terms that were significantly enriched at least once were used, which can suggest that also non-significant ones were used in some of these correlations (?). It was really hard to understand how this figure came about, and there is not enough information in the materials and methods to get a better understanding of this. It also was not clear if and how the numbers of herbivores per plant would tie into this figure. Officially each figure should be informative as a standalone piece of information. So, this information needs to be added concisely to the legend and also better explained in the materials and method section.

Correlating metabolite concentration with insect performance is indeed one way to identify potentially active compounds, as mentioned in the discussion. However, in this case, leaf metabolomes were assessed, whereas aphids suck on the phloem. Leaf and phloem metabolic profiles may differ substantially within plants. Another question is why allonin was chosen for the test with the aphids. I assume there must have been more significant negative correlations with other compounds – and for other compounds with other herbivores. It is not well motivated why this single compound-herbivore relationship was singled out.

In addition, there are some minor comments or corrections to make in a next version of this manuscript. For example, there are several figure legends, which comprise abbreviations, such as DAM or NC, which need to be explained under each figure. Second, the label f is missing in Figure 2 on the panel that it refers to. In the same figure, I suggest to add after how many days the plants were harvested (panel 2a). In figure 3, in the fourth line of the legend, it mentions: different alphabets on the boxes - that should be different letters.

Some phrasing may need to be adjusted. In line 267, the phrasing "enrichment of glucosinolates... was replaced by flavonoids" could be interpreted as anthropocentric. I suggest rephrasing this to be a direct comparison, for example: "Higher levels of flavonoids were observed in the SI-induced metabolome, in contrast to the higher levels of glucosinolates and open-chain polyketides found" Additionally, in line 272, the phrase "exhibited more diminished specificity" is slightly redundant. Simply stating that "bak 1-5 exhibited reduced specificity" would be more concise.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have thoroughly addressed all the points raised by the reviewers in this revised version. The responses are well-reasoned and satisfactorily resolve the previous concerns. In my view, the manuscript has been substantially improved and now meets the standards for publication. I therefore recommend that it be accepted.

Reviewer #2

(Remarks to the Author)

I thank the authors for implementing my comments and addressing my many questions so very carefully. After reading the revised version, which I found much clearer and complete, in particular the rationale behind selecting allantoin for the bioassays. Also, the limitations of the study have been discussed much better in the discussion now.

I must confess that, despite your best effort to explain figure 6 very clearly in your rebuttal letter, I still fail to see how these conclusions emerge from the figures (distributions) and computational analyses as they are presented. To be very honest: the text for supplementary Method 1 is much beyond my expertise and current knowledge. I am fully ready to accept that this is my limitation, and I hope that the other reviewer can say more about this.

Figure 7 and its legend has become much clearer now, thanks!

I have only a few comments/suggestions for improvement or clarification left.

These statements were added but were somewhat unclear:

Line 159 -160: "... number of aphids after 6 days of feeding showed that the actual attack intensities were increased by the initial treatments (Supplementary Fig. 2)". Do you mean: increased with the numbers of aphids placed on the plant ?

Line 162: "... feeding sites" Also in Fig. S2 : What is meant here? How many places they damaged the leaves (Irrespective of the size of the area removed)?

Line 813: resistive compounds....: this is an uncommon term, please replace by anti-herbivore compounds/defensive compounds

In general, the text was much improved, leaving only a few minor grammatical errors with respect to using a plural noun and a singular verb and vice versa. Nothing that a text editor could not fix.

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1 REVIEWER COMMENTS

3 Reviewer #1 (Remarks to the Author):

5 *Choi et al. investigate how Arabidopsis thaliana differentially adjusts its leaf*
6 *metabolome in response to four herbivore species (Lipaphis pseudobrassicae, Myzus*
7 *persicae, Plutella xylostella, Spodoptera litura) applied at three distinct attack*
8 *intensities. Using untargeted metabolomics combined with herbivore performance*
9 *assays, the authors show that plants mount species-specific and intensity-dependent*
10 *metabolic responses that go beyond simple feeding-guild or host-range classifications.*
11 *These responses are quantitatively characterized through chemical distance metrics,*
12 *chemical diversity, and MS/MS-based annotations. The study further dissects the*
13 *regulatory underpinnings using hormone (JA, SA) treatments and mutants impaired in*
14 *phytohormone signaling (myc2/3/4, npr1) as well as membrane co-receptors (bak1-5,*
15 *sobir1). Finally, the authors perform correlation-based screening to identify putatively*
16 *resistance-associated metabolites, validate allantoin as a novel resistance compound*
17 *against L. pseudobrassicae, and discuss the adaptive nature of the observed metabolic*
18 *reconfiguration.*

20 *This work provides a comprehensive metabolomics analysis. The mechanistic*
21 *dissection is particularly interesting. By comparing wild-type and mutant lines*
22 *impaired in JA/SA signaling and membrane receptor complexes, the study goes beyond*
23 *descriptive metabolomics and provides new evidence linking both upstream*
24 *receptor-mediated recognition (via BAK1 and SOBIR1) and downstream*
25 *phytohormonal pathways to herbivore-specific metabolic fingerprints. This adds a new*
26 *layer to the plant defense signaling paradigm, traditionally dominated by*
27 *phytohormones. Overall, this is an impressive study that could potentially advance our*
28 *understanding of plant defense specificity. I have only a few minor points that need to*
29 *be addressed to improve the paper.*

30 : We deeply appreciate your effort on reviewing our manuscript and valuable comments
31 that significantly improves our manuscript. We fully revised our manuscript based on
32 your comments to improve clarity and logical discussions. Point-by-point responses are
33 described below.

35 *1. The authors used different initial numbers of herbivores (1, 2, 3 for caterpillars; 2,*
36 *4, 6 for aphids) to create three intensity levels. However, the actual damage or feeding*
37 *pressure may not scale linearly with initial insect number, especially for aphids that*
38 *can reproduce during the 6-day feeding assays. Did the authors measure final insect*
39 *numbers or feeding damage (e.g., leaf area consumed, honeydew production) to verify*
40 *that the intended intensity levels were achieved?*

41 : We also agree that direct measures of feeding pressure are important. For the chewers,
42 we added the data quantifying damaged leaf area and the number of feeding sites per
43 plant in Supplementary Fig. 3; for the two aphids we added the final aphid number after 6
44 days in Supplementary Fig. 2. Both measures generally scaled with the initial intensity,
45 confirming that the intended level of attack was achieved. The expected non-linearity was
46 observed: the number of SI feeding sites number increased steadily across intensities,
47 while damaged area saturated at SI3. In contrast, damaged area by SI gradually increased
48 gradually with intensity in *npr1*, *bak1-5*, and *sobir1*. These results support that plants
49 attacked by stronger herbivore strongly deter further insect feeding, together with the
50 herbivore performance data (Supplementary Fig. S1).

51 Determining whether our initial treatments successfully applied gradually increasing
52 level of induced defense and/or fitness cost to plants remains difficult, since metabolic
53 induction is multidimensional and qualitative rather than a single dose. Moreover, it

could be more important for plants to anticipate the future attack than correlating the induction to the cumulative damage, as the degree of herbivory varies temporally in the natural habitat and plant defense can be phenologically tuned.

Revised manuscript L201-205: The number of aphids after 6 days of feeding showed the actual attack intensities increased by the initial treatments (Supplementary Fig. S2). The damaged leaf area and number of feeding sites was measured for plants attacked by chewing herbivores, which showed gradual increase of chewing damage through intensities (Supplementary Fig. S3).

2. The functional Hill number increased for Lp and Mp, whereas chewers (Px and Sl) decreased diversity (Fig. 3h). The decrease in metabolic diversity is consistent with previous results in *Nicotiana attenuata* consumed by chewing insects and with optimal defense theory. How do the authors interpret the increased diversity observed upon phloem-feeding insect attack?

: This contrast is one of our most interesting findings which we discuss explicitly in the revised manuscript. As you mentioned, a previous study¹ tested two mutually exclusive hypotheses, the optimal defense and moving target theories, finding that two chewing herbivores (one generalist, one specialist) both decreased chemical diversity, supporting optimal defense theory. In contrast, we found that attack by two aphid species increased chemical diversity, potentially consistent with the moving target theory. Higher mean pairwise DreaMS-embedding distance among aphid-induced features, reflecting greater chemical dissimilarity, supports this possibility. Furthermore, exclusiveness of this increase of chemical diversity only in chemically weighted metric (functional Hill number) but not in chemically unweighted metric (Hill number) also implies that aphids-induced metabolites comprise structurally distinct compounds. However, enrichment of specific chemical classes (e.g., flavonoids by Lp and saccharides by Mp) is not consistent with moving target theory which predicts structurally random induction of metabolites. Therefore, while our results do not fully adhere to the dichotomy between optimal defense and moving target, nevertheless providing several novel observations deserving further investigations. Another notable observation is the partial loss of this phenotype in *bak1-5*, *sobir1*, and *npr1*, suggesting that membrane receptors and salicylic acid-mediated processes are required for optimal defense theory. It is interesting that chewing herbivore-dependent chemical diversity decrease was dependent on jasmonic acid both in Li *et al* and in our results. We additionally interpreted our results in discussion.

Revised Manuscript L634-646: This aphid attack-induced increase in chemical diversity is partially consistent with moving target theory, which predicts unpredictable induction of metabolites upon herbivore attack. While enrichment of several chemical classes in aphid-induced metabolome blurs the dichotomy between optimal defense and moving target theories, induction of metabolites without significant class-level enrichment may still occur. Not significant changes in unweighted Hill number supports this possibility. Interestingly, *npr1*, *bak1-5*, and *sobir1* mutants exhibited partially weakened increase of chemical diversity by aphid attack, but methyl salicylate treatment alone did not significantly alter the chemical diversity. Our results suggest that SA signaling associated with membrane receptors might be necessary to mediate induction of chemically diverse compounds in response to aphid attacks^{43,62}.

3. SA levels were induced by Lp, Mp, and Px, but not by Sl (Fig. 2c). However, MeSA treatment significantly reduced diversity (Fig. 3i), a pattern contrasting with the increased diversity induced by Lp and Mp. This discrepancy should be further discussed.

: Thank you for the interesting point to be discussed. Discrepancy between herbivore and hormone treatment on chemical diversity of induced metabolome provides a test on necessity and sufficiency of hormonal signalling on mediating metabolic responses.

Indeed, MeSA treatment revealed that SA is not sufficient to increase chemical diversity, while results of *npr1* suggests that SA signalling is at least partially required to increase chemical diversity in response to aphid attack. Additional discussions with several previous studies suggesting that SA signalling broadly modifies transcriptome and possibly metabolism were added.

Revised manuscript L642-646: Interestingly, *npr1*, *bak1-5*, and *sobir1* exhibited partially weakened increase of chemical diversity by aphid attack, but methyl salicylate treatment alone did not significantly alter the chemical diversity. Our results suggest that SA signaling associated with membrane receptors might be necessary to mediate induction of chemically diverse compounds in response to aphid attacks^{43,62}.

4. The authors acknowledge that *bak1-5* and *sobir1* are pleiotropic co-receptors affecting many membrane receptors, including those for MAMPs/DAMPs. This limits the interpretability of their specificity loss, as the effect could stem from impaired general stress perception rather than herbivore-specific recognition. Do the authors have evidence (e.g., from early marker gene expression) that BAK1/SOBIR1 are specifically engaged by herbivore elicitors/treatment in their system? Such evidence would strengthen the conclusion that membrane receptors “fine-tune herbivore-specific metabolic responses”.

: We fully recognize the validity of this point on interpreting herbivore species-specific responses. We agree that our current results do not directly provide evidences supporting plant perceives species-specific ligands to exhibit specific responses. Since early signalling events of pattern-triggered immunity within several hours are largely shared across different ligands², herbivore species-specific insect-driven ligands, though none has been identified yet, may not be sufficient to induce species-specific responses. Accordingly, herbivore species-specific marker genes besides general PTI process-related (e.g., FRK1 and PAD3) or phytohormonal signalling (e.g., PR1 and VSP2) are not available. Our sampling for metabolomics was conducted after 6 days of herbivore feeding, comprising not only release of DAMPs and possibly HAMPs, but also the effectors³. Moreover, feeding guild of herbivores affect the types of damaged tissue and cells, potentially inducing specific responses. Damaged plant tissue can be an entry point for subsequent pathogens, which could jointly induce plant immune responses with herbivory. We believe that our results are therefore thought to be a resultant sum of multiple series of signalling events during plant-herbivore interactions, which are modulated by membrane co-receptors. Indeed, previous studies support that BAK1 and SOBIR1 (and their homologues in other plant species) are associated in perception of various herbivore-driven elicitors⁴⁻⁸. While there is little support for the early PTI responses are directly herbivore species-specific, we believe that the accumulation of PTI by various signals over an ecologically relevant duration of herbivore feeding would be sufficient to produce species-specific responses. We added these interpretations to the discussion to clarify the limitation of current results.

We additionally quantified allantoin in all genotypes, which showed that the induction of allantoin by Lp depends on NPR1 and BAK1, while the induction by Sl and Px was retained in all genotypes (Supplementary Fig. S12). Furthermore, in a preliminary work ongoing in our group, a *Spodoptera litura* extract elicits canonical early immune responses (e.g., PAD3 expression) that are attenuated in *sobir1*, directly indicating SOBIR1 involvement in HAMP perception.

Revised manuscript L684-692: As herbivore attack comprises not only the release of HAMPs and DAMPs but also actual cellular damage, release of effectors, and distal and systemic signaling among different parts of the attacked plants over a long-term period, at which layer the herbivore species-specificity is achieved remains elusive. Moreover,

molecular mechanism of signal detection leading to hormonal regulation and propagating into specific metabolic outcomes deserves further studies.

5. The authors show that allantoin is highly induced by Lp, Px, and Sl and acts as a resistance compound against Lp. Is the accumulation of allantoin affected in the phytohormone or co-receptor mutants? If so, this would further connect functional interpretation with mechanistic regulation and complement the correlation-based screening of resistance compounds.

: We thank the reviewer for this valuable suggestion, which strengthened the link between function and regulation in our study. Following this advice, we quantified allantoin accumulation across all phytohormone (*myc2/3/4*, *npr1*) and co-receptor (*bak1-5*, *sobir1*) mutants (Supplementary Fig. S12).

We found that the Lp-induced accumulation of allantoin was lost in *npr1* and *bak1-5*, but retained in *myc2/3/4* and *sobir1*, whereas the strong induction by the chewers Px and Sl was preserved across all four mutants. This result connects the functional identity of allantoin with its mechanistic regulation. Allantoin requires NPR1 (SA signaling) and BAK1 (membrane co-receptor) specifically for its Lp-responsive induction. This supports our broader finding that SA signaling and membrane-receptor function may jointly mediate herbivore-specific metabolic responses (Fig. 4). Moreover, because the same metabolite is induced by Px and Sl independently of NPR1/BAK1, its accumulation is regulated in an herbivore-specific manner, consistent with the central hypothesis of the manuscript. Notably, the loss of Lp-induced allantoin in *npr1* coincides with the loss of intensity-dependent resistance against Lp in the same mutant (Supplementary Fig. S1), linking this regulatory requirement to a functional resistance outcome.

Revised manuscript L733-743: Interestingly, induction of allantoin by Lp was impaired in *npr1* and *bak1-5* mutants, while those genotypes retained allantoin induction by Sl and Px. This exemplified our metabolome-wide observation that SA signaling and membrane co-receptors jointly shape herbivore-specific metabolic responses, thereby linking the correlation-based identification of allantoin to its herbivore-specific mechanistic regulation. The complex role of allantoin in plant-herbivore interactions may further reflect its interactions with phytohormones^{74,75}, supporting multiple functionalities of metabolites⁷⁶.

6. In line 932, “helimg” should be corrected to “helping”.

: Thank you for pointing out our mistake. This is corrected in the revised manuscript.

Revised manuscript L1293-1294: We also thank Byungho Kim, Yeonjee Ko, Ha Ryeong Lee, Seongmin Lee, and Ji-Yong Kim for helping the experiments.

7. In Figure 4, “Foldenrichment” should have a space (“Fold enrichment”).

: Corrected as well. The label now reads ‘Fold enrichment’.

Reviewer #2 (Remarks to the Author):

This manuscript details a study on the metabolomic effects of four different herbivores on Arabidopsis plants. The research aims to analyze the specificity of the plant's response based on the herbivore's feeding mode (sap-sucking vs. chewing) and diet breadth (specialist vs. generalist). To further understand the underlying mechanisms, the researchers also subjected mutant plants, defective in jasmonic or salicylic acid signaling and membrane receptors, to the same treatments. The authors correlated changes in the plant's metabolic profiles, and hormone levels (jasmonic, salicylic, and abscisic acid) after six days of herbivore feeding to herbivore and plant biomass, thereby identifying patterns related to the number of herbivores and their feeding style.

By correlating changes in the metabolome to herbivore feeding levels, They identified allotonin as a most responsive compound and experimentally tested its effect of on aphid performance. Based on their data, they concluded that plants respond specifically to particular herbivores, and also that some of the signaling pathways involved may not have to be intact for the plant to show a response. They critically discuss the issues related to their analysis, which is highly appreciated.

Overall, this paper presents a highly valuable dataset on the effect and specificity of herbivore-induced responses. The hypotheses are clearly introduced and visualized in figure 1. Several different statistical (multivariate) analyses have been performed and joined to interpret the data set and the figures elaborately show the results obtained. Overall, the text is well written and clear but it may need editing by a native English speaker.

: We deeply appreciate your review and helpful comments. Based on provided comments, we fully revised our manuscript and provided point-by-point responses below. Moreover, we extensively improved the grammar and vocabulary of the manuscript.

Having said that, the authors overstate the lack of studies comparing induced responses to multiple types of herbivores. One of the most comprehensive – even though on herbivore-induced volatile responses – may be Danner H, et al (2018) New Phytologist 220:726-738. doi: 10.1111/nph.14428. This one, and others (e.g. from the Poelman group at Wageningen University, Netherlands) are missing in the Supplementary excel table (minor comment; please give full bibliographic information, not only titles).

: We agree the original phrasing overstated the novelty of our comparative approach, and we have addressed this in the revised manuscript. We added several papers, including Danner *et al.* (2018) to the supplementary table and Figure 1. We also fully agree that studies from the Poelman group provides valuable insights into plant responses to multiple herbivores⁹. However, they generally include quantification of expression level of phytohormone-responsive marker genes rather than metabolites^{10–12}, therefore we decided not to include those studies in current analysis. The original overstatement has been revised to acknowledge ongoing efforts to study the ecological functions of plant metabolite diversity.

Revised manuscript L88-94: Given the clear adaptive value of specificity in plant defensive metabolism and the potential mechanisms driving it²⁷, there have been multiple attempts to reveal the metabolic specificity of plant response to multiple herbivores across diverse systems, revealing phenotypic plasticity of plant metabolomes in response to herbivores of different feeding guild and diet breadth (Fig. 1a)^{2–4,14,28–39}. However, few studies have integrated multiple species of herbivores, herbivory intensity gradients, and metabolome-wide annotation within a defense theory framework.

Similarly, the MS would benefit by looking at studies providing a strong conceptual framework as well as experimental evidence for the functional role of metabolic diversity, including: Petrén H, et al (2023) Quantifying chemodiversity considering biochemical and structural properties of compounds with the R package chemodiv. New Phytologist 237:2478-2492. doi: <https://doi.org/10.1111/nph.18685> ; Whitehead SR, et al (2021) Interaction diversity explains the maintenance of phytochemical diversity. Ecology Letters 24:1205-1214. doi: 10.1111/ele.13736; Walker et al (2023) Leaf metabolic traits reveal hidden dimensions of plant form and function. Science Advances 9:eadi4029. doi: [doi:10.1126/sciadv.adi4029](https://doi.org/10.1126/sciadv.adi4029); Walker et al (2022) Functional Traits 2.0: The power of the metabolome for ecology. Journal of Ecology 110:4-20. doi: <https://doi.org/10.1111/1365-2745.13826>). These previous studies would be a great

support to interpret the data in the proper conceptual framework to close the gap between ecology and metabolomics (lines 418-419).

: Thank you for these valuable suggestions, which have led to significant conceptual improvement in our manuscript. We have incorporated recommended studies into revised manuscript to contextualize our findings within the growing body of work on ecological functions of plant chemical diversity.

Revised manuscript L801-804: To close this gap, ecological metabolomics methods have been developed to quantify chemical diversity analogously to ecological diversity and interpret them in an ecologically relevant manner⁵²⁻⁵⁵. In parallel, complementary and trait-based approaches have revealed the ecological function of chemical diversity in controlled and natural systems^{56,57}.

Several aspects of the experimental setup require clarification in the materials and methods section. First, the number of plants used for each herbivore treatment is not specified. This information is essential for understanding the statistical power and robustness of the experiment. Second, it is unclear whether individual plants were harvested and analyzed separately or if plants were pooled into biological replicates. If pooling was performed, the number of plants constituting each pool must be detailed. Third, the manuscript does not state whether control plants were established for the methyl jasmonate and methyl salicylate treatments. The plants were sprayed with solutions of these compounds in 0.2% Tween and 10% ethanol. The solvent solution alone, containing only Tween and ethanol, may have elicited physiological or metabolic responses in the plants. Therefore, a control group treated only with the solvent solution is necessary to isolate the specific effects of methyl jasmonate and methyl salicylate.

: We apologize for the ambiguity in the method descriptions and have revised those sentences to clarify our experimental setups. We used individual plants for bioassay and sampling without pooling, and mock treatment for phytohormone treatment was indeed the 0.2% Tween-20 and 10% ethanol.

Revised manuscript L1142-1146: Each single plant was individually housed in a Phytohealth cage (SPL) with herbivores. After six days, insects were removed and measured, then the aboveground plant tissues were harvested, weighed, and flash-frozen in liquid nitrogen; each plant represented a single biological replicate. Eight biological replicates were sampled for each group.

Revised manuscript L1162-1163: Mock plants were treated by 10% EtOH aqueous solution. 0.01% Tween-20 was then supplemented.

It is also questionable how much of the primary metabolome was sufficiently captured with the metabolic approach used LC-MS). The excel list with the m/z data suggests that amino acids were detected but the masses suggest that most are not amino acids proper.

: We appreciate your valuable comments. While our metabolomic range covers primary metabolites, several analytic conditions limited access to primary metabolites. First, 80% methanol extract may not fully dissolve highly hydrophilic primary metabolites. Moreover, our chromatography condition limits our ability to detect primary metabolites. Although CSH C18 column covers a broad polarity range and particularly suited for less hydrophobic metabolites, it is not appropriate for retaining highly hydrophilic primary metabolites such as amino acids and sugars. Finally, the scan range of mass spectrometer was set as 100-1,500 m/z, excluding many primary metabolites including several amino acids. The class annotations of features from CANOPUS provides general classifications rather than exclusive annotations, as the classification relies on pre-defined chemical

class (NPCClassifier and Classyfire), with many compounds often belong to multiple classes¹³. Particularly, while the underlying annotation process of CANOPUS is not interpretable deep learning model, broad chemical class such as amino acid can serve as a fallback class where no better particular class for the feature exists in the database. Therefore, features annotated as amino acids might not be actual amino acids, while those could be derivatives from amino acids.

Regarding the herbivory treatments, particularly with the chewing herbivores, it is important to know if the amount of leaf mass removed was measured. These data would be valuable for several reasons. It could reveal whether the plant's response correlated with the amount of removed biomass or the total area of leaf damage. Furthermore, this measurement is highly relevant to the subsequent analysis presented in Figure 7D, where plant biomass is correlated with metabolomic profiles. While a higher degree of herbivory is expected to induce a stronger metabolic response, it also directly reduces plant biomass, especially in the case of chewing herbivores. This reduction in biomass, which increases with herbivore pressure, could act as a confounding factor in the analyses presented in figure 7D, which has not been accounted for in the analysis.

: We thank the reviewer for this thoughtful comment, which prompted us to both add data and clarify an important gap.

First, we quantified the leaf damage caused by the chewing herbivores (damaged leaf area and number of feeding sites), which increased gradually with attack intensity (Supplementary Fig. S3). Second, regarding the potential confounding effect on Fig. 7d, we fully agree with the reviewer. Because herbivory simultaneously induces metabolic changes and reduces plant biomass, the feature-plant aboveground biomass correlations are confounded by this shared intensity gradient, and we have taken care not to over-interpret this panel. We would respectfully note two points that, in our view, keep this from affecting our central conclusions. (1) Our main claim of adaptive metabolomic reconfiguration is based on the relationship between metabolite induction and herbivore performance (Fig. 7c), which comprises measurements made on the herbivore performance and therefore independent of plant biomass. Fig. 7d is presented as a complementary perspective of the growth-defense trade-off. (2) The same adaptive pattern is observed for the phloem sap-feeding aphids (Lp and Mp), which remove negligible leaf tissue, indicating that the pattern is not an artefact of physical biomass loss. We have nonetheless revised our manuscript to state this limitation.

Revised manuscript L998-1005: Because herbivory simultaneously induces metabolic responses and reduces plant biomass, and both effects intensify with herbivore pressure, the correlation between feature abundance and plant biomass is partly confounded by this shared intensity gradient, particularly for chewing herbivores. We cannot fully separate biomass reduction due to tissue loss from that reflecting growth-defense trade-off. Notably, the same adaptive metabolic reconfiguration was observed for the phloem-feeding aphids (Lp and Mp), indicating that it does not arise from physical biomass loss. Fully disentangling these contributions would require dedicated experiments such as controlled leaf removal.

There were two figures that I struggled with to understand or interpret properly. One is figure 6, presenting several network analyses converted in distribution plots. Without knowing the exact background of these analyses or which biological process they inform us on, their meaning is really difficult to understand.

: We appreciate the reviewer identifying this ambiguity, and we have addressed it in two complementary places. First, we added **Supplementary Text 1**, which provides the full background and formal definitions: how the metabolite co-association network is built as

a graphical-lasso-regularized Gaussian graphical model so that each edge represents a direct (conditional) association between two features after accounting for all others, and how the four node-centrality measures (degree, eigenvector, betweenness, closeness) are defined and computed. Second, we now state explicitly in the Results what these analyses inform us biologically: Mantel test (Fig. 6a) shows that structurally related metabolites (DreaMS molecular network) also tend to co-vary in abundance (GGM co-association network), and the centrality distributions (Fig. 6b–e) test whether the phytohormones occupy central positions. Instead of phytohormones, amino acids and glucosinolates emerge as central classes, suggesting that these metabolite groups, rather than the hormones themselves, sit at the regulatory core of the herbivore-induced metabolic response. We hope these additions make Figure 6 self-contained and interpretable without additional context.

Revised manuscript L538-540: The metabolite class-level specificity observed across herbivore treatments prompted us to investigate whether structurally similar compounds are also co-regulated, as well as whether phytohormones center at the regulation of metabolites.

Revised manuscript L545-548: A mantel test was then applied to test whether spectral similarity across features predicts correlations among them, resulting in significant overlap between the molecular network and the partial correlation network in herbivore-induced metabolome of WT plants (Fig. 6a).

Revised manuscript L1280-1291: To examine the structure of the herbivore-induced metabolome, we constructed two feature-feature networks: a molecular network from pairwise DreaMS-embedding similarities, and a co-association network inferred from metabolite abundances. The co-association network was a partial-correlation network estimated with a graphical-lasso-regularized Gaussian graphical model (`huge::huge` and `huge::huge.select` with EBIC), so that each edge represents a direct (conditional) association between two features after accounting for all others. The two networks were compared with a Mantel test (`vegan::mantel`). To identify features at central positions, we computed four node-centrality measures (degree, eigenvector, betweenness, and closeness) with the `igraph` package, and paired these with JA, SA and ABA quantifications to test whether the phytohormones occupy central positions. Full mathematical definitions, regularization parameters, and edge-weight/edge-length conventions are provided in Supplementary Method 1.

In figure 7, I was struggling with understanding panels c and d. Apart from the fact that I think that d may have suffered from a confounding factor (see above), it was not clear why there were no dots for m/z features in the middle. I assume it has something to do with the enrichment. In the figure legend, it said that only NC terms that were significantly enriched at least once were used, which can suggest that also non-significant ones were used in some of these correlations (?). It was really hard to understand how this figure came about, and there is not enough information in the materials and methods to get a better understanding of this. It also was not clear if and how the numbers of herbivores per plant would tie into this figure. Officially each figure should be informative as a standalone piece of information. So, this information needs to be added consisey to the legend and also better explained in the materials and method section.

: We have clarified all three points in the Methods. We agree that current presentation does not include sufficient information, thereby revised the description. We removed features whose peak area showed no significant correlation to perform linear regression between the resistance and log2 fold change using features that has significant correlation with herbivore performance (Fig. 7c) or plant aboveground biomass (Fig. 7d). As spearman correlation coefficient and the p-value is linearly related, filtering the features based on the p-value also resulted in filtering features exhibiting coefficient smaller than

certain value. To clarify the methodology, we added more detail in the method section and the figure legend of the revised manuscript. We also generated a Supplementary Fig 13 explaining analytic scheme of regression analysis.

Revised manuscript L668-684: Figure 7. Identities of putative herbivore-resistance metabolites identified by correlation-based screening. For each molecular feature, its abundance was correlated (Spearman) with herbivore performance and with plant aboveground biomass across all samples of a given herbivore species and genotype, pooling the three attack intensities; the negative of the feature–performance coefficient was taken as a *resistance value* (higher = associated with poorer herbivore performance). **(a)** Metabolite Set Enrichment Analysis (MSEA) of features ranked by their correlation with herbivore performance, shown as normalized enrichment scores (NES) for each NPC class (NC) term and herbivore. For visualization only NC terms significant for at least one herbivore are displayed, while the enrichment itself was computed on all annotated features. Symbols: ·, $p < 0.1$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **(b)** Spearman correlation coefficients (effect size; color) between each individual glucosinolate (rows) and each herbivore's performance (columns); asterisks as in (a). **(c, d)** Test of adaptive metabolomic reconfiguration. Each dot is one molecular feature, plotted by its inducibility (y-axis: $\log_2$ fold change of abundance, the highest-intensity group vs. control, Lp3/Mp3/Px3/Sl3) against **(c)** its resistance value (x-axis: $-1 \times$ the feature–herbivore performance correlation coefficient; higher = more resistance-associated) or **(d)** its growth association (x-axis: the feature–plant biomass correlation coefficient). Features without a significant correlation on the x-axis ($p \geq 0.1$) were excluded. Lines are per-genotype linear regressions (WT, *myc2/3/4*, *npr1*, *bak1-5*, *sobir1*; solid, $p < 0.05$; dashed, not significant).

Revised manuscript L1265-1277:

Correlation-based feature screening

The correlation coefficient of each molecular feature peak area and metadata (herbivore performance and plant aboveground biomass) was calculated using `ecomet::ScreenFeaturePhenotypeCorrelation` function. The coefficient of each feature was calculated based on Spearman correlation with p-value cutoff of 0.1 to broadly capture both linear and non-linear correlation. The correlation was performed for each feature per herbivore species per genotype, pooling data points from all attack intensities. The coefficient values were plotted with log-transformed fold change values from comparison between the control group and intensity 3 groups (Lp3, Mp3, Px3, and Sl3) to visualize whether there is a tendency for resistive or nutritional compounds to be induced or reduced upon herbivore attack (Supplementary Fig. 13). The significance of such tendency was tested by binomial test on proportions of features on 1st and 3rd quadrant to features on 2nd and 4th quadrant.

Correlating metabolite concentration with insect performance is indeed one way to identify potentially active compounds, as mentioned in the discussion. However, in this case, leaf metabolomes were assessed, whereas aphids suck on the phloem. Leaf and phloem metabolic profiles may differ substantially within plants.

: We appreciate your valuable comments to be addressed. Indeed, dedicated investigation on the phloem sap metabolome would provide interesting opportunity to study plant response to phloem sap feeders and identify putative defensive compounds that are specific to aphids. In current study, we sampled total leaf tissue rather than the phloem sap for two reasons. First, our primary goal was to directly compare response of plants against different herbivory contexts (feeding intensity, feeding guild, and diet breadth), which requires consistent sampling scheme across different herbivore treatments.

Secondly, while stylet sampling and EDTA-based exudate collection are available for phloem sap analysis, both can potentially affect metabolic responses during the treatment. Moreover, although aphid feeding is restricted to phloem sap, leaf metabolome and transcriptome can be significantly induced, providing sufficient metabolomic signals which allowed us to identify allantoin. Therefore, we have revised the manuscript to acknowledge this limitation.

Revised manuscript L988-997: It is notable that allantoin was identified by analyzing total leaf metabolome while aphids specifically feed on the phloem sap. Whether the concentration of allantoin is indeed increased in the phloem sap by aphid attack requires further validation. Interestingly, induction of allantoin by Lp was impaired in *npr1* and *bak1-5* mutants, while those genotypes retained allantoin induction by Sl and Px. This exemplifies our metabolome-wide observation that SA signaling and membrane co-receptors jointly shape herbivore-specific metabolic responses, thereby linking the correlation-based identification of allantoin to its herbivore-specific mechanistic regulation. The complex role of allantoin in plant-herbivore interactions may further reflect its interactions with phytohormones^{74,75}, supporting multiple functions of metabolites⁷⁶.

Another question is why allotonin was chosen for the test with the aphids. I assume there must have been more significant negative correlations with other compounds – and for other compounds with other herbivores. It is not well motivated why this single compound-herbivore relationship was singled out.

: Indeed, allantoin was one of several candidates showing strong negative correlations with herbivore performance. Among them, we selected allantoin based on three criteria: the reliability of its computational annotation by SIRIUS, the commercial availability of an authentic standard for validation, and the strength of its negative correlation with Lp performance. Moreover, it is also notable that this compound is known for its interaction with phytohormones^{14,15} but not for its direct bioactivity against insects. The rationale for this selection is now explicitly stated in the revised manuscript.

Revised manuscript L744-748: Among multiple candidate features showing strong negative correlations with herbivore performance, we focused on a feature of *m/z* 157.0362, which exhibited a strong negative correlation with the performance of Lp (Fig. 8a), reliable computational annotation, and commercial availability of an authentic standard. We tested whether this compound confers resistance against the herbivore species.

In addition, there are some minor comments or corrections to make in a next version of this manuscript. For example, there are several figure legends, which comprise abbreviations, such as DAM or NC, which need to be explained under each figure. Second, the, label f is missing in Figure 2 on the panel that it refers to. In the same figure, I suggest to add after how many days the plants were harvested (panel 2a). In figure 3, in the fourth line of the legend, it mentions: different alphabets on the boxes - that should be different letters.

: Thank you for detailed comments on our error on the manuscript. We revised the manuscript to include all the proper information. Regarding Fig. 2f, the panel is located at the right upper part of the figure (the stacked bar plot of feature counts with CANOPUS annotation)

Revised manuscript L248-249: The amount of (b) jasmonic acid, (c) salicylic acid, and (d) abscisic acid of WT plants attacked by different herbivore species and intensities.

Revised manuscript L424-425: Different letters on the boxes indicate statistically

significant differences

Revised manuscript L431-433: Mean pairwise DreaMS embedding-based distance within differentially accumulated molecular features induced by each herbivore in WT samples.

Revised manuscript L471-472: Figure 1. NPC Class (NC) term enrichment test of differentially accumulated molecular features induced by herbivores and hormones.

Revised manuscript L738: Different letters indicate statistically significant differences; one-way ANOVA followed by Tukey’s HSD)

Some phrasing may need to be adjusted. In line 267, the phrasing "enrichment of glucosinolates... was replaced by flavonoids" could be interpreted as anthropocentric. I suggest rephrasing this to be a direct comparison, for example: "Higher levels of flavonoids were observed in the SI-induced metabolome, in contrast to the higher levels of glucosinolates and open-chain polyketides found" Additionally, in line 272, the phrase "exhibited more diminished specificity" is slightly redundant. Simply stating that "bak 1-5 exhibited reduced specificity" would be more concise.

: Both sentences are revised for a more neutral and direct comparison.

Revised manuscript L478-479: Higher levels of enrichment of flavonols and tripeptides were observed in the *myc2/3/4* mutants, instead of the enrichment of glucosinolates by SI in WT plants.

Revised manuscript L483-485: *bak1-5* exhibited reduced specificity as glucosinolates, purine alkaloids, and imidazole alkaloids were enriched by more than one herbivore.

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590

Reviewer #1 (Remarks to the Author):

The authors have thoroughly addressed all the points raised by the reviewers in this revised version. The responses are well-reasoned and satisfactorily resolve the previous concerns. In my view, the manuscript has been substantially improved and now meets the standards for publication. I therefore recommend that it be accepted.

: We deeply appreciate your review that significantly improved our manuscript.

Reviewer #2 (Remarks to the Author):

I thank the authors for implementing my comments and addressing my many questions so very carefully. After reading the revised version, which I found much clearer and complete, in particular the rationale behind selecting allantoin for the bioassays. Also, the limitations of the study have been discussed much better in the discussion now.

I must confess that, despite your best effort to explain figure 6 very clearly in your rebuttal letter, I still fail to see how these conclusions emerge from the figures (distributions) and computational analyses as they are presented. To be very honest: the text for supplementary Method 1 is much beyond my expertise and current knowledge. I am fully ready to accept that this is my limitation, and I hope that the other reviewer can say more about this.

Figure 7 and its legend has become much clearer now, thanks!

I have only a few comments/suggestions for improvement or clarification left. These statements were added but were somewhat unclear:

Line 159 -160: "... number of aphids after 6 days of feeding showed that the actual attack intensities were increased by the initial treatments (Supplementary Fig. 2)". Do you mean: increased with the numbers of aphids placed on the plant ?

Line 162: “... feeding sites” Also in Fig. S2 : What is meant here? How many places they damaged the leaves (Irrespective of the size of the area removed)?

Line 813: resistive compounds...: this is an uncommon term, please replace by anti-herbivore compounds/defensive compounds

In general, the text was much improved, leaving only a few minor grammatical errors with respect to using a plural noun and a singular verb and vice versa. Nothing that a text editor could not fix.

: We deeply appreciate your thoughtful comments and efforts on reviewing our manuscript. We have clarified several points you mentioned.

Revised manuscript L146-149: The number of aphids remaining after 6 days of feeding increased with the initial number of released aphids, confirming that the three treatments produced a gradient of attack intensities. (Supplementary Fig. 2).

Revised manuscript L149-152: The damaged leaf area and number of feeding sites (individual leaf holes or separately damaged leaf margins) were measured for plants attacked by chewing herbivores, which showed a gradual increase in chewing damage through intensities (Supplementary Fig. 3).

Revised manuscript L710-714: The coefficient values were plotted with log-transformed fold change values from comparison between the control group and intensity 3 groups (Lp3, Mp3, Px3, and SI3) to visualize whether there is a tendency for defensive or nutritional compounds to be induced or reduced upon herbivore attack (Supplementary Fig. 13).