PLANT SIGNALING & BEHAVIOR
2021, VOL. 16, NO. 11, e1966586 (4 pages)
https://doi.org/10.1080/15592324.2021.1966586

Taylor & Francis
Taylor & Francis Group

SHORT COMMUNICATION

Metabolite analysis of Arabidopsis *CYP79A2* overexpression lines reveals turnover of benzyl glucosinolate and an additive effect of different aldoximes on phenylpropanoid repression

Veronica C. Perez[a], Ru Dai[b], Anna K. Block[c], and Jeongim Kim[a,b]

[a]Plant Molecular and Cellular Biology Graduate Program, University of Florida, Gainesville, FL, USA; [b]Horticultural Sciences Department, University of Florida, Gainesville, FL, USA; [c]Chemistry Research Unit, Center for Medical, Agricultural and Veterinary Entomology, U.S. Department of Agriculture-Agricultural Research Service, Gainesville, FL, USA

ABSTRACT

Indole-3-acetaldoxime (IAOx) and phenylacetaldoxime (PAOx) are precursors for the growth hormones indole-3-acetic acid (IAA) and phenylacetic acid (PAA) and the defense compounds glucosinolates in Brassicales. Our recent work has shown that Arabidopsis transgenic lines overexpressing AtCYP79A2, a PAOx-production enzyme, accumulate the PAOx-derived compounds benzyl glucosinolate and PAA. Here we report that they also accumulate the benzyl glucosinolate hydrolysis products benzyl isothiocyanate and benzyl cyanide, which indicates that the turnover of benzyl glucosinolate can occur in intact tissues. Myrosinases or β-glucosidases are known to catalyze glucosinolate breakdown. However, transcriptomics analysis detected no substantial increase in expression of known myrosinases or putative β-glucosidases in *AtCYP79A2* overexpressing lines. It was previously shown that accumulation of aldoximes or their derivatives represses the phenylpropanoid pathway. For instance, *ref2* mutant having a defect in one of the aldoxime catabolic enzymes decreases phenylpropanoid production. Considering that *AtCYP79A2* is not expressed in most organs under optimal growth condition, *ref2* accumulates aliphatic aldoximes but not PAOx. Interestingly, overexpression of *AtCYP79A2* in *ref2* resulted in a further decrease in sinapoylmalate content compared to *ref2*. This indicates that accumulation of PAOx has an additive effect on phenylpropanoid pathway suppression mediated by other aldoximes.

ARTICLE HISTORY
Received 13 July 2021
Revised 4 August 2021
Accepted 5 August 2021

KEYWORDS
Aldoximes; glucosinolates; phenylpropanoids; benzyl isothiocyanate

The amino acid derived aldoximes are hub metabolites in plants that branch into several pathways important for growth and defense. Tryptophan-derived indole-3-acetaldoxime (IAOx) and phenylalanine-derived phenylacetaldoxime (PAOx), are precursors of the growth regulating auxins indole-3-acetic acid (IAA) and phenylacetic acid (PAA) respectively.[1–5] Besides serving as auxin precursors, IAOx and PAOx are also intermediates of various defense-related compounds[6–8] including Brassicales-specific glucosinolates in Arabidopsis. In addition to their role as metabolic intermediates, the accumulation of aldoximes also represses phenylpropanoid biosynthesis via accelerated degradation of phenylalanine ammonia-lyase (PAL), the first step of the phenylpropanoid biosynthesis pathway.[5,9]

Aldoximes are synthesized by cytochrome P450 monooxygenases belonging to the 79 family in most plants. In Arabidopsis, PAOx is made from phenylalanine by the enzyme CYP79A2 (At5g05260).[8] PAOx can then be catalyzed by two enzymes, REF2 (CYP83A1) and REF5 (CYP83B1) to produce its aci-nitro intermediate which is further converted to produce benzyl glucosinonate (Figure 1a).[5,10] Although both REF2 and REF5 function redundantly to catalyze various aldoximes, REF5 has higher activity toward IAOx whereas REF2 is a major enzyme for aliphatic aldoximes. Previously, we have shown that Arabidopsis plants that overexpress *CYP79A2* in the wild-type and *ref2* genetic backgrounds accumulate high levels of the PAOx-derived benzyl glucosinolate although as expected the accumulation is higher in the wild-type background (*ox-1/WT*, *ox-2/WT*) than in the *ref2* background (*ox-21/ref2*, *ox-22/ref2*).[5] Interestingly, we found that *CYP79A2* overexpression lines accumulate benzyl glucosinolate hydrolysis products such as benzyl isothiocyanate and benzyl cyanide in intact seedlings. We analyzed metabolites in undamaged 2-week old whole seedlings following published protocol.[5,11] Sterilized seeds were vertically grown on agar plates containing Murashige and Skoog (MS) media[12] under long day condition (16 hour light/8 hour dark) for two weeks and then sampled in liquid nitrogen immediately. To quantify PAA, benzyl isothiocyanate and benzyl cyanide, we used a method adapted from Schmelz et.al.,[11] Briefly, tissues were flash frozen in liquid nitrogen and then 100 mg were extracted in 300 µl of H_2O:1-propanol:HCL(1:2:0.005) spiked with 100ng of d7-PAA as an internal standard. Then, samples were homogenized and further extracted and analyzed as stated in a published paper.[11] Benzyl glucosinolate was quantified as following published protocol.[5] The ions monitored and retention times for the compounds were as follows: PAA (151 m/z, RT 6.76 min), d7-PAA (158 m/z, RT 6.71 min), BC (118 m/z, 6.82 min), BTIC (150 m/z, RT 8.92 min). Compounds are confirmed and quantified using authentic standards. As

CONTACT Jeongim Kim jkim6@ufl.edu Plant Molecular and Cellular Biology Graduate Program, University of Florida, Gainesville, FL, USA

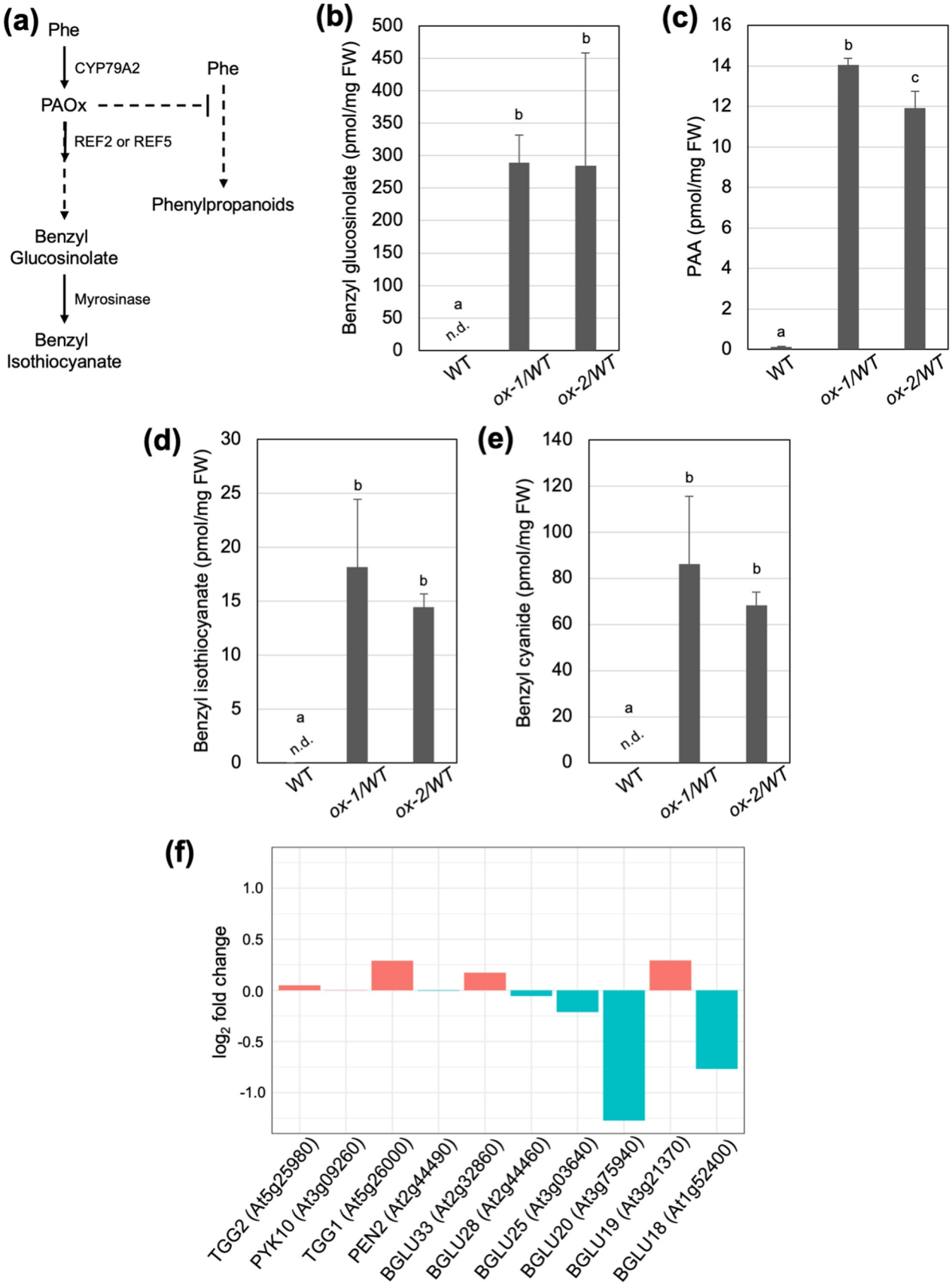

Figure 1. Glucosinolate turnover occurs *in vivo* without tissue disruption. (a) A schematic diagram of PAOx metabolism and benzyl glucosinolate degradation into benzyl isothiocyanate in Arabidopsis thaliana. In addition to the shown pathways, PAOx can also act as a precursor for PAA. (b) Benzyl glucosinolate, (c) PAA, (d) benzyl isothiocyanate, and (e) benzyl cyanide content of WT, *ref2*, and *CYP79A2* overexpression lines. All metabolite data was collected from 2-week-old whole seedlings grown on MS plates. Benzyl glucosinolate content was determined via HPLC, while all other metabolites were analyzed via GC-MS. Data represents mean ± SD (n = 3). The means were compared by one-way ANOVA and statistically significant differences ($P < .05$) were identified by Tukey's test and are indicated by letters to represent differences among groups. (f) Expression levels (log_2 fold change) of myrosinase and β-glucosidase genes in *CYP79A2* overexpression line *ox-2* compared with wild-type. TGG3, 4, 5, and 6 were not expressed in our samples. Expression levels were calculated using published RNAseq data (PRJNA682862).[5]

shown in Figure 1b–e, whole seedlings overexpressing *CYP79A2* increase PAA and benzyl glucosinolate compared to wild type similarly to soil-grown plants and were also found to produce benzyl glucosinolate hydrolysis products, benzyl isothiocyanate and benzyl cyanide. The activation of glucosinolates into cytotoxic products such as benzyl isothiocyanate is initiated by β-glucosidases known as myrosinases when pathogen/insect attack disrupts specialized cells or subcellular compartments separating glucosinolates from these enzymes.[13–15] However, several studies indicate that aliphatic or indole glucosinolates may also undergo this turnover even in the absence of tissue disruption.[13,15–19] The accumulation of benzyl isothiocyanate and benzyl cyanide, two products of benzyl glucosinolate hydrolysis, in intact Arabidopsis seedlings grown in aseptic condition supports *in vivo* turnover of benzyl glucosinolate (Figure 1d,e). Current dogma indicates that while a group of myrosinases known as 'classical myrosinases' (TGG1-6) are mainly involved in tissue disruption-mediated glucosinolate degradation, other β-glucosidases categorized as atypical myrosinases likely function in glucosinolate turnover not resulting from tissue disruption.[13,15–20] To identify β-glucosidases that might function in benzyl glucosinolate turnover, the transcriptomes[5] of wild-type and *ox-2/WT* were analyzed to determine if any known or candidate β-glucosidases were upregulated in the benzyl isothiocyanate-accumulating lines. However, TGG 3 ~ 6 did not express in our sample whereas TGG1 and TGG2 were expressed but not transcriptionally induced in *ox-2/WT* (Figure 1f). Nine putative β-glucosidases were expressed in our sample and PYK10, BGU19, and BGLU33 were slightly upregulated in *ox-2/WT*. However, the remaining β-glucosidases were down-regulated (Figure 1f). A recent study has shown that BGLU28 and BGLU30 are responsible for glucosinolate breakdown under sulfur deficiency.[19] In our samples, BGLU30 was not expressed and BGLU28 was down-regulated (Figure 1f), which further supports their roles in sulfur deficient condition as our samples were grown in sulfur sufficient media.[12,19] More work is needed to identify which enzymes are participating in benzyl glucosinolate turnover in Arabidopsis.

Despite differences in benzyl glucosinolate content dependent on the genetic background, the *CYP79A2* overexpression lines accumulated PAA to a similar degree.[5] It was therefore unexpected that *CYP79A2* overexpression in the *ref2* background resulted in more severe developmental changes than its overexpression in the wild-type background.[5] The *ox-21/ref2* and *ox-22/ref2* lines showed small dark green leaves compared to *ox-1/WT* and *ox-2/WT* lines (Figure 2a). Given their similarity in PAA content, this difference in growth may be due to an alteration in phenylpropanoid content rather than auxin accumulation (Figure 1a). The *ref2* mutant accumulates less phenylpropanoids and has an altered lignin profile, possibly due to the accumulation of aldoximes derived from mostly aliphatic amino acids.[10] Since *CYP79A2* is not expressed in most organs, it is unlikely that this alteration in phenylpropanoid biosynthesis and profile is due to PAOx in the *ref2* background. This circumstance gave us the opportunity to determine how the accumulation of various aldoximes (PAOx and aliphatic aldoximes) impacts phenylpropanoid metabolism. To determine whether different types of aldoximes have an additive effect on phenylpropanoid repression, the level of sinapoylmalate (a major phenylpropanoid of Arabidopsis leaves) was determined in *ox-21/ref2* and *ox-22/ref2*. A statistically significant decrease in sinapoylmalate content was observed in *ox-21/ref2* and *ox-22/ref2* compared to *ref2* (Figure 2b), which suggests that the accumulation of multiple aldoximes has an additive effect on repression of phenylpropanoid biosynthesis. Given that aldoxime-mediated phenylpropanoid repression includes accelerated degradation of PAL[9] which can affect the production of all phenylpropanoids including lignin, a pivotal structure compound, this combined repression in phenylpropanoid biosynthesis by both PAOx and aliphatic aldoximes may contribute to the altered growth phenotype of *ox-21/ref2* and *ox-22/ref* compared to *ox-1/WT* and *ox-2/WT*. Further study awaits to determine if the repression caused by PAOx and aliphatic aldoximes acts synergistically with IAOx-mediated phenylpropanoid repression and to identify

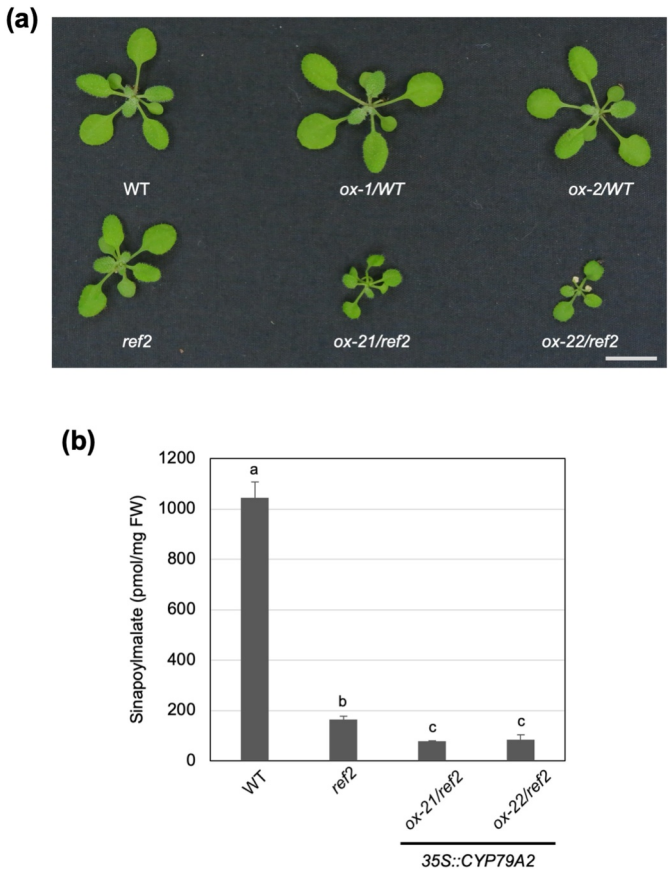

Figure 2. Aldoxime-derived repression of phenylpropanoid biosynthesis is additive. (a) Representative 2-week-old *CYP79A2* overexpression lines in the WT and *ref2* genetic backgrounds compared with their controls. Bar, 1 cm. (b) Sinapoylmalate content of WT, *ref2*, and *CYP79A/ref2* overexpression lines (*ox-21*, *ox-22*). Sinapoylmalate in 2-week-old whole aerial parts was analyzed via HPLC. Data represents mean ± SD (n = 4). The means were compared by one-way ANOVA and statistically significant differences ($P < .05$) were identified by Tukey's test and indicated by letters to represent differences among groups.

aldoxime-mediated repression mechanisms besides PAL degradation.

Acknowledgments

We thank ICBR Proteomics & Mass Spectrometry Facility at UF with LTS operation.

Disclosure statement

No potential conflicts of interest were disclosed.

Funding

This work was supported by the United States Department of Agriculture (USDA)-National Institute of Food and Agriculture Hatch (005681) and a startup fund from the Horticultural Sciences Department and Institute of Food and Agricultural Sciences at the University of Florida to JK. The United States Department of Agriculture (USDA)-Agricultural Research Service Project number 6036-11210-001-00D and the USDA- National Institute of Food and Agriculture-Specialty Crop Research Initiative grant 2018-51181-28419 to AB

ORCID

Veronica C. Perez http://orcid.org/0000-0002-3526-2487
Ru Dai http://orcid.org/0000-0002-2040-7049
Anna K. Block http://orcid.org/0000-0003-1689-4005
Jeongim Kim http://orcid.org/0000-0002-5618-3948

References

1. Zhao Y. Auxin biosynthesis and its role in plant development. Annu Rev Plant Biol. 2010;61:49–64. doi:10.1146/annurev-arplant-042809-112308.
2. Zhao Y. Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. Genes Dev. 2002;16:3100–3112. doi:10.1101/gad.1035402.
3. Sugawara S, Hishiyama S., Jikumaru Y, Hanada A, Nishimura T, Koshiba T, Zhao Y, Kamiya Y, Kasahara H. Biochemical analyses of índole-3-acetaldoxime-dependent auxin biosynthesis in Arabidopsis. PNAS. 2009;106:5430–5435. doi:10.1073/pnas.0811226106.
4. Aoi Y, Tanaka K, Cook SD, Hayashi KI, Kasahara H. GH3 auxin-amido synthetases alter the ratio of indole-3-acetic acid and phenylacetic acid in arabidopsis. Plant Cell Physiol. 2020;61:596–605. doi:10.1093/pcp/pcz223.
5. Perez VC, Dai R, Bai B, Tomiczek B, Askey BC, Zhang Y, Rubin GM, Ding Y, Grenning A, Block AK, Kim J. Aldoximes are precursors of auxins in Arabidopsis and maize. New Phytologist 231, 1449–1461.
6. Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA. Cytochrome P450 CYP79B2 from Arabidopsis catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. J Biol Chem. 2000;275:33712–33717. doi:10.1074/jbc.M001667200.
7. Glawischnig E, Hansen BG, Olsen CE, Halkier BA. Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. PNAS. 2004;101:8245–8250. doi:10.1073/pnas.0305876101.
8. Wittstock U, Halkier BA. Cytochrome P450 CYP79A2 from *Arabidopsis thaliana L.* Catalyzes the conversion of L-phenylalanine to phenylacetaldoxime in the biosynthesis of benzylglucosinolate. J Biol Chem. 2000;275:14659–14666. doi:10.1074/jbc.275.19.14659.
9. Kim JI, Zhang X, Pascuzzi PE, Liu C, Chapple C. Glucosinolate and phenylpropanoid biosynthesis are linked by proteasome-dependent degradation of PAL. New Phytol. 2020;225:154–168. doi:10.1111/nph.16108.
10. Hemm MR, Ruegger MO, Chapple C. The Arabidopsis *ref2* mutant is defective in the gene encoding CYP83A1 and shows both phenylpropanoid and glucosinolate phenotypes. Plant Cell. 2003;15:179–194. doi:10.1105/tpc.006544.
11. Schmelz EA, Engelberth J, Tumlinson JH, Block A, Alborn HT. The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. Plant J L. 2004;39(5):790–808. doi:10.1111/j.1365-313X.2004.02168.x.
12. Murashige T, Skoog F, Revised A. Medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473–497. doi:10.1111/j.1399-3054.1962.tb08052.x.
13. Wittstock U, Kurbach E, Herfurth AM, Stauber EJ. Glucosinolate breakdown. Advances in Botanical Research. 80:125–169.
14. Bones AM, Rossiter JT. The myrosinase-glucosinolate system, its organisation and biochemistry. Physiol Plant. 1996;97:194–208. doi:10.1111/j.1399-3054.1996.tb00497.x.
15. Sugiyama R, Hirai MY. Atypical myrosinase as a mediator of glucosinolate functions in plants. Front Plant Sci. 2019;10:1008. doi:10.3389/fpls.2019.01008.
16. Petersen B, Chen S, Hansen C, Olsen C, Halkier B. Composition and content of glucosinolates in developing. *Arabidopsis thaliana*. Planta. 2002;214:562–571.
17. Jeschke V, Weber K, Moore SS, Burow M. Coordination of glucosinolate biosynthesis and turnover under different nutrient conditions. Front Plant Sci. 2019;10. doi:10.3389/fpls.2019.01560.
18. Wittstock U, Burow M. Glucosinolate breakdown in arabidopsis: mechanism, regulation and biological significance. Arabidopsis Book. 2010;8. doi:10.1199/tab.0134.
19. Sugiyama R, et al. Retrograde sulfur flow from glucosinolates to cysteine in *Arabidopsis thaliana*. PNAS. 2021;118:22. doi:10.1073/pnas.2017890118.
20. Harun S, Abdullah-Zawawi M-R, Goh -H-H, Mohamed-Hussein Z-A. A comprehensive gene inventory for glucosinolate biosynthetic pathway in arabidopsis thaliana. J Agric Food Chem. 2020;68(28):7281–7297. doi:10.1021/acs.jafc.0c01916.